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Microfabricated Capillary Electrophoresis Amino Acid Chirality Analyzer for Extraterrestrial Exploration

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Chiral separations of fluorescein isothiocyanate-labeled amino acids have been performed on a microfabricated capillary electrophoresis chip to explore the feasibility of using such devices to analyze for extinct or extant life signs in extraterrestrial environments. The test system consists of a folded electrophoresis channel (19.0 cm long × 150 μm wide × 20 μm deep) that was photolithographically fabricated in a 10-cm-diameter glass wafer sandwich, coupled to a laser-excited confocal fluorescence detection apparatus providing subattomole sensitivity. Using a sodium dodecyl sulfate/γ-cyclodextrin pH 10.0 carbonate electrophoresis buffer and a separation voltage of 550 V/cm at 10 °C, baseline resolution was observed for Val, Ala, Glu, and Asp enantiomers and Gly in only 4 min. Enantiomeric ratios were determined for amino acids extracted from the Murchison meteorite, and these values closely matched values determined by HPLC. These results demonstrate the feasibility of using microfabricated lab-on-a-chip systems to analyze extraterrestrial samples for amino acids.

A major goal of future missions to Mars by NASA and the European Space Agency is to search for chemical signatures that might reveal the presence of extinct or possibly extant extraterrestrial life forms. What molecules should we look for that will unambiguously answer whether those molecules are of biological origin? It has been argued that amino acids are the biomolecules of choice because of their essential role in biochemistry as we know it and because they are readily synthesized under plausible prebiotic conditions.[1,2] Since laboratory-based abiotic syntheses of amino acids result in racemic mixtures,[3] the homochirality of amino acids offers a way of distinguishing between abiotic and biotic origins. Although both sample return and in situ analyses can address these goals, the rapid development of microfluidic lab-on-a-chip systems,[4] the complex logistics of sample return compared with in situ missions, and back-contamination issues[5] suggest that in situ analysis is an approach that must be seriously considered.

Amino acid composition and enantiomeric analysis have been performed using gas chromatography (GC),[6] high-performance liquid chromatography (HPLC),[6,7] and capillary electrophoresis (CE).[8,9] In each case, chiral resolution is achieved either by reacting the amino acids with a homochiral reagent, thus creating diastereomers that can be separated by chromatography, or by using a chiral stationary phase. In the case of CE, chiral buffer additives can also be used to differentially alter the mobilities of enantiomers. In general, all of these methods have demonstrated excellent separations of amino acids and their enantiomers. The development of miniaturized versions of these instruments that can perform chiral analyses and that are compatible with the size of current in situ space probes will be required.

Recently, innovative progress has been made in the development of microfabricated chemical analysis systems that might have utility for extraterrestrial exploration. Early work in this area included the development of a gas chromatograph[10] and a liquid chromatograph[11] using micromachined silicon substrates, but these devices have not been widely used. More recently, CE channels have been photolithographically fabricated in glass sandwich structures, and electrophoretic separations of fluorescent dyes,[12] fluorescently labeled amino acids,[13] double-stranded DNA fragments,[14,15] and single-stranded DNA sequencing fragments[16,17]

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have been demonstrated. Chromatographic separations can also be performed on these microchips by derivatizing the channel walls with a stationary phase[18,19] or by including micelles in the separation buffer.[20] Sample preparation, such as PCR, can also be integrated onto these microfabricated devices, as well as pre- and postcolumn derivatization.[21−23] However, chiral analysis using microchip-based CE has not been reported.

We were therefore interested in developing a prototype microfabricated analysis device that could determine amino acid composition and provide chiral resolution of relevant enantiomers. Here, we report the microfabrication of such a CE system and the optimization of the separation conditions for fluorescein isothiocyanate (FITC)-labeled amino acids. Challenges included the development of a long, folded channel design that produces high-resolution separations and the development of an effective fluorescent labeling and separation method tailored for 488-nm excitation. The accuracy and precision of the enantiomeric ratios determined with this device and method are evaluated. Finally, we present analyses of amino acid extracts taken from the Murchison meteorite to demonstrate the feasibility of analyzing extraterrestrial material for amino acid composition and chirality.

EXPERIMENTAL SECTION

Microfabrication. The photolithography and etching of the CE wafers were performed at the UC Berkeley Microfabrication Laboratory, with minor modifications to previous procedures.[17,24] Borofloat glass wafers (10-cm diameter, Precision Glass & Optics, Santa Ana, CA) were pre-etched in concentrated HF for 15 s and cleaned before depositing a 1500-Å amorphous silicon sacrificial layer by low-pressure chemical vapor deposition. These wafers were then primed with hexamethyldisilazane, spin-coated with photoresist (Shipley 1818, Marlborough, MA) at 5000 rpm for 30 s, and soft-baked at 90 °C for 30 min. A contact mask aligner (Quintel Corp., San Jose, CA) was used to expose the photoresist layer with UV light through the mask design shown in Figure 1a (the masked channel width was 110 μm), and the exposed channel pattern was developed with Microposit developer (Shipley). The exposed amorphous silicon was removed using a CF_4 plasma etch. The wafers were chemically etched with concentrated HF at room temperature for 3 min, producing channels 20 μm deep × 150 μm wide. The remaining photoresist was stripped off, and the amorphous silicon was removed by a CF_4 plasma etch. Access holes (2.1-mm diameter) were drilled at the reservoir sites using a diamond-coated drill bit (Crystalite, Westerville, OH). The etched and drilled wafer was thermally bonded to a flat wafer of the same dimensions to form capillaries, as described earlier.[24] To create deeper wells over the drilled reservoirs, 3-mm-thick PDMS elastomer gaskets (Corning, Sylgard 184) with 3.5-mm-diameter holes were laid over the access holes.

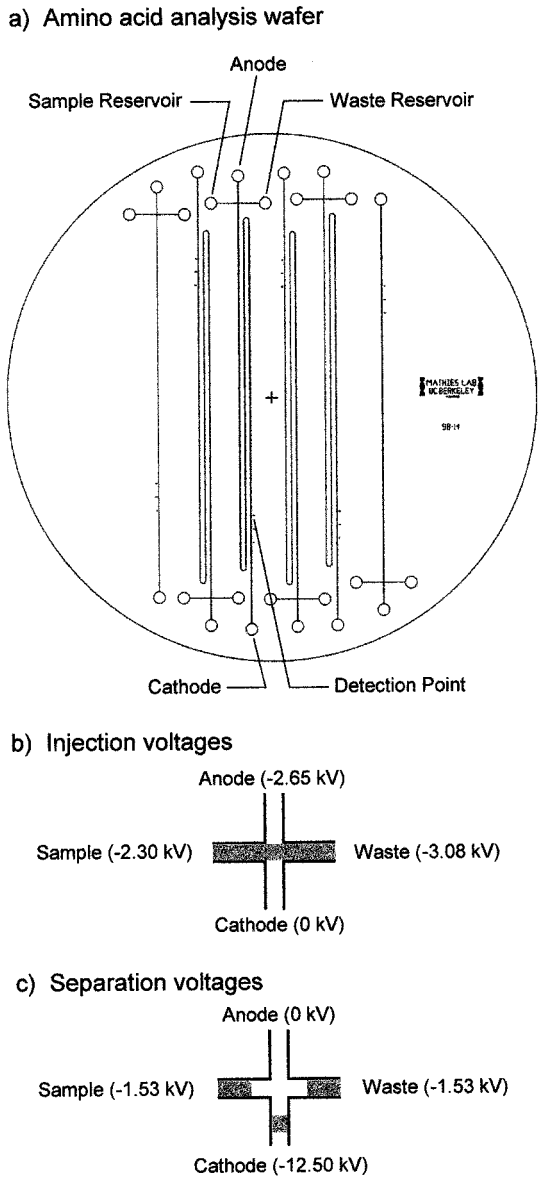

Figure 1. (a) Mask design for the amino acid analysis wafer. Pre-etch channel dimensions for the doubly folded channels used in this work were 110 μm wide × 21.4 cm long with 19.0 cm from the injection cross to the detection point. The radius of curvature around the turns was 500 μm. Distance markers were etched outside of and orthogonal to the separation channel for accurate and reproducible positioning of the confocal fluorescence detector. Channels connecting the injection intersection to the sample, anode, and waste reservoirs were all 0.4 cm in length. (b) Illustration of the voltages applied during the sample injection process. (c) Illustration of the voltages applied during the sample analysis process.

Sample Preparation and Labeling. Amino acids were fluorescently labeled by adding 10 μL of 1 mM FITC in acetone with 0.1% pyridine to 90 μL of an amino acid standard in sodium carbonate buffer (10 mM, pH 10.0).[25] The standard contained equimolar amounts of Gly and the D- and L-forms of Val, Ala, Ser, Glu, and Asp that were diluted to a total amino acid concentration of 1 mM. These protein amino acids were chosen because of their

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abundance in carbonaceous meteorites.[26] After the reaction was carried out in the dark at room temperature for approximately 12 h, the solution was diluted 1/50 with sample buffer consisting of 10 mM carbonate and 12 mM sodium dodecyl sulfate (SDS) at pH 10.0.

Because of coeluting Ala and Ser peaks, it was necessary to remove Ser from the standard by reaction with periodate to quantitate Ser and Ala enantiomeric ratios.[27] The periodate treatment included reaction of the amino acid standard with 1 mM $NaIO_4$ for 15 min, followed by quenching (15 min) with 1% glycerol to remove excess IO_4^-. Standard FITC derivatization was then performed.

Portions of the same Murchison meteorite sample that have been analyzed previously[28,29] were also analyzed using the microfabricated CE separation system and HPLC. Approximately 100 mg of pulverized interior and exterior Murchison meteorite samples was transferred to test tubes, sealed with 1 mL of double-distilled water, and heated at 100 °C for 24 h. The resultant supernatant was removed, dried under vacuum, and hydrolyzed with 6 N HCl at 100 °C for 24 h in sealed test tubes. The HCl was removed under vacuum, and the hydrolysate was desalted using a Bio-Rad AG50W-X8 cation-exchange resin. The final desalted residue was resuspended in 100 μL of double-distilled water and stored at −2 °C. For the HPLC analyses performed at the Scripps Institution of Oceanography, 10 μL of the amino acid extracts was dried down under vacuum in 10 μL of 0.4 M sodium borate buffer (pH 9.5) to remove ammonia, resuspended in 20 μL of double distilled water, and then derivatized by *o*-phthaldialdehyde/*N*-acetyl-L-cysteine (OPA/NAC, 99% chiral purity) for 1−15 min.[30,31] For the microchip CE analyses, the UC Berkeley laboratory received 50-μL aliquots of the amino acid extracts that had been dried down for shipment. These samples were redissolved in 50 μL of carbonate buffer and labeled by adding FITC stock solution in a 1:4 proportion to the extract solutions. After reaction for approximately 12 h, the derivatized solutions were diluted 1/10 with sample buffer for analysis. Blanks were taken through the entire extraction and derivatization procedures as well.

Separation and Detection Procedures. For the microchip CE analyses, enantiomeric resolution of the D- and L-forms of amino acids was accomplished by including chirally discriminating cyclodextrin (CD, Sigma, >99% pure) in the run buffer. CDs are cyclic oligosaccharides containing six, seven, or eight D(+)-glucopyranose units, identified as α-, β-, and γ-CD, respectively. The inner cavity of CD is hydrophobic, while the outside rim has exposed hydroxyl groups, attached to chiral centers, that allow for the enantioseparation of fluorescently labeled amino acids by stereoselective complex formation.

Separation and injection cross channels were filled by placing 20 μL of run buffer (10 mM carbonate, 12 mM SDS, pH 10.0) containing CD in the anode and waste reservoirs and applying a vacuum at the cathode to draw solution through the separation channel. Then, the cathode reservoir was filled with 20 μL of run buffer, while the sample reservoir was filled with 20 μL of derivatized sample. The chip was placed on a temperature-controlled mount that has been described previously.[17] Electrodes were inserted into all of the reservoirs, and the separation channel was aligned with the excitation laser which was focused to a 10-μm spot through a 32× NA 0.4 objective. The confocal fluorescence detection system has been described.[14,17] Sample was loaded into the separation channel using the 30-s pinched injection procedure illustrated in Figure 1b. Subsequent application of a −520 V/cm separation field and back-biasing voltages (Figure 1c) was used to separate the derivatized amino acids while preventing bleeding of sample into the separation channel from the sample and waste reservoirs. The channels were flushed between runs with 1 M NaOH, followed by a deionized water rinse.

For the HPLC separations, the OPA/NAC-derivatized amino acids were analyzed by a C-18 reversed-phase Phenomenex column coupled to a Shimadzu RF-535 UV−fluorescence detector (detection limit ~10^{-15} mol). The HPLC conditions are described in detail elsewhere.[30,31]

RESULTS AND DISCUSSION

The goal of our research is to explore the feasibility of using microfabricated CE systems for in situ amino acid composition and enantiomeric quantitation analyses in extraterrestrial environments. Initially, we focused on optimizing the conditions for the chiral separation of derivatized amino acids in microfabricated CE channels. Due to the previous success of using micellar electrokinetic chromatography (MEKC) with the chiral buffer additive γ-CD for enantiomerically resolving FITC-labeled amino acids[32] and the compatibility of this fluorescent labeling format with our detection system, we decided to take a similar approach. The native forms of α-, β-, and γ-CD were used as chiral selectors, and it was found that γ-CD produced the best selectivity presumably because of the complimentary fit between its large hydrophobic pocket and the fluorescein functional group. Use of mixed CDs in the run buffer did not improve the enantiomeric resolution beyond that obtained with γ-CD alone. Furthermore, the 19.0-cm folded channel gave superior enantiomeric resolution compared to the 5.0-cm straight channel geometry; consequently, we used only long, folded channels in subsequent experiments to optimize the separation.

The chiral resolution of enantiomers by CD-containing electrophoresis buffers is temperature dependent.[33] This parameter is explored in Figure 2 for microchannel-based separations. In the 24 °C separation, D- and L-Glu are well resolved, D- and L-Asp are nearly resolved, while D-Ala and D-Ser migrate together, as do the L-forms of Ala and Ser. Reducing the temperature to 15 °C gives baseline resolution of Asp enantiomers and also induces a splitting of the D-Ala and D-Ser coelution peak. Further reduction of the temperature to 10 °C and then to 5 °C improves the chiral

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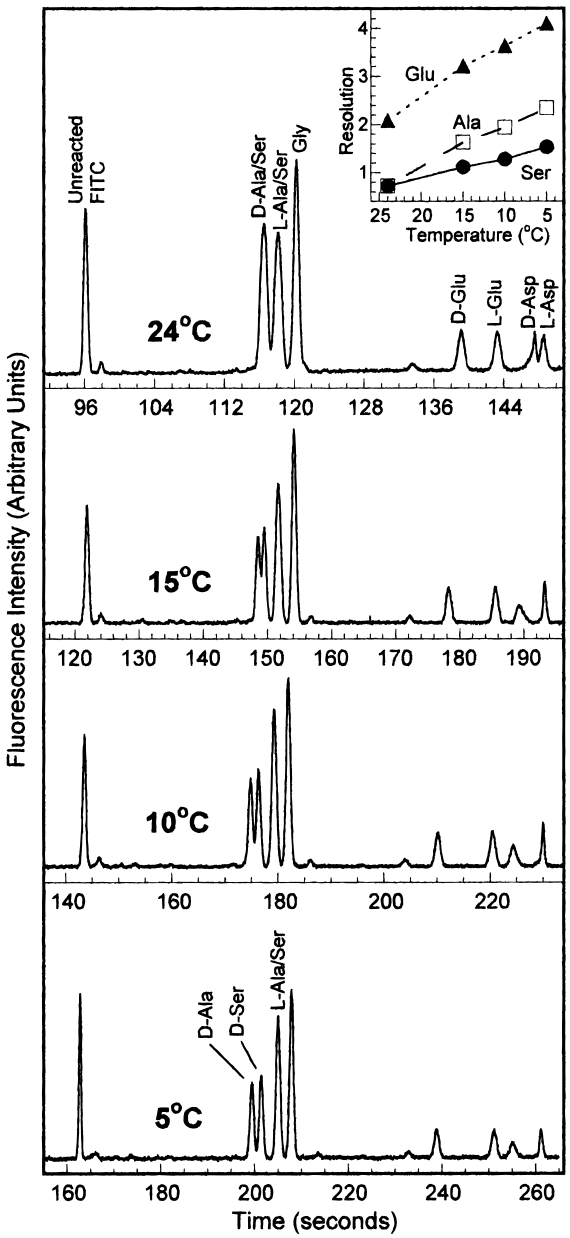

Figure 2. Electropherograms demonstrating the effect of temperature on the resolution of four chiral pairs of FITC-labeled amino acids and Gly. The inset plots the inverse relationship between chiral resolution of each pair of amino acid enantiomers and temperature. The pH 10.0 run buffer consisted of 10 mM carbonate, 12 mM SDS, and 5 mM γ-CD. The separation distance using the folded channel design was 19.0 cm; the applied injection and separation voltages are given in Figure 1.

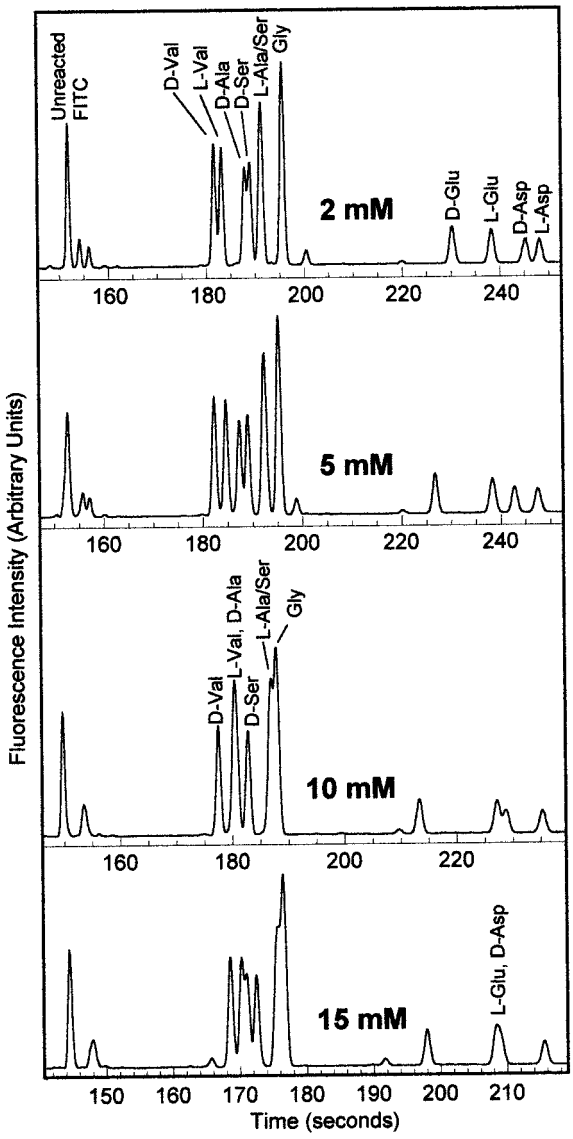

Figure 3. Electropherograms demonstrating the effect of γ-CD concentration on the enantioselectivity of five chiral pairs of FITC-labeled amino acids and Gly. Temperature was 10 °C.

resolution of all amino acid enantiomers and results in near-baseline resolution of D-Ala and D-Ser at the lowest temperature, but the L-forms of Ala and Ser continue to coelute. Although 5 °C gave the best enantiomeric resolution, we performed subsequent experiments at 10 °C because of excessive condensation and consequent arcing from the cathode electrode in the 5 °C separations.

The concentration of CD in the electrophoresis buffer is also important in achieving optimal chiral resolution.[33] In Figure 3, a γ-CD concentration of 2 mM results in baseline resolution of all amino acid enantiomers except D- and L-Val; a slight splitting of D-Ala and D-Ser is also observed. Increasing the concentration to 5 mM improves the enantiomic resolution of all the chiral amino acids. Note in particular that D-Ala and D-Ser are well resolved. Increasing the γ-CD concentration further to 10 mM improves resolution between each pair of amino acid enantiomers, but the coelutions of L-Val with D-Ala, of L-Ala and L-Ser with Gly, and of L-Glu with D-Asp make this concentration undesirable. γ-CD concentrations of 15 mM and greater produce separations with poor enantiomeric resolution. Because of the excellent separation provided by 5 mM γ-CD, we performed subsequent electrophoretic runs at this concentration.

The coelution of L-Ala with L-Ser in our electropherograms under all conditions made it difficult to determine accurate enantiomeric ratios for these two amino acids. Therefore, a periodate treatment was employed to selectively remove D- and L-Ser. Complete removal of the Ser peaks by this method is shown in Figure 4, while all other amino acids remain unaffected. This procedure allows us to determine the enantiomeric ratios for both

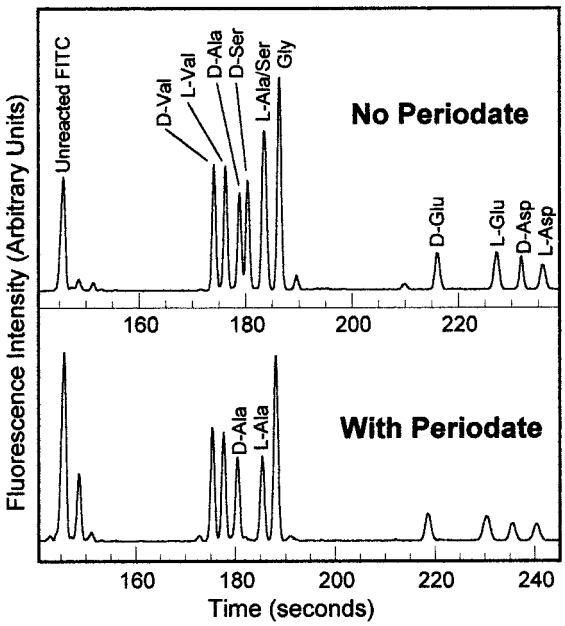

Figure 4. Electropherograms of the amino acid standard separations without and with periodate treatment to remove the D- and L-Ser peaks. Temperature was 10 °C, and the γ-CD concentration was 5 mM.

Ala and Ser, although two separate runs of treated and untreated samples are required.

We then evaluated the accuracy and precision with which enantiomeric ratios could be determined. Standards were separated that contained known percentages of the L-form (ranging from 30 to 90%) for each chiral pair of amino acids. From the electrophoretic data, we subtracted best-fit baselines and determined peak parameters from fitted Gaussians. The percentages of the L-form were calculated from peak areas for each enantiomeric pair and plotted against the known percentages of the L-form in the standards. Linear regressions from such plots (data not shown) had R^2 values of >0.999, slope values ranging from 0.94 to 1.02, and *y*-intercept values ranging from 4.6 to −3.9%. Relative standard deviations for each experimental L-percentage were less than 3% ($n = 4$). These results demonstrate the analytical feasibility of quantifying enantiomeric ratios for all of the chiral amino acids tested using the microplate CE method.

Previously, enantiomeric separations of FITC-labeled Val and Ser have been demonstrated in under 14 min using a cyclodextrin buffer additive and MEKC on conventional capillaries.[32] Here, we show that these separation methods can be transitioned to microfabricated CE chips, and we demonstrate the first chiral analyses on microfabricated devices. When 10 mM γ-CD is included in the run buffer at 10 °C (Figure 3), superior chiral resolution of the FITC-labeled Ser and Val enantiomers is achieved over conventional CE. Also, the separation of these four analytes is complete in under 200 s, 4 times faster than conventional CE systems.

As a further test of our system, chiral amino acid separations were performed on hot water extracts taken from interior and exterior samples of the Murchison meteorite (Figure 5). In the exterior sample, all of the protein amino acids present in the standard are identifiable and well resolved from one another. Interestingly, we also observe new peaks that coelute with the protein amino acids containing neutral side groups. For instance, isovaline (Iva) enantiomers comigrate with D- and L-Val, while α-aminoisobutyric acid (AIB) coelutes with D-Ala. Numerous other peaks of unknown identity also interfere, making it difficult to identify peaks and to quantitate enantiomeric ratios in this region of the electropherogram accurately. Removal of Ser by the periodate treatment did not significantly improve these coelution problems. However, in the acidic amino acid area, there are no obvious peaks interfering with Glu and Asp for which enantiomeric ratios were determined (Table 1). In the interior Murchison sample, fewer interfering peaks are present in the neutral amino acid region, but coelutions of these peaks with the Val, Ala, and Ser enantiomers still prevented the accurate determination of enantiomeric ratios for these amino acids. The acidic area was free of observable peak interferences and was thus used to determine enantiomeric ratios for Glu and Asp.

Table 1 presents the D/L ratios calculated for Glu and Asp for the exterior and interior Murchison samples and compares our values to other measurements on the same Murchison meteorite sample. Using the Glu peaks, we determined average ($n = 3$) D/L ratios of 0.33 ± 0.04 and 0.65 ± 0.07 for the exterior and interior samples, respectively. Using the Asp peaks, the D/L values were 0.21 ± 0.03 and 0.30 ± 0.06 for the exterior and interior samples. For both amino acids, there are significant excesses of the L-form, although the enantiomeric ratios shift toward racemic values in the interior samples. HPLC analyses are in agreement with our microchip CE values within the error limits. GC/MS analyses performed more than 25 years ago on the same interior Murchison sample examined here[28,29] show similar D/L ratios for Glu, but the D/L ratio for Asp is more than twice our value. The results presented in Table 1 may be affected by terrestrial contamination. The more racemic values found in the interior versus the exterior samples support the hypothesis that there was contamination by terrestrial L-amino acids after the meteorite's fall to Earth in 1969.[34] Thus, the discrepancy between our D/L ratios for Asp in the interior compared with the 1971 GC/MS analyses of the Murchison samples[29] can be explained.

The main motivation for our examination of the Murchison meteorite was to see if our D/L ratios agree with available values that were determined by other established chiral separation methods. The best comparison is with the HPLC results since these analyses were performed on the same amino acid extracts. It is satisfying that our D/L ratios for both Asp and Glu match within the error estimates. There is also acceptable agreement with previous GC/MS analyses for Glu. This overall agreement establishes the feasibility of using microfabricated CE devices for amino acid chirality analysis on extraterrestrial samples.

The data in Figure 5 demonstrate chiral separation of FITC-derivatized amino acids extracted from the Murchison meteorite with enantiomeric resolution comparable to HPLC and GC/MS methods.[1,28−31] Although we were unable to determine enantiomeric ratios for Ala, Ser, and Val because of interfering peaks, manipulation of the type and concentration of micelles included in the run buffer should help resolve some of the coelutions. More detailed peak-fitting procedures could also be used to deconvolute overlapping peaks. Another important consideration is that the

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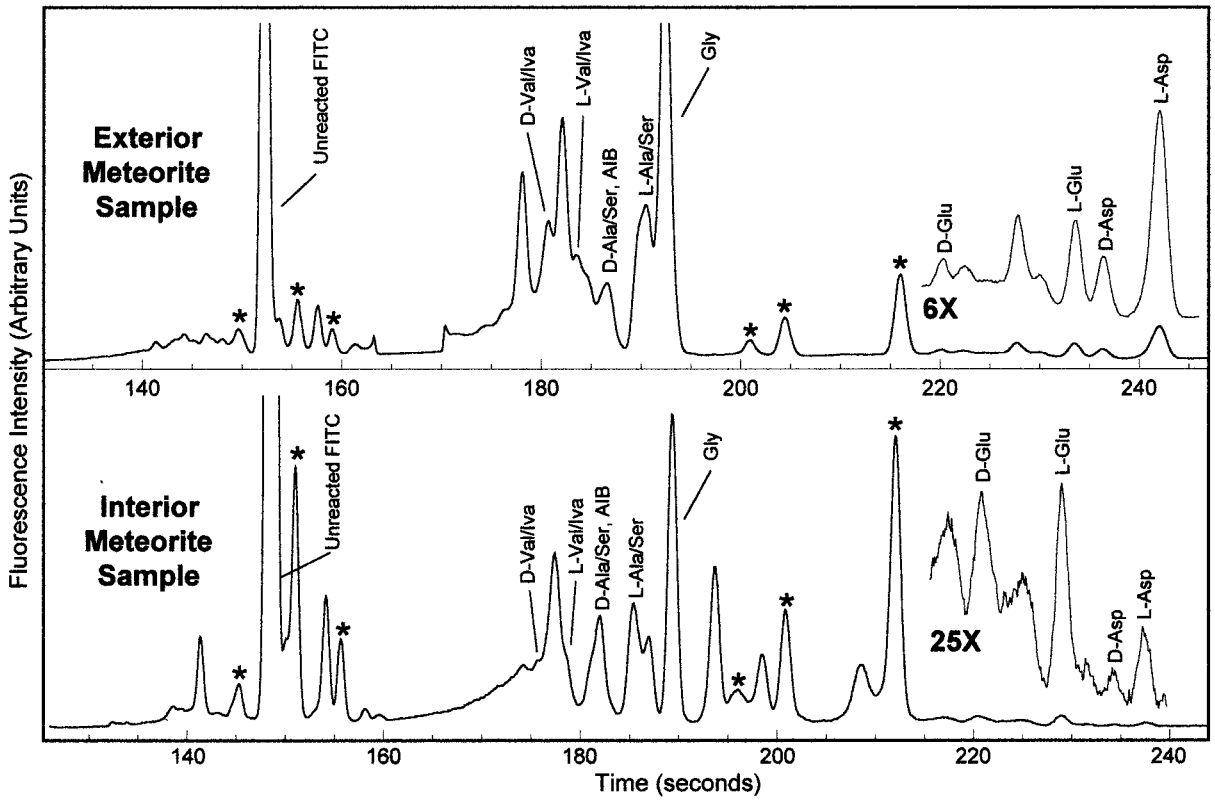

Figure 5. Electropherograms of FITC-labeled amino acid extracts of samples taken from the interior and exterior of the Murchison meteorite. The labeled bands were identified by co-injection of amino acid standards (all protein amino acid enantiomers) or by retention times (Gly, AIB, and D/L Iva). The insets present magnifications of the acidic regions of the electropherograms. Peaks that were present in the derivatized blank sample have been marked by an *. Separation conditions are identical to those in Figure 4; the periodate reaction was not performed on the extracts for these electropherograms. (AIB = α-aminoisobutyric acid; Iva = isovaline).

Table 1. Murchison Meteorite Asp and Glu D/L Values

amino acid	microchip CE[a]	HPLC[b]	GC/MS[c]
Glu			
exterior	0.33 ± 0.04	0.3 ± 0.1	na
interior	0.65 ± 0.07	0.7 ± 0.1	0.8
Asp			
exterior	0.21 ± 0.03	0.3 ± 0.1	na
interior	0.30 ± 0.06	0.3 ± 0.1	0.7−0.8

[a] D/L ratios were calculated by subtracting best-fit baselines from the electropherograms, determining peak parameters from fitted Gaussians, and taking the ratio of the area of the D-form to that of the L-form. Averages listed are from at least three replicate FITC derivatization reactions. [b] HPLC values from the Scripps Institution of Oceanography and confirmed by G. D. McDonald and K. L. F. Brinton at the Cal Tech Jet Propulsion Laboratory (personal communication). Chromatograms of Murchison amino acid extracts as well as details of the separation conditions have been published previously.[30,31] [c] GC/MS values for the same interior Murchison sample.[28,29] The enantiomeric ratio for Asp was estimated from GC peak heights.[29] Experimental error was not determined. na, not available.

FITC reagent used in the chip-based separations reacted with both primary and secondary amines, thus creating a more complex mixture of detectable analytes than the OPA/NAC derivatization method utilized by the HPLC analyses, which labeled only primary amines. Use of a fluorescent label that derivatizes only primary amines should help eliminate interfering peaks.

On the other hand, chip-based amino acid analyzers have a number of fundamental advantages compared with traditional methods. The speed of chiral analyses using the microfabricated CE system is ∼8 times faster than the HPLC analyses and 20 times faster than the GC/MS analyses conducted in 1971. Such fast separation times are an inherent advantage for extraterrestrial, in situ measurements.[1] Furthermore, while the HPLC method required the injection of 50 microliters on column, our CE chip device has an injected volume of ∼100 pL, and only a few μL are typically required for loading. The ability to work with smaller volumes is another important advantage for the development of in situ space probes.

CONCLUSION

Although the feasibility of our prototype microfabricated amino acid analysis system has been established, the development of a complete lab-on-a-chip device that is compatible with current Mars probe configurations for in situ analyses will be a significant challenge. Such a device will be required to perform amino acid extractions from extraterrestrial samples, purification of the extracts, derivatization, chiral separation, and detection of the amino acids. A first important step is the miniaturization of the detection system to a wafer-scale device. The use of fluorescent labels such as OPA and naphthalene-2,3-dicarboxaldehyde[35,36] that can be excited by available doubled diodes and that react quickly with amino acids is one possible step. Another exciting possibility is the use of electrochemical detection. It has recently been shown

(35) Seiler, N. In *Handbook of Derivatives for Chromatography*; Blau, K., Halket, J. M., Eds.; John Wiley & Sons: Chichester, 1993; p 175.
(36) de Montigny, P.; Stobaugh, J. F.; Givens, R. S.; Carlson, R. G.; Srinivasachar, K.; Sternson, L. A.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1096−1101.

that electrochemical detectors can be integrated into CE wafers and used for sensitive neurotransmitter and nucleic acid detection.[37] An additional challenge will be the development of an integrated microfluidic system for extraction and derivatization of the amino acids. The current rapid advancement of lab-on-a-chip technology makes us optimistic about this challenging task.[4] The analyses performed on such devices need not be limited to amino acids, but could be expanded to include other organic molecules that have been analyzed by CE techniques, such as polycyclic aromatic hydrocarbons,[38] pyrimidines, and purines.[39] Because of their extreme miniaturization potential, microfabricated CE devices would be well-suited for in situ probes on Mars or other celestial bodies such as Europa, comets and asteroids where sample return missions are less feasible and where organic analyses would be fascinating.

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