Anal. Chem. **2002,** *74,* 1702−1711

Analysis of Lipoproteins by Capillary Zone Electrophoresis in Microfluidic Devices: Assay Development and Surface Roughness Measurements

Bruce H. Weiller,*,†,‡ Laura Ceriotti,† Takayuki Shibata,† Dietrich Rein,§ Matthew A. Roberts,*,§ Jan Lichtenberg,† J. Bruce German,§ Nico F. de Rooij,† and Elisabeth Verpoorte*,†

Institute of Microtechnology, University of Neuchâtel, CH-2007 Neuchâtel, Switzerland, and Nestlé Research Center, Vers-Chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

The development of a new assay for lipoproteins by capillary electrophoresis in fused-silica capillaries and in glass microdevices is described in this paper. The separation of low-density (LDL) and high-density (HDL) lipoproteins by capillary zone electrophoresis is demonstrated in fused-silica capillaries with both UV absorption and laser-induced fluorescence detection. This separation was accomplished using Tricine buffer (pH 9.0) with methylglucamine added as a dynamic coating. With UV detection, LDL eluted as a relatively sharp peak with a migration time of ∼11 min and HDL eluted as a broad peak with a migration time of 12.5 min. Fluorescence detection of lipoproteins stained with NBD-ceramide was used with the same buffer system to give comparable results. Furthermore, fluorescence staining of human serum samples yielded results similar to the fluorescently stained LDL and HDL fractions, showing that this method can be used to quantify lipoproteins in serum samples. The method was also used to detect lipoproteins in glass micro-CE devices. Very similar results were obtained in microdevices although with much faster analysis times, LDL eluted as a sharp peak at ∼25 s and HDL as a broad peak at slightly longer time. In addition, higher resolution was obtained on chips. To our knowledge, these results show the first separation and detection of lipoproteins in a microfluidic device using native serum samples. Atomic force microscopy was used to characterize the rms surface roughness (R_q) of microfluidic channels directly. Devices with different surface roughness values were fabricated using two different etchants for Pyrex wafers with a polysilicon masking layer. Using 49% HF, the measured roughness is $R_q = 10.9 \pm 1.6$ nm and with buffered HF (NH_4F + HF) the roughness is $R_q = 2.4 \pm 0.7$ nm. At this level of surface roughness, there is no observable effect on the performance of the devices for this lipoprotein separation.

One of the most important and ubiquitous clinical measurements made today is the quantification of cholesterol in low-density (LDL) and high-density (HDL) lipoproteins from blood. The ratio of cholesterol in LDL ("bad") to that in HDL ("good") is the most commonly accepted indicator of risk for coronary artery disease.[1] The currently used methods for the measurement of LDL and HDL include ultracentrifugation,[2] selective precipitation, immunoassay,[3] and lipoprotein separation by size exclusion chromatography.[4] There is interest in developing a microfluidic device for this measurement in order to decrease the measurement time and to reduce the cost and sample volume required.[5] In addition, such a device could in principle allow sample preparation and labeling to be performed on chip as well as increased parallel processing of many samples. Both aspects would greatly increase sample throughput in a clinical laboratory.[6]

Lipoproteins such as LDL and HDL are complex, nanometer-sized particles consisting of apolipoproteins, cholesterol, and a phospholipid monolayer on the surface and an interior consisting of triglycerols, cholesterol, and cholesterol esters.[7,8] The number of apolipoproteins on the surface varies, with one in the case of LDL and as many as eight in the case of HDL. LDL and HDL are defined by their densities in ultracentrifugation: 1.006−1.063 g/cm^3 for LDL and 1.063−1.210 g/cm^3 for HDL.[9] This corresponds to particles with size distributions of 20−25 nm for LDL and 8−12 nm for HDL and apparent molecular weights of (2−3) × 10^6 for LDL and (1.50 −3.0) × 10^5 for HDL.[10−12] The analysis

† Institute of Microtechnology.

‡ Permanent address: The Aerospace Corp., P.O. Box 92957/M2-248, Los Angeles, CA 90009.

§ Nestlé Research Center.

(1) (a) Kannel, W. B. *J. Atheroscler. Thromb.* **2000**, *6*, 60−6. (b) Buchwald, H.; Boen, J. R.; Nguyen, P. A.; Williams, S. E.; Matts, J. P. *Atherosclerosis* **2001**, *154*, 221−7.

(2) Havel, R. J.; Eder, H. A.; Bragdon, J. H. *J. Clin. Invest.* **1955**, *34*, 1345.

(3) Measurement methods for lipoproteins: Friedewald, W. T.; Levy, R. I.; Fredrickson, D. S. *Clin. Chem.* **1972**, *18*, 499−502.

(4) Okazaki, M.; Ohno, Y.; Hara, I. *J. Biochem. (Tokyo)* **1981**, *89*, 879−87.

(5) Roberts, M.; Geiger, W.; German, J. B. *Am. J. Clin. Nutr.* **2000**, *71*, 434−7.

(6) Colyer, C. L.; Tang, T.; Chiem, N.; Harrison, D. J. *Electrophoresis* **1997**, *18*, 1733−41.

(7) Lehninger, A. L.; Nelson, D. L.; Cox, M. M. *Principles of Biochemistry*, 2nd ed.; Worth Publishers: New York, 1993.

(8) Smith, L. C.; Massey, J. B.; Sparrow, J. T.; Gotto, A. M.; Pownall, H. J. In *Supramolecular Structure and Function*; Pifat, G., Herak, J. N., Eds.; Plenum Press: New York, 1983; pp 205−44.

(9) Havel, R.; Eder, H.; Bragdon, J. *J. Clin. Invest.* **1955**, *34*, 1345.

(10) Esterbauer, H.; Waeg, G.; Puhl, H.; Dieber-Rotheneder, M.; Tatzber, F. In *Free Radicals and Aging*; Emerit, I., Chance, B., Eds.; Birkhaeuser Verlag: Basel, Switzerland, 1992; pp 145−157.

(11) Rudel, L. L.; Marzetta, C. A.; Johnson, F. L. *Methods Enzymol* **1986**, *129*, 45−57.

10.1021/ac011096y CCC: $22.00
Published on Web 03/06/2002

of lipoproteins is difficult; they are not single molecules but complex particles with distributions of sizes and with a variety of different molecules on their surfaces and interiors. Furthermore, the commercial sources of lipoprotein samples are limited and vary in quality. This provides additional challenges in the development of new analytical methods and devices.

An important recent development in the analysis of lipoproteins is the use of capillary electrophoresis (CE).[13−17] The implementation of this powerful analytical technique for this application has been limited by surface adsorption of lipoprotein particles on the walls of fused-silica capillaries. Two approaches for CE of lipoproteins have been used. One approach recently developed by Schmitz et al. uses isotachophoresis in a capillary coated with poly(dimethylsiloxane) (PDMS).[14] In this method, serum is stained with a fluorescent dye, either Sudan black or NBD-ceramide, and lipoproteins are separated according to their electrophoretic mobilities at constant velocity. It gives sharp peaks and excellent resolution of all major lipoproteins and subfractions. However, isotachophoresis is a relatively complex technique that requires the use of many spacer compounds (nine in this case) added to the running buffer as well as separate leading and terminating buffers.[18−22] A second approach involves the use of a dynamic coating in simple capillary zone electrophoresis (CZE) to reduce the interaction of lipoproteins with the walls. A dynamic coating is of benefit due to the inherent difficulties in applying fixed coatings and their low, long-term reliability. Stocks and Miller showed that, using Tricine buffer with added methylglucamine as a dynamic coating, LDL gives a single sharp peak with UV absorption and that LDL and oxidized LDL can be separated.[16] However, they did not analyze for HDL and so the separation of LDL and HDL was not demonstrated with this system. Sodium docecyl sulfate (SDS) has also been used at low concentrations with some success to measure LDL and HDL by CZE; however, lipoprotein particle separation was not demonstrated. Another drawback of this approach is that delipidation and rupture of lipoprotein particles can occur with the use of detergents, which might confuse the interpretation of results.[23,24]

Microfluidic devices for capillary electrophoresis have been very successful for a wide range of bioanalytical measurements.[25] The potential of micro-CE devices for clinical applications has been recognized by several groups, and some progress in this area has been reported.[26] However, there are relatively few reported measurements of samples from human blood using micro-CE devices. Some notable examples are homogeneous immunoassays for serum theophylline[27,28] and serum cortisol[29] and the separation of serum proteins in a non-native matrix.[30] The analysis of blood samples with microfluidic devices is particularly difficult due to surface adsorption of blood components that causes sample to be lost to the walls and changes the surface properties of the microchannels.

This work describes the development of a CZE separation method for lipoprotein samples from native blood, with subsequent application to a microchip-based assay format. The analytical performance of the method is then compared and contrasted between the two formats. It is shown that LDL and HDL can be separated, using Tricine buffer and methylglucamine as a dynamic coating, and detected by direct UV detection or by fluorescence detection after staining with NBD-ceramide. Similar results were obtained with serum samples showing that the method should work for quantification of lipoproteins in serum samples. Furthermore, this method was used to detect and separate LDL and HDL on micro-CE devices using samples from native human blood. To our knowledge, these results represent the first separation and detection of lipoproteins in a microfluidic device. Surface roughness of the microchannels was measured directly by atomic force microscopy for devices produced by two different etching processes. Surface roughness in the range of 2.4−10.9 nm has no significant effect on the performance of the devices for this separation.

EXPERIMENTAL SECTION

Lipoprotein Fractionation by Micro-ultracentrifugation. Fresh blood was collected in tubes without additive and allowed to clot for 20 min at 22 °C before separation of serum (1500*g*, 15 min, 4 °C). One male, borderline-hypercholesterolemic blood donor volunteered throughout the study (total serum cholesterol 5.2−6.2 mmol/L). A 500-μL sample of fresh serum was mixed with 250 μL of very low-density lipoprotein (VLDL) density solution (0.195 M NaCl, $d_{20} = 1.006$). After centrifugation (Sorvall micro-ultracentrifuge M150, Sorvall S150AT rotor, Sorvall 2-mL microfuge tubes) at 900000*g* and 16 °C for 1 h, 250 μL of the VLDL layer was removed using the fine tip of an elongated glass Pasteur pipet. LDL density solution (250 μL, 0.195 M NaCl, 2.44 M NaBr, $d_{20} = 1.182$) was added and mixed. After a second centrifugation (900000*g*, 16 °C, 2 h), 250 μL of the LDL layer was removed and replaced by 250 μL of the HDL density solution (0.195 M NaCl, 6.37 M NaBr, $d_{20} = 1.442$). After a third centrifugation (900000*g*, 16 °C, 2.5 h), 250 μL of the HDL layer was removed. Samples were pooled, and the cholesterol of each fraction was measured on a Cobas Bio (Hoffmann-La Roche, Basel, Switzerland) using a cholesterol reagent according to the manufacturer's specifications (CHOD-PAP, Roche Diagnostics No. 1489232, Rotkreuz, Switzerland).

(12) Rye, K. A.; Clay, M. A.; Barter, P. J. *Atherosclerosis* **1999**, *145*, 227−38.
(13) Schmitz, G.; Mollers, C. *Electrophoresis* **1994**, *15*, 31−9.
(14) Schmitz, G.; Mollers, C.; Richter, V. *Electrophoresis* **1997**, *18*, 1807−13.
(15) Bottcher, A.; Schlosser, J.; Kronenberg, F.; Dieplinger, H.; Knipping, G.; Lackner, K. J.; Schmitz, G. *J. Lipid Res.* **2000**, *41*, 905−15.
(16) Stocks, J.; Miller, N. E. *J. Lipid Res.* **1998**, *39*, 1305−9.
(17) Stocks, J.; Nanjee, M. N.; Miller, N. E. *J. Lipid Res.* **1998**, *39*, 218−27.
(18) Several groups have also begun to implement this approach; see: refs 19−22.
(19) Bittolo-Bon, G.; Cazzolato, G. *J. Lipid Res.* **1999**, *40*, 170−7.
(20) Schlenck, A.; Herbeth, B.; Siest, G.; Visvikis, S. *J. Lipid Res.* **1999**, *40*, 2125−33.
(21) Zorn, U.; Wolf, C. F.; Wennauer, R.; Bachem, M. G.; Grünert, A. *Electrophoresis* **1999**, *20*, 1619−26.
(22) Inano, K.; Tezuka, S.; Miida, T.; Okada, M. *Ann. Clin. Biochem.* **2000**, *37*, 708−16.
(23) Macfarlane, R. D.; Bondarenko, P. V.; Cockrill, S. L.; Cruzado, I. D.; Koss, W.; McNeal, C. J.; Spiekerman, A. M.; Watkins, L. K. *Electrophoresis* **1997**, *18*, 1796−806.
(24) Cruzado, I. D.; Hu, A. Z.; Macfarlane, R. D. *J. Capillary Electrophor.* **1996**, *3*, 25−9.
(25) Effenhauser, C. S.; Bruin, G. J. M.; Paulus, A. *Electrophoresis* **1997**, *18*, 2203−2213. Bruin, G. J. M. *Electrophoresis* **2000**, *21*, 3931−3951.
(26) von Heeren, F.; Verpoorte, E.; Manz, A.; Thormann, W. *Anal. Chem* **1996**, *68*, 2044−53.
(27) Chiem, N. H.; Harrison, D. J. *Clin. Chem.* **1998**, *44*, 591−8.
(28) Chiem, N.; Harrison, D. J. *Anal. Chem* **1997**, *69*, 373−8.
(29) Koutny, L. B.; Schmalzing, D.; Taylor, T. A.; Fuchs, M. *Anal. Chem.* **1996**, *68*, 18−22.
(30) Colyer, C. L.; Mangru, S. D.; Harrision, D. J. *J. Chromatogr., A* **1997**, *781*, 271−6.

The lipoprotein fractions were dialyzed as follows to remove excess salt from the ultracentrifugation process: 15 h at 4 °C against 1000 volumes of phosphate-buffered saline (PBS, Catalog No. 79382, Fluka Chemie GmbH) using dialysis bags with a molecular weight cutoff of 6000−8000 (Spectra/Por, CA, No. 132 665).

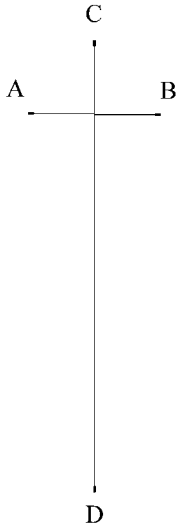
Lipoprotein Characterization by Size Exclusion Chromatography (SEC). Lipoprotein characterization was performed using size exclusion HPLC to separate the lipoproteins on the basis of size. The procedure was modified from methods described previously.[31,32] Briefly, 20 μL of diluted serum (1:1 with running buffer) was injected onto a Superose 6HR FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). Lipoproteins were eluted with running buffer (Dulbecco's phosphate-buffered saline, pH 7.4, 0.02% sodium azide) at a flow rate of 0.6 mL/min (Dionex GP500 HPLC system with an AD20 detector). Lipoprotein cholesterol was determined on-line using a postcolumn reactor. The reactor consisted of a T-connector, through which cholesterol reagent (CHOD-PAP, Roche Diagnostics) was delivered via an HPLC pump (Syknm S2100, Syknm, Gilching, Germany) at a rate of 0.1 mL/min. A mixing coil in a temperature-controlled water bath at 40 °C was used to regulate the reaction. The absorbance was recorded at 500 nm, and lipoprotein cholesterol ratios were calculated. SEC data were also collected without cholesterol determination using direct UV absorption at 280 nm.

Capillary Zone Electrophoresis. All experiments were performed on a Beckman MDQ instrument equipped with P/ACE workstation software and a commercial UV absorption or a laser-induced fluorescence (LIF) detector. The excitation source was an argon ion laser operating at 488 nm, and fluorescence was measured at 520 nm. The detector rise time was set to 1 s and the data rate to 4 Hz. Standard fused-silica capillaries (60 cm, 75 μm internal diameter) were purchased in bulk (Polymicro Technologies, Phoenix, AZ) and cut to size. The instrument was run at 20−28 kV in normal polarity (negative electrode at the detector end). Capillaries were washed with deionized (DI) water, NaOH (0.25 M), DI water, and buffer between sample injections. The running buffer was 40 mM Tricine (Sigma) with 40 mM of methylglucamine (Sigma) and adjusted to pH 9.0 with NaOH. Samples were injected in pressure mode, and the capillary temperature was set to 20.0 °C. Sample injection conditions (pressure and temperature) were fixed to keep sample plug lengths to less than 1% of total column length.

Lipoprotein fractions or sera were stained as follows. Lipoprotein solution (25 μL) was diluted with DI water (75 μL, 1:3 v/v) and incubated with a half volume (50 μL) of NBD-ceramide (Molecular Probes, OR) solution (0.5 mg/mL in ethylene glycol/DMSO, 9:1 v/v) for 1.0 min. The solution was then mixed with buffer (250 μL, 3:5 v/v). The final stained solution (400 μL) was used without filtering for both capillary and microchannel electrophoresis.

Microdevices. Two types of microdevices were used, one fabricated in-house and another purchased commercially (Figure 1). The in-house microdevices (designated IMT-1) were fabricated in 100-mm-diameter borosilicate glass wafers (500 μm thick, Pyrex 7740, Bullen Ultrasonics) using standard photolithographic techniques described previously.[33] The structure used had a double-tee injector (200 μm long), side channels (10 mm), and an overall separation length of 67.5 mm. Briefly, polysilicon (400 nm thick) was deposited on the wafer by low-pressure chemical vapor deposition at 570 °C in two separate depositions of 200 nm each. This was necessary to prevent pinhole formation in the wet chemical etching procedure. Following silanization with hexamethyldisilazane, positive photoresist (AZ-1518, Clariant) was spun on the wafer, prebaked, and exposed using a Cr mask with 10-μm line widths (Delta Mask, Enschede, Netherlands). After development and postbake, reactive ion etching (RIE) was used to transfer the pattern to the polysilicon. The photoresist was stripped in MOS-grade acetone, and the glass was etched in 49% HF (9 μm/min) or buffered HF solution (1 part 49% HF/7 parts 40% NH4F solution, 0.03 μm/min) to give channel depths of 20 ± 2 μm. Channel depths were confirmed by profilometry (Alphastep). The polysilicon was removed in a KOH bath (40% w/w) at 60 °C for ∼5 min. A Pyrex cover plate with diamond-drilled holes (Stecher AG, Thun, Switzerland) was bonded to the etched wafer at a temperature of 650 °C for 5 h.[34] Cross-sectional SEM measurement of a sealed channel gave a depth of 18 μm and a width at the top of 42 μm, which is within error of the expected channel dimensions of 18 μm × 46 μm. With the sealed channels, there may be some deformation of the top of the channel due to the fusion bonding process. Solution reservoirs were formed in different ways: holes (∼4-mm i.d.) in a thick (∼3 mm) sheet of PDMS (Sylgard 184, Dow Corning) reversibly sealed onto the cover plate, or by pipet tips or short pieces of tubing glued to the wafer.

The commercial microdevices (Part No. MC-BF4-100TT, Micralyne, Alberta, Canada) were also fabricated in borosilicate



Channel Dimensions	IMT-1	MC-1
AB (mm)	20	10
CD (mm)	67.5	85
Double tee (μm)	200	100
Depth (μm)	20 ± 2	20
Width (μm)	50 ± 4	50

Figure 1. Diagram of the microfluidic devices. Two types of devices were used in this study, one fabricated in-house (IMT-1) and one purchased commercially (MC-1). The dimensions are listed in the table. The double-tee length is the offset between ports A and B. The distance of ports A, B, and C to the intersection are the same. Details on the materials and fabrication methods are provided in the Experimental Section.

(31) German, B.; Xu, R.; Walzem, R. L.; et al. *Nutr. Res.* **1996**, *16*, 1239−49.
(32) Kieft, K. A.; Bocan, T. M.; Krause, B. R. *J. Lipid Res.* **1991**, *32*, 859−66.
(33) Dodge, A.; Fluri, K.; Verpoorte, E.; de Rooij, N. F. *Anal. Chem.* **2001**, *73*, 3400−9.
(34) Lichtenberg, J.; Verpoorte, E.; de Rooij, N. F. *Electrophoresis* **2001**, *22*, 258−71.

glass (Schott Borofloat) and are designated MC-1. These devices were very similar to the IMT-1 devices with a channel depth of 20 μm, a 100-μm-long double-tee injector, side channels of 5-mm length, and an overall separation length of 85 mm. For these devices, solution reservoirs were formed using a Plexiglas plate (5 mm thick) with 6-mm-diameter holes that was aligned and sealed to the cover plate with Viton O-rings. Atomic force microscopy (AFM) measurements were carried out at the bottom of the etched reservoirs in diced pieces of unbonded wafers using a commercial instrument (Nanoscope) operated in tapping mode. The wafers were run through the same thermal cycle used for bonding to control for annealing from this step.

Micro-CE Instrumentation. For micro-CE measurements instrumentation was used similar to that described in an earlier report.[34] It consisted of two high-voltage power supplies (HCN 7E-12500, F.u.G. Elektronik, Rosenheim, Germany) and high-voltage relays (Gunther, Nurnberg, Germany). Data acquisition and control cards (AT-MIO-16-XE−50, PC-DIO-24, National Instruments) were used. Data acquisition and instrument control were accomplished using in-house software written in LabView (National Instruments). Electrical contacts with solutions were made using Pt electrodes. LIF detection used an Ar^+ laser (Ion Technologies, Salt Lake City, UT) at 488 nm coupled to a fiber optic (200-μm i.d.) for excitation at 45° from the bottom of the wafer. The laser beam was collimated and focused to a ~50-μm spot with glass lenses (l = 8 cm). The fluorescence signal was collected and detected using an inverted microscope with objective (NPL FL, 25×, NA 0.35, Leitz), band-pass filter (Melles-Griot 03FIL004, 514 nm, 10 nm fwhm), pinhole (1-mm diameter, Melles Griot), and photomultiplier tube, (Hamamatsu, model H5701-50). To remove high-frequency noise, the acquired signal was filtered with a RC low-pass filter (cutoff at 33 Hz) and a numerical algorithm (cutoff at 50 Hz). Typical conditions were as follows: sample injection, 1000 V for 30 s; separation, 4000−5000 V applied to the separation channel with 800−1000 V applied to each arm of the double tee to prevent leakage of sample into the separation channel. This translates into separation field strengths of 593−741 V/cm. (Here and throughout the paper, all voltages are negative.)

For mobility measurements, the current monitoring method was used.[35] Diluted buffer (90% with DI) was placed in the sample well and buffer was placed in all other wells. Voltage was applied between sample and buffer waste and then switched to the buffer well and buffer waste. The time to clear the channel was measured by monitoring the current. For 40 mM Tricine with 40 mM methylglucamine at pH 9.0, the measured mobility in the IMT-1 device is $\mu_{eof} = 3.9 \times 10^{-4}$ cm^2/V·s.

RESULTS AND DISCUSSION

Lipoprotein Fractionation and Characterization. The data from size exclusion chromatography of LDL and HDL fractions are shown in Figure 2. The fractions were prepared by ultracentrifugation as described in the Experimental Section and were dialyzed against PBS overnight. The traces show the retention times on a Superose 6HR column with absorbance detection of derivatized cholesterol at 500 nm. Cholesterol was derivatized in a postcolumn reactor with cholesterol reagent as described in the Experimental Section. Panel a shows the data for the HDL fraction and panel b shows the data for the LDL fraction. The large peak at later time in Figure 2a is HDL and the smaller peak is a small amount of LDL contamination. The fractional peak areas are 5.9 and 94.1% for LDL and HDL respectively (Table 1). Since the method relies on sequential ultracentrifugation and manual pipetting in microcentrifuge tubes, it is not uncommon to observe a small amount of contamination from the next lighter fraction. It should be noted that, because the detection is based on cholesterol determination, the peak areas represent relative amounts of cholesterol in the particles but not particle concentrations. In fact, since there is more cholesterol in the LDL particles, the method is more sensitive to LDL and the relative particle number of LDL is smaller than apparent. Given typical weight percents of 45 and 17% cholesterol, and estimated molecular weights of 2.5×10^6 and 2.65×10^5 of LDL and HDL respectively,[8] the fractional concentrations of LDL and HDL particles are 0.3 and 99.7%, respectively. For the LDL fraction in Figure 2b, the fractional cholesterol peak areas are 99.7 and 0.3% and the particle concentrations for LDL and HDL are 93 and 7%. These data show

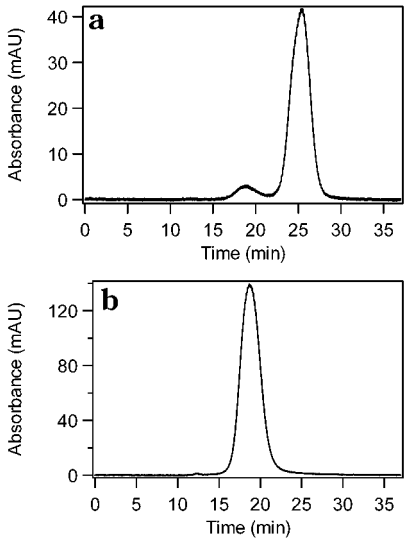


Figure 2. Size exclusion chromatography of lipoprotein fractions: (a) HDL; (b) LDL. Fractions were produced by ultracentrifugation of fresh serum samples. The lipoproteins were detected by postcolumn reaction determination of cholesterol by absorbance at 500 nm.

Table 1. Lipoprotein Size Exclusion Chromatography Data

figure	species	cholesterol peak area	protein peak area	particle concn
2a	HDL	94.1		99.7
	LDL	5.9		0.3
2b	HDL	0.3		7.0
	LDL	99.7		93.0
3a	HDL	4.4		53.7
	LDL	93.1		45.5
	VLDL	2.5		0.8
3b	HDL	51.8		96.4
	LDL	46.6		3.5
	VLDL	1.6		0.1
3c	HDL		81.2	95.2
	LDL		16.6	4.4
	VLDL		1.9	0.3

(35) Huang, X.; Gordon, M. J.; Zare, R. N. *Anal. Chem.* **1988**, *60*, 1837−8.

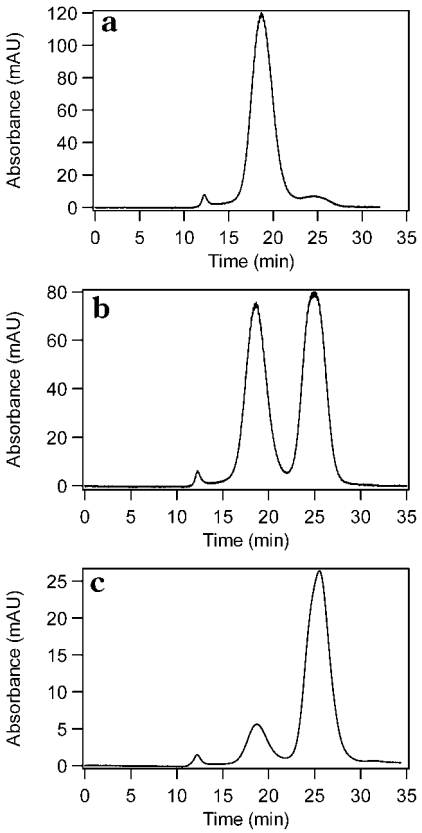


Figure 3. Size exclusion chromatography of lipoprotein samples: (a) LDL fraction, cholesterol detection by postcolumn reaction; (b) HDL fraction, cholesterol detection by postcolumn reaction; (c) HDL fraction, direct protein detection by UV absorption (at 280 nm). These samples are different from the ones used in Figure 2.

that, when implemented properly, the ultracentrifugation method used here produces good fractionation of lipoproteins with relatively small amounts of cross contamination.

Similar data from LDL and HDL fractions that are not as well separated are shown in Figure 3. Panel a shows the LDL fraction and panel b shows the HDL fraction; both are cholesterol measurements as in Figure 2. In both traces, three peaks are observed corresponding to VLDL, LDL, and HDL cholesterol, respectively. For the LDL fraction, the fractional peak areas are 2.5, 93.1, and 4.4% for VLDL, LDL, and HDL respectively, with corresponding fractional concentrations of 0.8, 45.5, and 53.7%. The HDL fraction shown in Figure 3b is contaminated with a considerable amount of LDL. There are three peaks corresponding again to VLDL, LDL, and HDL cholesterol with fractional peak areas of 1.6, 46.6, and 51.8% and relative particle concentrations of 0.1, 3.5, and 96.4%, respectively. Again contamination from the lighter fraction is due to manual pipetting. It should be noted that these data were obtained before the fractionation procedure was optimized. Also shown for comparison is data for the same samples and column without cholesterol derivatization but with direct UV absorption detection at 280 nm (Figure 3c). There are also three peaks with the same elution times, but now the fractional peak areas are 1.9, 16.6, and 81.2% for VLDL, LDL, and HDL, respectively. Assuming UV absorption at 280 nm is mainly due to aromatic amino acids of the apolipoproteins and assuming 8, 22, and 47% protein in VLDL, LDL, and HDL, we can compare Figure 3b and c. Calculating particle distribution starting from cholesterol or protein measurements yields similar particle ratios, 0.1 versus 0.3%, 3.5 versus 4.4%, and 96.4 versus 95.2%, for VLDL, LDL, and HDL respectively (Table 1). This is relevant to the CZE measurements with UV detection described below.

CZE Separation of LDL and HDL. The CZE trace obtained with UV absorption from LDL and HDL fractions and mixtures is shown in Figure 4. The conditions were chosen to be similar to that presented by Stocks and Miller;[16] the buffer was 40 mM Tricine (pH 9.0) with methylglucamine at 40 mM with a separation voltage of 20 kV in normal polarity with a 75-μm-diameter capillary. The lipoprotein sample (undialyzed) was diluted with water (1:5 v/v) and then with buffer (3:5 v/v). Figure 4a shows the LDL fraction, Figure 4b shows the HDL fraction, and Figure 4c shows a 1:1 mixture (v/v) of the two. The LDL peak observed is similar in migration time and peak shape to that obtained by Stocks and Miller. In that study, they observed a peak for LDL at 9.2 min with a width of 0.1 min ($N = 4.7 \times 10^4$, HETP = 11 μm, $L_{eff} = 50$ cm), while we observe a peak at ~11.5 min with a width of 0.3 min ($N = 8.1 \times 10^3$, HETP = 62 μm, $L_{eff} = 50$ cm).[36] The fractions used for this experiment are the same ones used for the data in Figure 3. There is a small amount of HDL in the LDL fraction, which corresponds to the small broad peak after the LDL peak. The center trace shows the HDL fraction as a later broad peak with migration time of 12.5 min and a width of 1.4 min ($N = 440$, $L_{eff} = 50$ cm). Finally, Figure 4c shows a mixture of LDL and HDL (1:1).

The intensities and positions of the peaks in the electropherogram confirm the assignment to LDL and HDL. First, when these fractions are mixed, there are no new peaks; therefore all components elute as these two peaks. This removes any doubt that may arise due to variations in absolute migration times. Second, the intensities of the peaks are in accord with expectations from the volume ratios and the intensities in the separate traces. For example, for the sharp peak, the expected peak height is 0.12 and the measured height is 0.13. For the broad peak, the expected height is 0.30 and the measured height is 0.24. While there are other components in the sample, all of the major species that absorb at 214 nm elute as these two peaks.

The relative peak shapes for LDL and HDL are interesting to consider. LDL elutes as a relatively sharp peak whereas HDL elutes as a broad peak. There are two primary factors affecting the peak width of these species, size distribution and particle homogeneity. Both lipoproteins consist of a distribution of particle sizes, 20−25 nm for LDL and 8−12 nm for HDL, as measured by electron microscopy.[7] Therefore, the distribution of sizes for HDL are almost twice as broad as for LDL. Data on the capillary electrophoresis of liposome particles, a related chemical system, have been presented.[37] For a broad distribution of liposome sizes, 355 ± 210 nm, the observed peak width with UV absorption was quite broad, 0.7 min with a migration time of 5.5 min. Interestingly, the peak width appears to correlate with the size distribution as measured by laser light scattering. For the case of lipoproteins, the size distribution of particles should also affect the peak width and a broader peak for HDL is expected. In addition to the size

(36) All of the peak widths presented are the measured full width at half-maximum (fwhm). N denotes the number of theoretical plates as calculated by $N = 5.54(t/w)^2$, where t is the migration time and w is the peak width.

(37) Roberts, M. A.; Locascio-Brown, L.; MacCrehan, W. A.; Durst, R. A. *Anal. Chem.* **1996**, *68*, 3434−40.

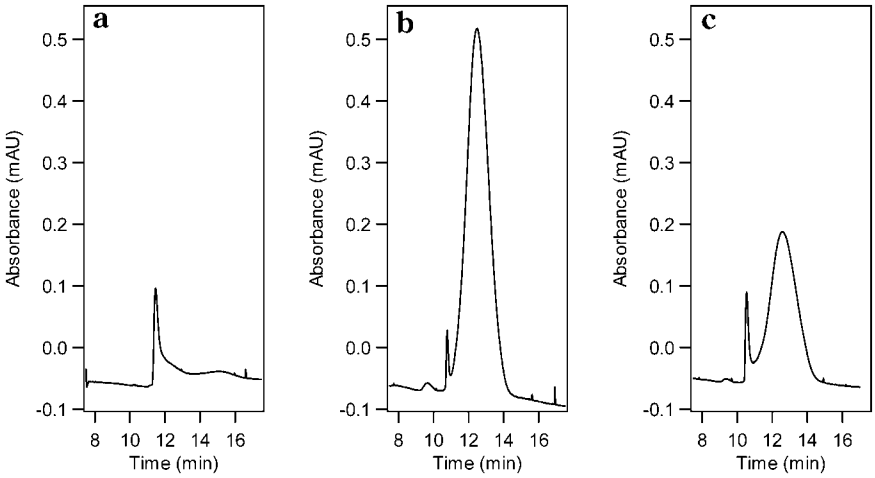


Figure 4. Separation of LDL and HDL by CZE and detection by UV absorbance: (a) LDL; (b) HDL; (c) 1:1 mixture of HDL and LDL. The detection is at 214 nm, and the separation conditions were as follows: 333 V/cm, $L_{eff} = 50$ cm, 40 mM Tricine, 40 mM methylglucamine, pH 9.0, and 75-μm-i.d. fused-silica capillary.

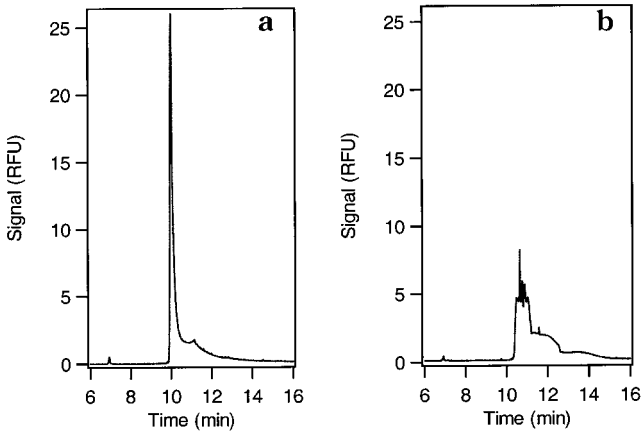


Figure 5. CE peaks for LDL and HDL with fluorescent detection: (a) LDL; (b) HDL. Each fraction was prepared as described in the Experimental Section and was stained with NBD-ceramide. Detection was LIF with excitation at 488 nm and detection at 520 nm. The separation conditions were as follows: 333 V/cm, $L_{eff} = 50$ cm, 40 mM Tricine, 40 mM methylglucamine, pH 9.0, and 75-μm-i.d. fused-silica capillary.

distribution, the width of the lipoprotein peaks could also be affected by the number of apolipoproteins on the surface; HDL has many more than LDL. This could lead to a greater range of electrophoretic mobilities for HDL compared to LDL, and the combination of these factors should give a much greater peak width for HDL compared to LDL as observed.

The electropherograms for LDL and HDL with fluorescent detection are shown in Figure 5. The LDL and HDL fractions were dialyzed overnight against PBS and were stained with NBD-ceramide as described in the Experimental Section. The voltage was 20 kV, and pressure-assisted injection was used. As Figure 5a shows, fluorescently stained LDL (LDL*) elutes as a sharp peak with migration time of 10.00 min with a width of 0.12 min ($N = 3.9 \times 10^4$, HETP = 13 μm, $L_{eff} = 50$ cm).[38] Figure 5b shows that fluorescently stained HDL (HDL*) elutes as a broad peak with a migration time of ~10.8 min. The HDL* peak is typically not as symmetric as with the UV absorption detection (Figure 4), and there are typically some features at the trailing edge that could be an indication of a small amount of differentiation in the staining process. It should also be noted that the migration times for labeled lipoproteins detected by LIF are significantly different from the unlabeled ones detected by UV absorption. This is probably due to the NBD-ceramide stain, which causes a chemical modification of the lipoprotein particles.

Shown in Figure 6 are CZE data for mixtures of LDL and HDL using fluorescent staining for the same samples in Figure 5. Mixtures with HDL/LDL ratios of 2:1, 1:2, and 1:1 (v/v) are shown in Figure 6a−c, respectively. The mixtures were prepared prior to staining. It is worthwhile to compare the ratio of peak areas for the LDL* peak with those expected from the volume ratios. When the LDL* peak areas are normalized with respect to the LDL* peak in the 1:1 mixture, the relative LDL* peak areas for the 2:1 and 1:2 mixtures are 1.50 and 0.72, respectively, in agreement with the expected values of 1.34 and 0.66. Peak areas were integrated using a estimated linear cutoff for the peak separation. This does result in significant error for the peak areas. In any event, the data clearly show that LDL and HDL can be separated using NBD staining with this buffer system in simple CZE.[39]

CZE data for a serum sample that was stained with the same procedure used for the lipoprotein fractions is shown in Figure 7. Significantly, a sharp early peak and a broad later peak are observed that are very similar to the peaks observed for LDL and HDL separately. Higher voltage was used for these data (28 kV), which is partly responsible for the shorter migration times and for the increased resolution. However, the migration times do not correlate as expected with field strength. It is possible that other components in the serum could influence the mobility of the lipoprotein particles.[40] While the assignment of these peaks is not conclusive, these data suggest that it is possible to use this simple

(38) Here we use the superscript * to denote fluorescently stained lipoproteins.

(39) It should be noted that we do observe significant variations in migration times from run to run and from sample to sample. We have not been able to determine the exact cause, but it is most likely due to surface modification from sample adsorption and variations in the samples.

(40) The authors thank reviewer I for the thoughtful comments that are incorporated here.

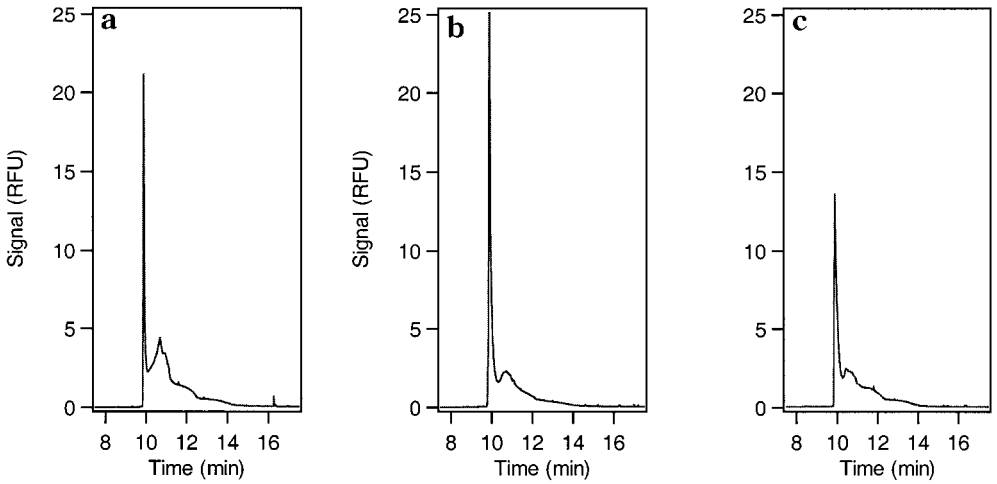


Figure 6. Electropherograms of mixtures of HDL and LDL with fluorescence detection: (a) HDL:LDL = 2:1; (b) HDL:LDL = 1:2; (c) HDL:LDL = 1:1. The mixtures were prepared before staining. The separation conditions were as follows: 333 V/cm, L_{eff} = 50 cm, 40 mM Tricine, 40 mM methylglucamine, pH 9.0, and 75-μm-i.d. fused-silica capillary.

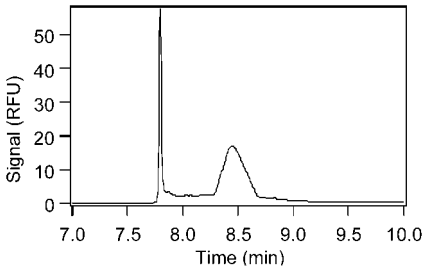


Figure 7. Electropherogram of fluorescently stained serum. Serum was prepared as described in the Experimental Section and was stained with the same method used for the lipoprotein fractions. The separation conditions were as follows: 467 V/cm, L_{eff} = 50 cm, 40 mM Tricine, 40 mM methylglucamine, pH 9.0, and 75-μm-i.d. fused-silica capillary.

method to separate and quantify lipoproteins in serum samples. It should be noted that NBD-ceramide is a lipophilic dye and will specifically stain lipoproteins over other components of serum.[41] For example, separate experiments have confirmed that IgG is not detectable with this staining procedure.[42]

Integrated Capillary Zone Electrophoresis. To verify that the procedures and instrumentation with the chips give reliable separations and good detection sensitivity, a standard control mixture of amino acids was used. Typically, a mixture of FITC-labeled arginine (Arg), serine (Ser), glycine (Gly), and phenylalanine (Phe), each at 120 nM concentration, was used and labeling was carried out using the procedure described in the literature.[43] The injection and separation conditions were as described in the Experimental Section. The resulting electropherograms are qualititatively similar to those presented in the literature.[34,43] For the unreacted FITC peak, which is at a concentration of 500 nM, the calculated signal-to-noise ratio is SNR = 83 and $N = 1.2 \times 10^4$ theoretical plates. The effective separation length is 4.2 cm, which gives 3.0×10^5 plates/m or a plate height (HETP) of 3.4 μm. From these data a minimum detectable concentration of 20 nM (SNR = 3) is calculated and was confirmed by dilution experiments. Prior to every run with the microdevices, this mixture was run to confirm chip condition and instrumental performance.

The results for LDL and HDL samples using the IMT-1 chips are shown in Figure 8. The lipoprotein samples were prepared and stained using the same procedure as for the capillary column data. The LDL fraction is shown in Figure 8a, the HDL fraction is shown Figure 8b, and a 1:1 mixture in shown in Figure 8c. Comparing the data in Figure 8 to the capillary column data shown in Figures 5 and 6, good agreement is observed. The same qualitative peak shapes for LDL and HDL are observed, with a sharp early peak for LDL and broad later peak for HDL. The respective migration times measured for LDL and HDL are much shorter on chip than in columns, with 24.6 and 27.6 s on chip versus 11.5 and 12 min in columns. The calculated theoretical plates for the LDL and HDL peaks are 2.9×10^4 and 1.3×10^3, respectively, in the chips. With the effective column length of 4.2 cm, these convert to plate heights of 1.4 and 33 μm for LDL and HDL, respectively. Recall that, for LDL* in capillary column electrophoresis, the plate height was 13 μm at a field strength of 333 V/cm. Assuming a linear relationship between field strength and efficiency, the calculated plate height for the chip at 333 V/cm is 3 μm. Therefore, the efficiency is higher on chip even when the greater separation voltage is accounted for.

The SNR for the data from IMT-1 chips is considerably lower than for the columns. For the LDL peak in Figure 6a, the SNR = 9.2×10^3 (based on peak-to-peak values), whereas for the LDL peak in Figure 8a, the SNR = 22, or ~420 times smaller. There are many instrumental factors that will affect the SNR such as relative peak width, detection bandwidth, collection optics, laser power, probe volume, and scattered light. Undoubtedly our instrumentation could be improved with respect to the SNR. However, our primary interest is the performance of the chip with respect to the capillary. A factor that is of concern is the sample recovery on chips relative to capillaries.

Sample recovery in chips could be lower than in fused-silica capillaries if there is increased surface adsorption. It is well known that proteins and other biomolecules readily adsorb to surfaces.

(41) Technical literature, Molecular Probes, Inc.
(42) Unpublished data, L. Ceriotti and T. Shibata.
(43) Effenhauser, C. S.; Manz, A.; Widmer, H. M. *Anal. Chem.* **1993**, *65*, 2637−42.

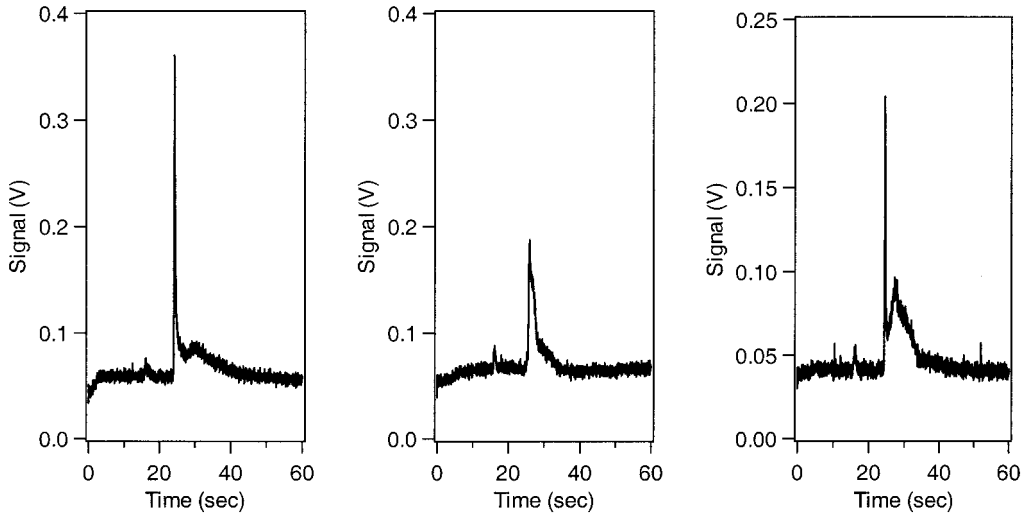


Figure 8. Electropherograms of lipoproteins on chip: (a) LDL; (b) HDL; (c) HDL:LDL = 1:1. The separation conditions were as follows: 714 V/cm, L_{eff} = 42 mm, and 40 mM Tricine, 40 mM methylglucamine, pH 9.0. The chip used was IMT-1.

Lipoprotein particles are known to adsorb to fused-silica surfaces quite readily. For example, without added methylglucamine, surface adsorption of LDL to fused-silica capillaries is so problematic that no usable peaks in CZE are observed.[16] In addition, LDL has been observed to have a greater affinity for untreated fused-silica over silanized fused-silica surfaces.[44] The surface-area-to-volume ratio of the microfluidic channels is much greater than for the 75-μm-diameter capillaries. If a surface adsorption process does occur, then it could be enhanced in microfluidic channels.

Enhancement of surface adsorption processes is possible if the chips have rougher surfaces. If surface adsorption does occur, it could be greatly affected by an increased number of surface adsorption sites. The surface roughness of fused-silica capillaries has been measured by AFM, and in general, they are very smooth with root-mean-square (rms) surface roughness values (R_q) of $R_q = 0.42 \pm 0.06$ nm.[45] The microfluidic channels are produced by an etching process which is expected to give a rougher surface. To determine whether this is a potential problem in the microdevices, the surface roughness of the microfluidic channels was measured directly with AFM.

Surface Roughness Measurements of Microdevices. AFM data for etched Pyrex microchannels are shown in Figure 9a. The data were obtained at the bottom of a channel produced by the process described in the Experimental Section. The measurement was performed in the region of the reservoir in a small piece cut from a processed but unbonded wafer. The wafer was subjected to the same thermal cycle as used for bonding in order to eliminate annealing effects. The data for unetched Pyrex were obtained in the region that was masked by polysilicon. Three separate measurements for the unetched and four separate measurements for the etched piece were obtained. The mean rms values ($R_q \pm \sigma$) are for unetched, $R_q = 1.0 \pm 0.2$ nm, and for etched, $R_q = 10.9 \pm 1.6$ nm, pieces. Compared with the fused-silica capillaries, the surface roughness of the etched Pyrex channels is ~26 times rougher. Therefore, it is probable that the surface area of these microchannels is significantly greater than the fused-silica capillary columns.

To determine whether reduced surface roughness can improve the micro-CZE of lipoproteins, devices were produced with smoother surfaces. As described in the Experimental Section, by simply changing the etchant from 49% HF to buffered HF, we find that smoother surfaces are produced. All other process steps were kept the same. AFM measurements of the resulting channels etched for 660 min to a depth of 20.3 μm are shown in Figure 9b. The measured surface roughness is $R_q = 2.4 \pm 0.7$ nm for a 3-fold reduction in surface roughness.

We have also examined commercially available chips (MC-1) with design and materials similar to that of the IMT-1 chips (Figure 1). They consist of a double-tee injector with length of 100 μm, channel depth of 20 μm, side channels 5 mm long, and an overall separation channel length of 85 mm. These devices are also fabricated from a borosilicate glass, Borofloat,[46] which is very similar to Pyrex[47] in chemical composition. A slightly different fabrication process was used.[48,49]

AFM measurements for the etched channels in the MC-1 devices are shown in Figure 9c. The data were taken in the same way as in Figure 9a and b for the IMT-1 devices. The unetched data show a surface roughness of 0.5 ± 0.1 nm, and the etched data show a surface roughness of 2.1 ± 0.2 nm. The underlying causes for the resulting surface roughness values are related to the etchant used as well as the other process steps that can affect the surface and bulk properties of the material. We hope to address these issues in a future publication.

(44) (a) Ho, C.-H.; Hlady, V. In *Proteins At Interfaces II: Fundamentals and Applications*; Horbett, T. A., Brash, J. L., Eds.; ACS Symposium Series 602: American Chemical Society: Washington, DC, 1995. (b) Hlady, V.; Ho, C.-H. *Materialwiss. Werkstofftech.* **2001**, *32*, 185−92.

(45) Kaupp, S.; Wätzig, H. *Electrophoresis* **1999**, *20*, 2566-74.

(46) The composition of Borofloat is: SiO_2 (81%), B_2O_3 (13%), $Na_2O + K_2O$ (4%), and Al_2O_3 (2%). Borofloat has the same chemical composition as Duran, another Schott product, Schott company literature, www.schottglass.com/Duranb1.htm.

(47) The composition of Pyrex is: SiO_2 (81%), B_2O_3 (13%), Na_2O (4%), and Al_2O_3 (2%), Stockdale, B. *J. Assoc. Lab. Autom.* **1999**, *4*, 35−9.

(48) The MC-1 chips were etched using the a mixture of $HF:HNO_3:H_2O$ = 20:14:66 by volume; Glen Fitzpatrick, Micralyne, Inc., personal communication. This etchant is not compatible with the polysilicon mask we use. See ref 49 for more details on this etchant.

(49) Vossen, J. L.; Kern, W. *Thin Film Processes*; Academic Press: New York, 1978; p 437.

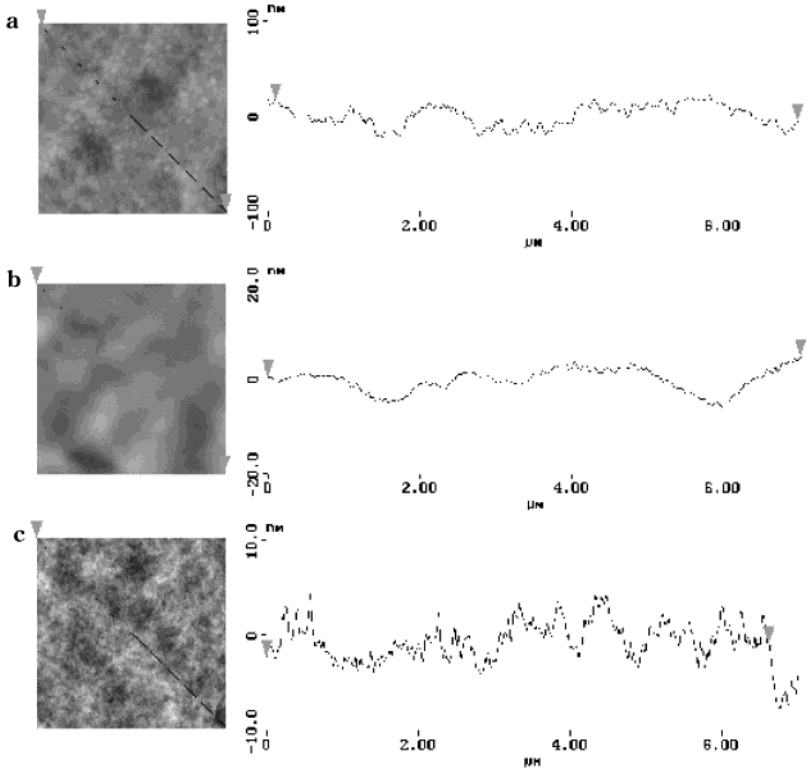


Figure 9. AFM data for etched microfluidic channels. (a) Fabricated from Pyrex 7740 and etched with 49% HF. The *y*-axis is 200 nm full scale, and the mean rms surface roughness is $R_q = 10.9 \pm 1.6$ nm. (b) Fabricated from Pyrex 7740 and etched with buffered HF. The *y*-axis is 40 nm full scale and $R_q = 2.4 \pm 0.7$ nm. (c) Fabricated from Borofloat and etched with $HNO_3/HF/H_2O$. The *y*-axis is 20 nm full scale and $R_q = 2.1 \pm 0.2$ nm. In each case, the data were taken in three places at the bottom of etched channels from unsealed witness plates. For the Pyrex wafers, each was annealed at 650 °C for 5 h to simulate the fusion bonding step.

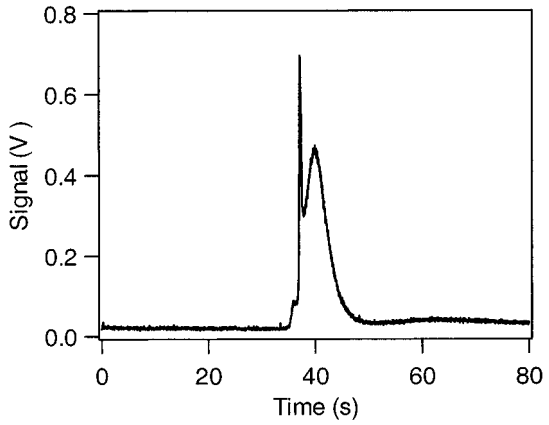


Figure 10. Electropherograms of an HDL Fraction on the MC-1 Chip. HDL fraction with a small amount of LDL contamination. The sharp peak is LDL and the broad peak is assigned to HDL. The separation conditions were as follows: 706 V/cm, $L_{eff} = 60$ mm, and 40 mM Tricine, 40 mM methylglucamine, pH 9.0.

Effect of Surface Roughness on CZE of Lipoproteins in Microchannels. Using the smooth IMT-1 chips fabricated with buffered HF, we did not observe any significant difference between the two types of chips for this separation. We examined the performance of the chips with respect to sample throughput and efficiency. Both LDL and HDL were examined using fractionated samples from the same batch but stained on different days. The resulting data are very similar to that presented in Figure 8 with minor differences that are within experimental and systematic errors. It should be noted that this does not rule out the effects of surface roughness on CE performance for other systems. We hope to explore this in future studies with a more ideal system than lipoproteins.

The MC-1 chips result in data that are significantly different from that obtained with the IMT-1 chips. Data for the MC-1 chip are shown in Figure 10 for an HDL fraction with a significant LDL contamination. The samples were prepared and stained the same way as in Figure 8 and the detection parameters were also identical. Note the qualitative similarity to the capillary column data in Figures 4 and 6. The difference between these data and that from the IMT-1 devices is also notable with respect to the SNR and peak area for the same detection sensitivity and sample preparation conditions. It should be noted that the injection plug for the MC-1 chips is shorter by a factor of 2. We have already ruled out the effects of surface roughness from the measurements with the IMT-1 chips. Therefore, the differences between the MC-1 and IMT-1 chips must be attributed to factors other than surface roughness.

The major difference between the MC-1 chips and the IMT-1 chips is the channel layout as shown in Figure 1. The MC-1 chip has sidearms that are half as long as the IMT-1 chips. It may be that surface adsorption of lipoproteins in the sidearms is significant and the longer sidearms in the IMT-1 chips enhance this process. However, the short sidearms make the MC-1 chips very susceptible to pressure effects due to differences in liquid levels and surface tension developed in the small-diameter fluid reservoirs in the cover plates.[50] Related issues with these devices have been

reported.[51] Pressure effects could influence the amount of sample that is injected with these devices. However, control experiments with fluorescein dye do not give similar results but are completely consistent with the volume of sample injected as determined by the double-tee length. Further experiments are required to determine the exact cause of this observation.

CONCLUSIONS

A new assay for lipoproteins has been developed based on capillary zone electrophoresis using Tricine buffer, methylglucamine as a dynamic coating, fluorescence staining with NBD-ceramide, and laser-induced fluorescence detection. Data from fused-silica capillary columns demonstrate the viability of the method to separate LDL and HDL from native serum samples.

The assay has been shown to function well in the glass microchip format using native serum samples. Analysis times are 26 times shorter on chip than in capillary columns, and the resolution is higher as well. Height equivalent theoretical plates for LDL samples have been observed as small as 1.4 μm in chips.

The surface roughness of the microfluidic channels was measured directly and no effect on chip performance was observed in this case. Channels with surface roughness of $R_q = 10.9 \pm 1.6$ nm and $R_q = 2.4 \pm 0.7$ nm were produced with two etchants. Rougher surfaces result from concentrated HF, while the smoother surfaces are produced with buffered HF solution. Over this range of surface roughness, no effect could be observed on the sample recovery or efficiency of the chips for LDL or HDL samples.

ACKNOWLEDGMENT

The very capable assistance of the technical staff at the Institute of Microtechnology is greatly appreciated. Assistance from Vincent Linder with the design of the high-voltage power supply is acknowledged. Financial support was received from the Swiss government under Grant CTI-3969.2. B.H.W. acknowledges support from The Aerospace Corp. during a foreign assignment at the Institute of Microtechnology.

Received for review October 16, 2001. Accepted January 4, 2002.

AC011096Y

(50) For a good discussion of these issues, see: Fletcher, P. D. I.; Haswell, S. J.; Paunov, V. N. *Analyst* **1999**, *124*, 1273−82.

(51) Crabtree, H. J.; Cheong, E. C. S.; Tilroe, D. A.; Backhouse, C. J. *Anal. Chem.* **2001**, *73*, 4079−86.