Mars Organic Detector III: A Versatile Instrument for Detection of Bio-organic Signatures on Mars

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ABSTRACT

Recent advances in the development of microfabricated lab-on-a-chip analysis systems have enhanced the feasibility and capabilities of *in situ* chemical and biochemical analyzers. While a wide variety of bio-organic molecules can be probed, we have focused our initial studies on the development of an amino acid analyzer with the hypothesis that extraterrestrial life would be based on homochiral amino acid polymers. In previous work, we developed a prototype electrophoresis chip, detection system and analysis method where the hydrolyzed amino acids were labeled with fluorescein and then analyzed in minutes via a capillary zone electrophoresis (CZE) separation in the presence of γ-cyclodextrin as the chiral recognition agent.[1] In more recent work, we have demonstrated the feasibility of performing amino acid composition and chirality analyses using fluorescamine as the labeling reagent. Fluorescamine is advantageous because it reacts more rapidly with amino acids, has a low fluorescence background and because such a chemistry would interface directly with the Mars Organic Detector (MOD-I) concept being developed at Scripps. A more advanced analysis system called MOD-III is introduced here with the ability to analyze zwitterionic amino acids, nucleobases, sugars, and organic acids and bases using novel capture matrix chemistries. MOD-III, which is enabled by the nanoliter valves, pumps and reactors presented here, will provide a wide spectrum of organic chemical analyses and is suitable for a variety of *in situ* missions.

Keywords: Mars organic detector, microfabricated chemical analyzer, PDMS membrane valves, microfabricated pumps, amino acid analysis

1. INTRODUCTION

The question of the existence of life on Mars is a central focus of many of the space missions planned by NASA and other space agencies. When the next generation of landers are sent to Mars, one of the main objectives will be to search for signs of extant or extinct life. What chemical measurement is best indicative of life when the nature of that life form is unknown? A reasonable hypothesis is that life would exploit homochiral molecules, such as amino acids, to produce well-defined 3-dimensional polymeric structures capable of enzymatic-like functions.[2] The detection of these macromolecules would therefore be indicative of life. In the case of extinct life, macromolecules such as DNA and proteins are likely to have been degraded; however, amino acids would still be present in a non-racemic ratio due to their low isomerization rates.[3,4] Martian meteorites (such as ALH84001) and other meteorites (such as Murchison) have been thoroughly studied and shown to contain a variety of organic molecules including amino acids and sugars.[5,6,7,8] However, the observed amino acid composition and chirality are best explained by a mixture of racemic abiotic amino acids contaminated by terrestrial sources. In future work it will be important to perform *in situ* analyses to avoid these contamination ambiguities and to broaden our hypothesis to include the analysis of other biomolecules, such as sugars, nucleobases, and organic acids and bases to provide a more thorough biochemical investigation of Mars.

The challenge for *in situ* analysis is the development of a compact device capable of delivering significant analysis capabilities. Our proposal utilizes powerful lab-on-a-chip technologies to create a microfabricated analysis device that will sample, concentrate, and analyze multiple classes of biomolecules. The MOD-III concept (see Figure 1) can accept samples from a variety of sources such as a sublimation cold finger (MOD-I), an aqueous extraction system, or melt water. The fluid is moved within the chip using microfabricated pumps to capture chambers that contain affinity matrices specific to the classes of biomolecules being analyzed. The target molecules are captured, concentrated and purified followed by release for analysis. Here we present the MOD-III concept in more detail and introduce technologies that establish the feasibility of the MOD-III analyzer.

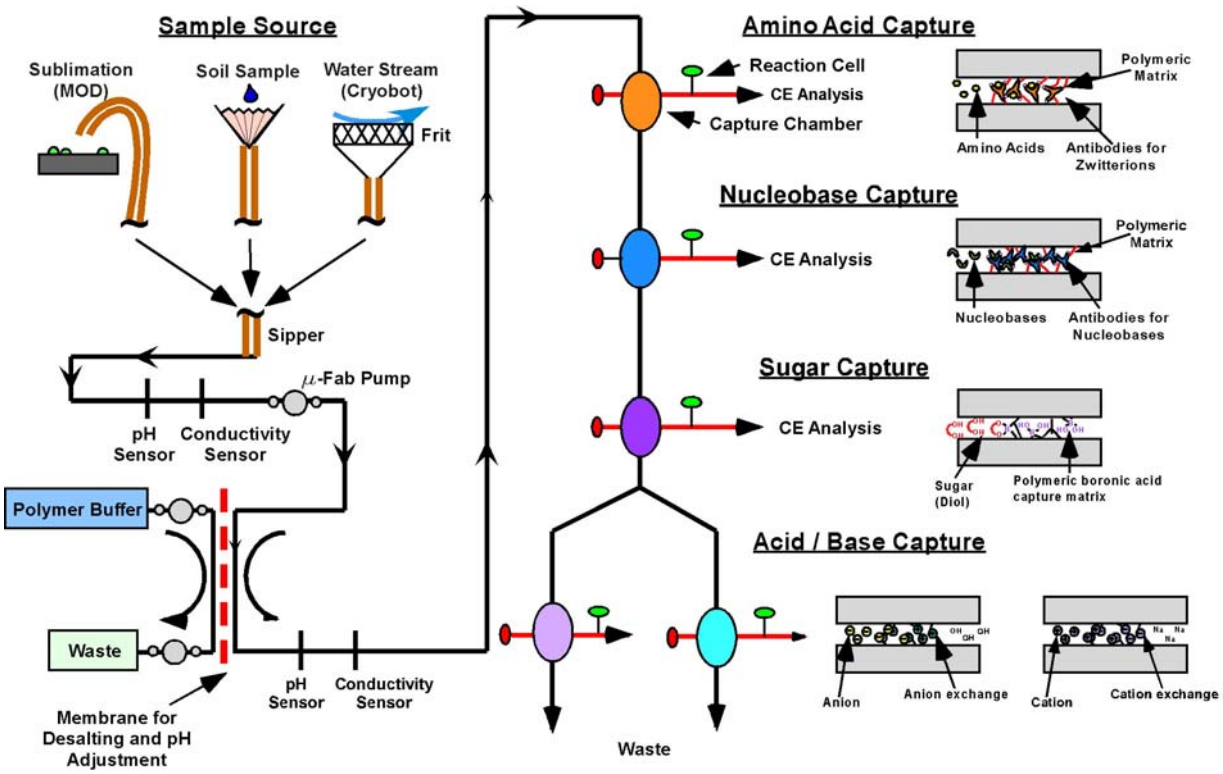

Figure 1. Schematic of the MOD-III chemical analyzer. Aqueous sample is obtained, pH and conductivity adjusted, and pumped through a system of affinity capture matrices by microfabricated pumps. After capture/concentration/purification, each sample class is released and sent to a CE analysis device for a second dimension of molecular characterization.

2. TECHNOLOGY DEVELOPMENT

2.1 Photolithography and chip fabrication

The separation channels (19 cm long, 150 μm wide and 20 μm deep) used here for chemical analysis are microfabricated in 10-cm diameter glass wafers by standard photolithographic techniques.[9] A 70 μm wide, 1 cm long injection channel crossing the separation channel allows for precise control of sample plug dimensions. Four electrodes are used; one at each end of the separation and sample injection channels. During the injection stage, electro-osmotic flow (EOF) drives the sample from the sample well across to the waste well, filling the intersection. During the run stage, the EOF is directed down the separation channel, and the sample contained at the intersection is swept along and analyzed by CZE. The sample plug is well defined by the dimensions of the cross, and excellent resolution can be achieved in seconds. In addition, multiple separation channels can be microfabricated on one chip to create high-density arrays of channels, allowing simultaneous analysis of several samples or multiple analyses on the same sample.[10]

2.2 Microfabricated electrodes and heaters

During the fabrication process, metal can be put on the chip through Low Pressure Chemical Vapor Deposition (LPCVD) to create monolithic electrodes, heaters and temperature sensors. Such heaters and temperature sensors have been used to control the temperature in nL-volume reaction chambers for applications such as PCR.[11] Also, integrated metal electrodes are fabricated for use in controlling the electrophoretic injection and separation processes. The practical utility of the metal electrodes and integrated heaters has been demonstrated by performing on-chip PCR followed by separation and analysis of the amplified sample. This technology is sensitive enough for single-molecule amplification and detection.[12]

2.3 Capture chambers

Capture chambers are essential for performing chemical analysis in the MOD-III device. The capture chamber selectively binds the target molecules as they enter the chamber, while the other molecules and contaminants pass through. Sampling can be performed over an extended period of time so that the target molecules are highly concentrated (1000-fold or more) from a potentially large but dilute sample volume. Release of the captured materials is accomplished by heat or by eluting with an appropriate buffer, resulting in a concentrated and purified sample that is ready for subsequent chemical processing and electrophoretic analysis.

The feasibility of this affinity capture principle has recently been demonstrated through purification of DNA sequencing reaction products. The sample, containing polymerase reagents and primers in a high salt concentration solution is introduced onto the chip directly after thermal cycling. Using an electric field, the sample is driven through a 60 nL capture chamber containing an immobilized acrylamide matrix decorated with an oligonucleotide probe. The product DNA is captured in the matrix, while the PCR primers and high salt solution pass through. After capture, the chamber is heated to melt the DNA duplex and release the sample, which is then driven out of the chamber and into the separation channel. This method has been shown to clean the sample in 120 seconds, resulting in better separations while concentrating the sample by a factor of 200.[13]

Similar matrices have been developed for immobilizing antibodies and ion-exchange molecules in a polymer monolith within a capillary.[14] These matrices are robust, and the matrix format allows superior activity over surface immobilized and bead-immobilized techniques due to higher mass transfer and capture matrix activity. In addition, varying the contents of the polymerization mixture can easily control the pore size of the matrix.

2.4 Microfluidic valves and pumps

In order to move sample fluid in the chip, microfluidic valves and pumps have been developed (Figure 2). These valves are made by etching a discontinuous channel in the top glass fluid layer, covering it with a PDMS membrane, and then sealing the other side of the PDMS membrane with the manifold layer containing a large chamber which sits directly opposite the channel discontinuity. The result is a three-layer glass/PDMS/glass structure. When a vacuum is applied within the etched chamber, the PDMS membrane deflects downward into the manifold layer, and the valve is opened. When the vacuum is released, or a slight manifold pressure applied, the PDMS membrane returns to its original position, and the valve is sealed. The four-layer valve functions in the same manner; the fluid layer with a discontinuous

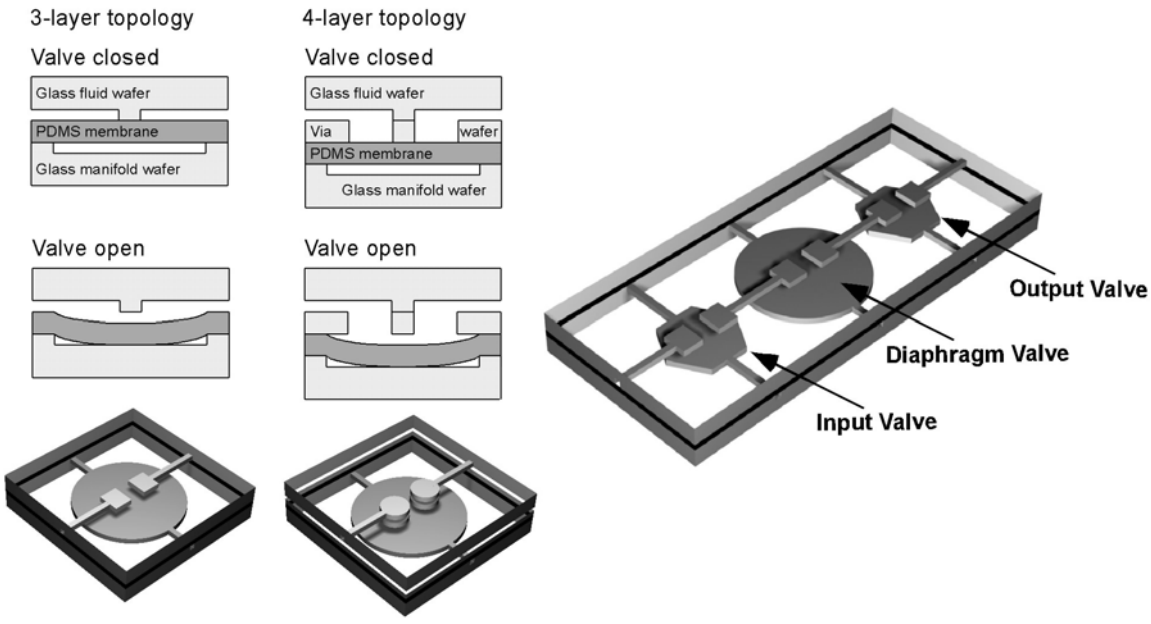

Figure 2. Microfabricated valves and pumps formed by binding an etched fluid layer, a PDMS membrane, and an etched manifold layer. The 3-layer valve structure requires no thermal bond. The four-layer structure is formed by thermally bonding a glass fluid wafer with a via wafer before assembling with the PDMS and manifold wafers. Adapted from ref. 15.

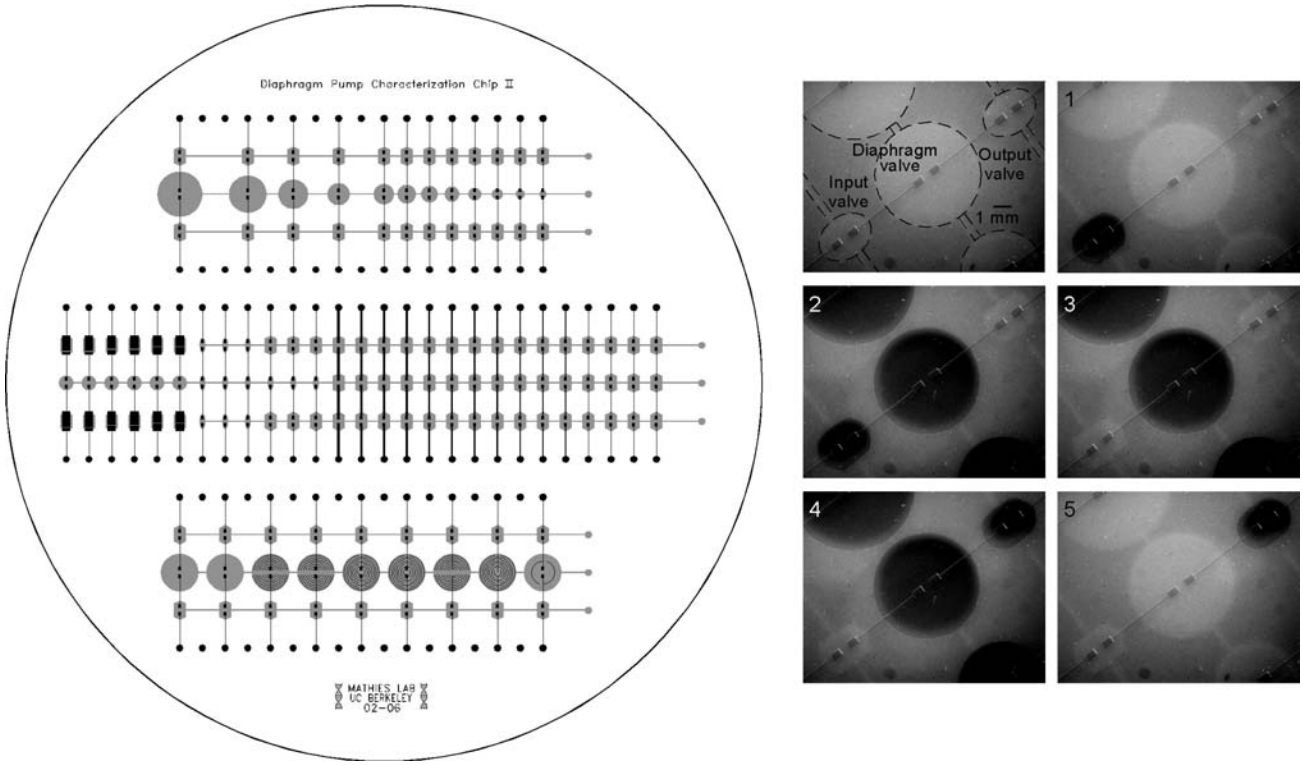

Figure 3. Pump test chip and illustration of pumping program: (1) input valve is opened (output valve is closed), (2) diaphragm valve is opened, (3) input valve is closed, (4) output valve is opened, (5) diaphragm valve is closed. Adapted from ref. 15.

channel is etched in the top glass layer, then thermally bonded to a glass plate containing drilled vias. This 2-layer glass structure is then sealed with the PDMS membrane and a manifold layer to create a valve, with the advantage of having a fluid channel completely enclosed in glass.

When three valves are placed in series, a microfabricated pump is created consisting of an input, output, and diaphragm valve, each independently addressable in the manifold (Figure 3). The input and output valves seal the fluid channel in one direction while the diaphragm valve is actuating, determining the direction of flow through the pump. The pump functions through a five-step program, as shown in Figure 3. In the first step, the input valve is opened, opening up the channel to the sample source. In the second step, the diaphragm valve is opened, drawing in fluid from the sample. In the third step, the input valve is closed, and the source is sealed off. The volume pumped in this actuation cycle is now completely contained within the open diaphragm valve. In the fourth step, the output valve is opened, and the fifth step, the diaphragm valve is closed, expelling the fluid through the open output valve. The pump design can move fluid in either direction by switching the actuation order of the input and output valves.

The design of this pump is advantageous because the pump does not need to be primed. The valves can also seal against pressures higher than that pressurizing the manifold, and the pump can pump against a pressure head equivalent to the manifold pressure. In addition, manifolds can be designed such that multiple pumps can actuate in parallel, as is demonstrated by the pump test chip shown in Figure 3. This chip contains 144 valves, 58 pumps, and requires only 9 pneumatic controls to actuate all valves. The flow rates of the pumps shown are dependent upon the volume of the diaphragm valve and the cross-sectional area of the fluid channel. The microfabricated valves can pump from 10 nL to 10 μL per actuation, and the flow rates can range from 1 nL/sec to 300 nL/sec.[15]

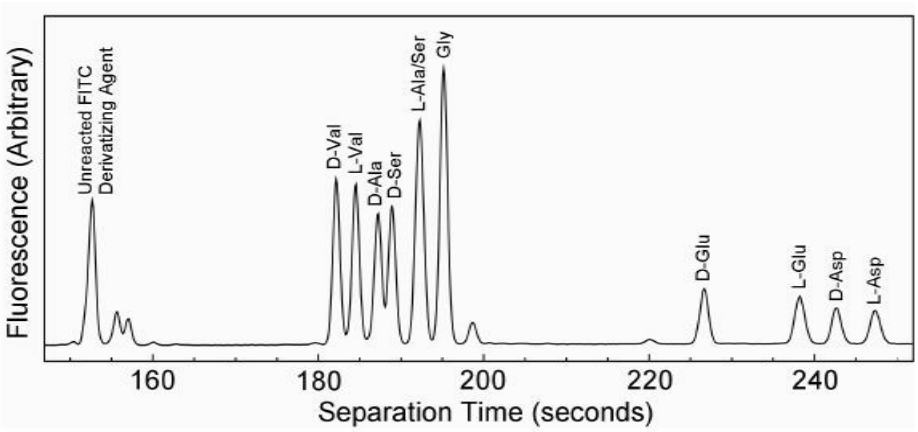

Figure 4. Chiral separation of fluorescein-labeled amino acids on a CE microchip. Run conditions: sodium dodecyl sulfate/ γ-cyclodextrin, pH 10.0, carbonate electrophoresis buffer, separation voltage of 550 V/cm at 10° C. From ref. 1.

3. AMINO ACID ANALYSIS

3.1 Amino acid composition and chirality determination

Laser-induced fluorescence provides a sensitive means of amino acid detection in a chip-based amino acid analyzer. The amino acids are labeled with a fluorescent dye and the sample is run through a capillary and separated based on charge/size ratio using a CZE process. We have previously demonstrated the separation of a Mars 7 standard containing valine, alanine, serine, glycine, aminoisobutyric acid, glutamic acid, and aspartic acid on a microfabricated glass chip.[1] To perform a chiral separation for enantiomeric analysis, a chiral recognition agent such as γ-cyclodextrin was included in the running buffer. The amino acids complex with the cyclodextrin by entering the hydrophobic core, thereby altering their mobilities. Enantiomeric resolution is obtained by optimizing the cyclodextrin cavity size and concentration such that one enantiomer will preferentially complex with the cyclodextrin, and will have a different net mobility. The chiral separation and enantiomeric resolution of the Mars 7 standard, using γ-cyclodextrin, is shown in Figure 4. This labeling technique was used to analyze an extract the Murchison meteorite, and the results obtained gave equivalent, if not better, accuracy than those obtained by HPLC.[1]

3.2 Fluorescamine labeling

Although fluorescein labeling has been used to effectively analyze real samples, the labeling reaction is slow (8-12 hours) and unreacted fluorescent FITC complicates the electropherogram. In order to develop a procedure more suitable to high-throughput, *in situ* analysis, the fluorescamine dye system was investigated (Figure 5). Fluorescamine reacts quickly with amino acids (under 1 minute) and is otherwise non-fluorescent. Excess reagent is hydrolyzed by water to yield a non-fluorescent product. In addition, fluorescamine absorbs maximally at 404 nm, which is compatible with a compact blue diode excitation source. Finally, since the MOD-I concept is based on sublimation of amino acids onto a fluorescamine-coated cold finger for labeling, this protocol can be directly interfaced with MOD-I.[16]

The separation of fluorescamine-labeled amino acids was performed using the same device used for fluorescein-labeled amino acids. Figure 6A presents the separation of the Mars 7 standard, 4 neutral and 2 acidic amino acids that are resolved in 180 seconds. Figure 6C presents the separation of arginine and phenylalanine, a basic and an aromatic amino acid, in less than 110 seconds. The amino acids were labeled in 1 minute, and the separations (including injection) were performed in less than 250 seconds. The elution order is identical to that found with fluorescein-labeled amino acids, and the detection limit for this label system is in the pico-molar range.[17]

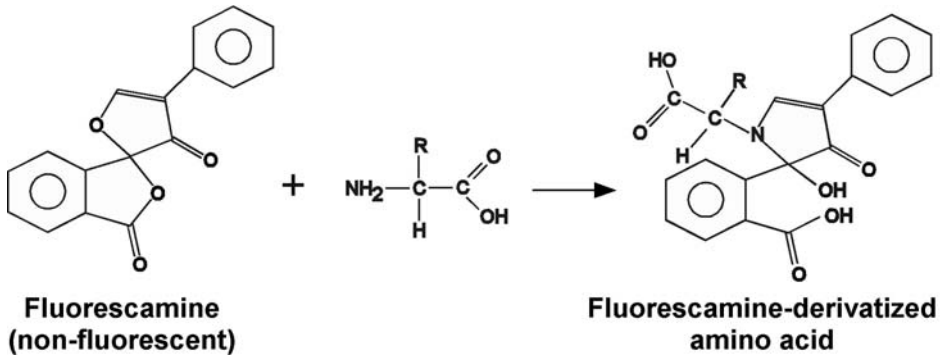

Figure 5. Reaction of fluorescamine with an amino acid. Dye is dissolved in acetone (1 mM), added to a reaction mixture containing amino acid and 50 mM borate buffer, pH 10.0.

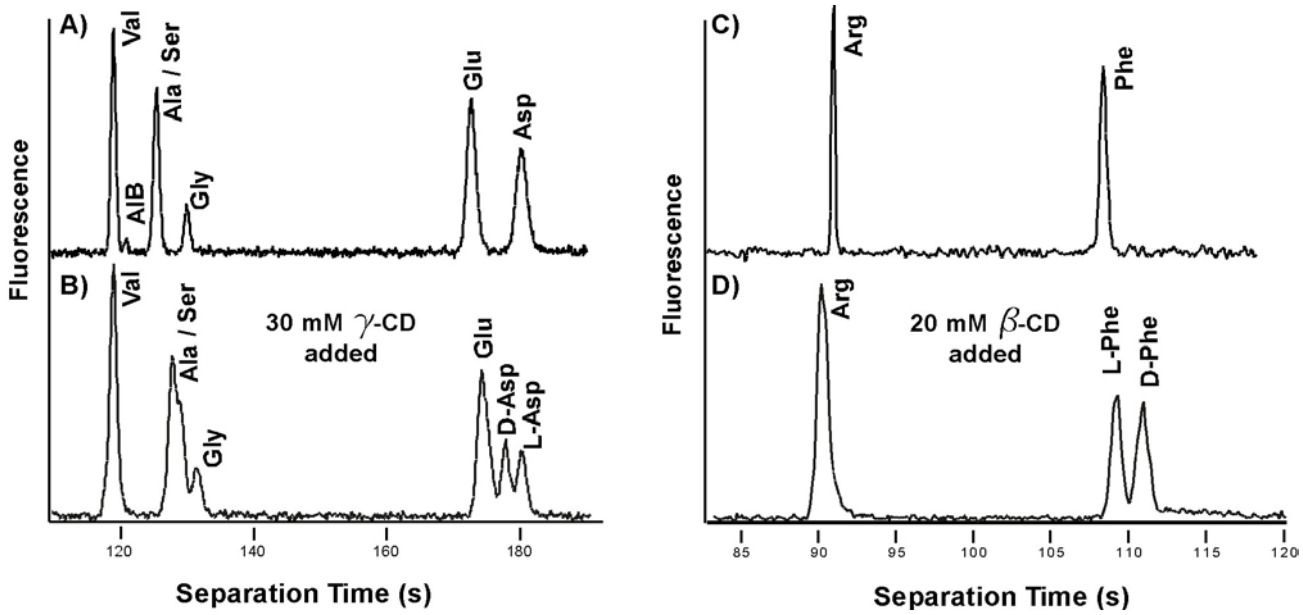

Figure 6. Separations of fluorescamine-derivatized amino acids. A) Separation of Mars 7 standard with 10 mM CO_3^{2-}, pH 9.0 and B) with γ-cyclodextrin added to running buffer. C) Separation of arginine and phenylalanine with 10 mM CO_3^{2-}, pH 9.0 and D) with β-cyclodextrin added to running buffer.

The chiral separation of fluorescamine-labeled amino acids has also been demonstrated. Figure 6B presents the chiral separation of the Mars 7 standard in γ-cyclodextrin. Figure 6D presents the chiral separation of arginine and phenylalanine in β-cyclodextrin. The amino acids are resolved in less than 200 seconds. By including γ-cyclodextrin in the running buffer, the enantiomers of aspartic acid are resolved, and by including β-cyclodextrin in the buffer, the enantiomers of phenylalanine are resolved. Chiral separations have also been demonstrated in α-cyclodextrin. The further optimization of the reagents and conditions with mixtures of cyclodextrins to obtain full chiral resolution is in progress. Nevertheless the combination of the separation capabilities demonstrated here and microfabrication technologies discussed above provide the basis for the MOD-III design.

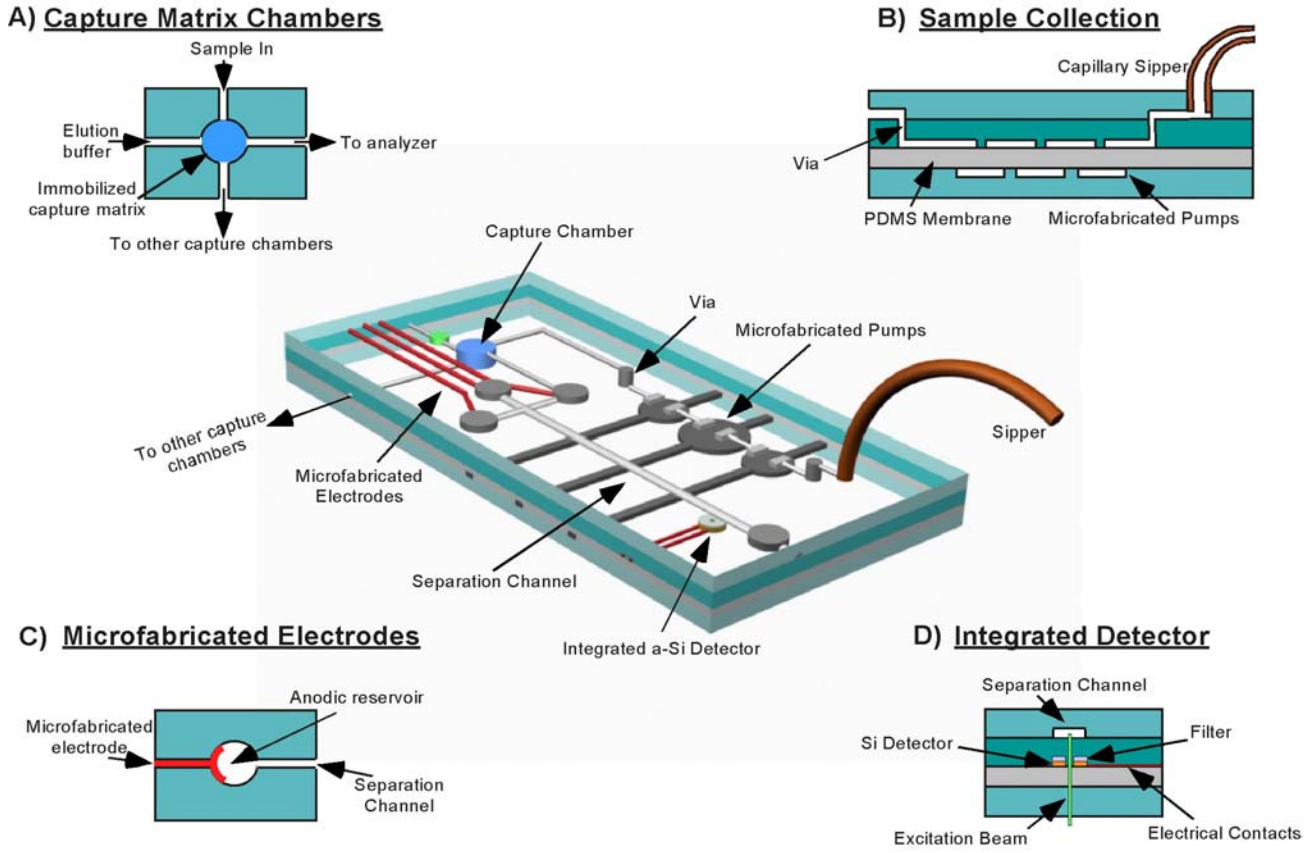

Figure 7. Design of the MOD III analyzer. Sample is collected by sipper (A), pumped to capture chamber (B) for concentration and purification and then released to the separation channel for analysis. The electric field is supplied by microfabricated electrodes (C), and the separation is detected by an integrated amorphous Si:H PIN photodiode (D).[24]

4. The MOD-III Analyzer

The MOD III analyzer shown in Figure 7 draws upon all of the technologies we have developed. It contains a sipper for sampling, microfabricated pumps to drive fluid flow, capture chambers for isolation and concentration of different bio-organic molecules, and separation channels for analysis. The design also includes integrated electrodes and detectors. In order to combine all of these microfabricated elements, the device consists of 4 layers. The top 2 layers are thermally bonded glass in which the separation channels and capture chambers are fabricated. Beneath the two glass plates is a PDMS membrane, and then finally the manifold layer is sealed to the bottom. The last layers only require manual assembly as the PDMS membrane holds the glass-PDMS-glass sandwich together. The fluid is drawn in, travels through a via to the bottom layers where it is pumped forward, and then travels back up to the separation layer for capture and analysis. This multi-layer design allows for a chemically compatible analysis layer composed entirely of glass with the pumping layers on the bottom for on-chip microfluidic control.

The MOD-III design contains capture chambers specific to five main classes of bio-organic molecules: amino acids, nucleobases, sugars, and organic acids and bases. The capture chamber is located between the top two glass plates of the MOD-III device and is filled with an organic macroporous polymeric matrix. The monomer building blocks can be hydrophobic or hydrophilic in nature, such as styrene and acrylamide respectively, and can contain reactive groups for further chemical modification or grafting once the monolith is polymerized.[14] The pore size can be controlled from 10 to 1000 nm, and the surface area can be up to 300 m^2/g.[18]

The capture of the five classes of organic molecules is done sequentially, as shown in Figure 1. Amino acids, then nucleobases are first removed from the sample stream. Amino acid and nucleobase capture is performed by

immobilizing zwitterionic- and nucleobase-specific antibodies within two different polymeric matrices. Protein immobilization within the matrix on poly(glycidyl methacrylate-co-ethylene dimethacrylate) has been demonstrated with trypsin, and was found superior over immobilization onto macroporous beads.[19] High mass transfer rates are achievable, and protein activity is maintained at high flow rates. Release of the target molecules is performed by altering the binding equilibrium through heating, pH or salt alterations to increase the off-rate from the antibody, together with pumping or electrophoresis to sweep the target molecules from the capture matrix.

Following amino acid and nucleobase capture, sugars are removed from the sample stream. Sugars (diols) are captured by a boronic acid capture matrix. The boronic acid group shown in Figure 1 reacts reversibly with a diol to form a cyclic boronate ester structure, immobilizing the sugar within the matrix and releasing water.[20] The sugar is released from the capture matrix for analysis by lowering the pH.

After sugar removal, the sample stream splits so that organic acid and base capture can occur in parallel. Acid capture is performed by an anion exchanger immobilized within the monolith, formed by reacting a glycidyl methacrylate monolith with diethylamine, leading to 3-diethylamino-2-hydroxy-propyl-functionalized porous monolith.[21] Base capture is performed by a cation exchanger, formed by grafting a poly(2-acrylamido-2-methyl-1-propanesulfonic acid) chain onto the glycidyl methacrylate monomer.[22]

Following capture and concentration, all target molecules are released from the chambers either by heat or by the use of an elution buffer, and sent to the CE system for secondary analyses (see Figure 7). Each molecular class is labeled, separated under optimal conditions and detected by an integrated detector. MOD-III can function in 2 ways: long-term capture in which large volumes are sent through the chambers before analysis, as would be necessary for low-concentration samples such as polar ice[23], or high-throughput in which multiple captures and analyses are performed as would be needed for profiling. In this manner MOD-III will be able to perform simultaneous and continuous separation, concentration, and analysis of five classes of bio-organic molecules.

5. CONCLUSIONS

The MOD III analysis system presented here utilizes existing proven chemical and microfabrication technologies to create a miniaturized analysis system capable of detecting 5 different classes of bio-organic molecules. The device is compact enough to fit into any current lander designs, and the on-board components for sample capture, concentration and preparation allow this analysis device to interface with different sources and to analyze low concentration samples. Chiral amino acid analysis has already been performed on-chip, demonstrating a sensitive means of determining enantiomeric ratios. We thus have demonstrated the feasibility of our first generation life detection assay system. Integration of the capture and concentration chemistries discussed above will make MOD-III a compact, portable yet extremely powerful device for analyzing bio-organic molecules in a variety of extraterrestrial environments.

6. ACKNOWLEDGEMENTS

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