The non-destructive separation of diverse astrobiologically relevant organic molecules by customizable capillary zone electrophoresis and monolithic capillary electrochromatography

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Abstract

The *in situ* detection of organic molecules in space is key to understanding the variety and the distribution of the building blocks of life, and possibly the detection of extraterrestrial life itself. Gas chromatography mass spectrometry (GC-MS) has been the most sensitive analytical instrument for organic analyses in flight, and was used on missions from NASA's Viking, Phoenix, Curiosity missions to ESA's Rosetta space probe. While pyrolysis GC-MS revealed the first organics on Mars, this step alters or degrades certain fragile molecules that are excellent biosignatures including polypeptides, oligonucleotides and polysaccharides, rendering the intact precursors undetectable. We have identified a solution tailored to the detection of biopolymers and other biomarkers by the use of liquid-based capillary electrophoresis and electrochromatography. In this study, we show that a capillary electrochromatography (CEC) approach using monolithic stationary phases with tailor-made surface chemistry can separate and identify various polycyclic aromatic hydrocarbons (PAHs), nucleobases and aromatic acids that could be formed under astrophysically relevant conditions. In order to simulate flyby organic sample capture, we conducted hypervelocity impact experiments which consisted of accelerating peptide-soaked montmorillonite particles to a speed of 5.6 km/sec, and capturing them in an amorphous silica aerogel of 10 and 30 mg/cc bulk density. Bulk peptide extraction from aerogel followed by capillary zone electrophoresis led to the detection of only two stereoisomeric peptide peaks. The recovery rates of each step of the extraction procedure after the hypervelocity impact suggest that major peptide loss occurred during the impact. Our study provides initial exploration of feasibility of this approach for capturing intact peptides, and subsequently detecting candidate biomolecules during flight missions that would be missed by GC-MS alone. As the monolith-based electrochromatography technology could be customized to detect specific classes of compounds as well as miniaturized, these results demonstrate the potential of the instrumentation for future astrobiology-related spaceflight missions.

Introduction

There is arguably no more intriguing scientific question than whether we are the only life forms that have ever evolved. We know that some of the building blocks for life that led to the origin of life on Earth are still elsewhere, even in our own solar system. What about more complex molecules, such as polypeptides? And, if there are polymers present, are they homochiral as they are in terrestrial life? To answer these critical questions requires missions that deploy non-destructive analytical instrumentation beyond the current state-of-the-art for flight. We know that the Earth has been a home to chemical evolution and still is. These precursor organic materials were synthesized endogenously on the Earth, as well as likely delivered by extraterrestrial objects including carbonaceous meteorites, comets and interplanetary dust particles (1). Diverse organic molecules such as amino acids, alcohols, sugar, ketones, lipids, various hydrocarbons have been discovered from the direct analysis of carbonaceous meteorites (2-6), and through the observation of interstellar clouds using near infrared spectroscopy (7). Other weakly polar organic compounds such as aliphatic hydrocarbons and monocarboxylic acids also have been identified from carbonaceous meteorites. These findings show that extraterrestrial environments can produce complex molecules. Among these extraterrestrial organics, a family of hydrocarbon molecules known as polycyclic aromatic hydrocarbons (PAH) were found to be dominant and ubiquitous in the interstellar medium (ISM), contributing to the formation of small hydrocarbon radicals (8). Thus, PAHs are considered the major organic carbon supplier for Earth and other planetary bodies, although the direct contribution of aromatic structures to the origin of life is still under debate (9).

What molecules would astrobiologists ideally like to detect? Purines and pyrimidine bases are the essential units of nucleotides, molecules that form base pairs to store and copy genetic information to pass on to the next generation. Although a complete nucleotide molecule has not yet been found in any prebiotic context, several possible chemical pathways that lead to nucleotide formation are known (10,11). Biologically-common purines such as adenine, guanine, hypoxanthine, and xanthine have been reported from various carbonaceous meteorites along with nucleobase analogs that are rare on the Earth (12). The mechanism of purine formation has been predicted as the oligomerization of HCN or from other observed interstellar precursor molecules such as C3NH and HNCNH (13).

A major class of organic compounds fundamental to terrestrial life is the amino acids and their precursors. It has been proposed that the Strecker reaction can occur in the interstellar medium forming amino acids during the aqueous alteration of the parent body (14), suggesting that amino acids are found in space. Well over 80 different amino acids have been detected from various carbonaceous meteorites (15). Further, outer planet icy moons such as Europa and Enceladus are made of icy planetesimals initially produced in the solar nebula, and likely harbor amino acids and their precursors in their subsurface oceans (16). Amino acids have been long considered as one of the plausible molecular biosignatures for extraterrestrial life detection on Mars and other planetary bodies (17), however chemical studies of extraterrestrial matter have often been challenged due to the risk of terrestrial contamination. For example, several criteria including amino acid distribution, enantiomeric ratio and stable isotopic analysis have been suggested to assess the true origins of amino acids from a Murchison meteorite (18). The attempt to capture and return extraterrestrial organic materials, as a part of cometary dust grains to the earth was first achieved by the NASA Stardust spacecraft targeting the comet 81P/Wild 2 (19). The Stardust spacecraft carried an aerogel known as a low-density amorphous SiO2 to decelerate and capture particles intact. The idea of using aerogel for hypervelocity capture and recovery of particles such as interplanetary dust has been previously investigated from the 90's with early studies and development of techniques conducted in a laboratory set up as well as in space (20-28). The analysis of the small cometary dust particle by Stardust revealed signatures of diverse suites of cometary organic compounds including simplest form of amino acid glycine (29,30). Hence series of HIE have been conducted after Stardust mission focusing on the fate of organic molecules during and after the impact using aerogel as a capture media (31-35). However, aerogel capture is a complicated process and thus impact induced alteration and inclusion (trapped inside melted silica) of carbon-bearing molecules needs to be treated cautiously to access the original organic material.

One of the potential biosignature is peptides, which are the chains of amino acids linked by a peptide bond. Terrestrial life produces polypeptides that are homochiral, thus indicative of a biological synthesis on Earth. The exogenous abiotic synthesis of peptides is known by the detection of diglycine in Murchison meteorite, and irradiation of interstellar model ices have demonstrated free radical formation of several dipeptide and amino acids species upon irradiation (36). Impact synthesis of alanine tripeptides has been shown with no enantiomeric selection (37). Peptide serves as an attractive candidate to evaluate the ongoing state of abiotic chemical evolution or possible biological reaction in extraterrestrial context. However, unlike terrestrial polypeptides consist of homochiral L-amino acids, separation and characterization of numerous peptide stereoisomers remains technically challenging. For space mission instruments, gas chromatography combined with mass spectrometry (GC-MS) has a long and distinguished heritage as a flight instrument, and is still one the most sensitive bioanalytical instruments. GC-MS has played a major analytical role in space missions such as NASA's Viking, Phoenix, Curiosity missions to the martian surface and ESA's Rosetta/Philae mission to a comet nucleus. However, the pyrolytic procedure required in gas chromatography destroys thermally labile molecules including biologically significant polymers such as polypeptides, oligonucleotides and polysaccharides. Recently this became the major issue for detecting organics from the Martian soil, since chlorinated organics were detected through Curiosity's SAM instrument which are likely to be a derivative of organic molecules reacting with perchlorate inside the oven. Thus, the very polymers that are indicative of life are rendered undetectable with GC-MS (38).

Other analytical strategies proposed for space missions include biosensors (39) and antibody-based systems such as the Life Marker Chip (40). However, these approaches lack flexibility as the instruments must be built with specific molecular targets. Thus far, liquid-based separation techniques are the only flexible alternative to GC.

Electro-migration techniques are especially advantageous due to their unquestionable separation power, low consumption of solvents and ease of miniaturization. The Urey instrument, known as Mars Organic Analyzer (MOA), was the first fabricated microchip electrophoresis (µCE) system proposed for the first liquid based organic detection on Mars (41). Microchip electrophoresis using a combination of laser-induced fluorescence (LIF) with a fluorescent amine reactive probe can detect amino acids with minute concentration of sub-parts-per-trillion as well as aldehydes, ketones, organic acids and thiols (42). This system has now been adopted for exploring potential life in the Planetary In Situ Capillary Electrophoresis System (PISCES) (43,44), and on icy moons (45,46). The drawback is it lacks the capability to prove the structure and mass of detected peaks. The downstream addition of MS detection is the most convenient solution of this problem. However, the MS approach excludes the usage of surfactants like in the Urey prototype (41) while simple capillary zone electrophoresis (CZE) mode faces difficulty to separate neutral species and enantiomers that are potential biomarkers (47).

To overcome these difficulties, we have been developing a hybrid technique known as capillary electrochromatography (CEC), allowing a combination of electro-driven migration and chromatographic separation mechanism using newly developed monolithic stationary phases. Electrochromatography provides a chemical separation with a combination of chromatographic selectivity and electrophoretic mobility. The ability of the monolithic column to withstand radiation, and its ability to separate neutral, hydrophilic, hydrophobic and chiral molecules as well as amino acids and proteins, suggest a clear application for in situ analysis during the space flight missions (48-51). Here we demonstrate the utility of capillary electrochromatography through two case studies. First, we introduced the CEC technology to separate neutral and biologically relevant organic molecules to evaluate the performance of CEC for future biosignature detection in space. Second, we performed a diastereomeric separation of various tripeptides under CE with hypervelocity impact experiments (HIE) to explore the feasibility of capture and detection of peptides using ultra-low density aerogel. This report describes combinatorial technical basis for liquid-based separation and detection of various astrobiologically relevant organic compounds as well as assessment on post impact alteration of short peptides as a potential biosignature.

Materials and Methods

Synthesis of generic monolith P(EDMA-co-NAS).

A generic monolith was synthesized in a capillary tube via photo-triggered free radical polymerization of 200 mg N-acryloxysuccinimide (NAS) and 110 μL ethylene glycol dimethacrylate (EDMA) using (700 μL) dry toluene as porogen. Azobisisobutyronitrile (AIBN) was added to the monomer solution as initiator with a fraction of 1% in mass with respect to the total number of monomers. The polymerization mixture was sonicated for about 5 min at room temperature to obtain a homogeneous solution and then filtered through a polytetrafluoroethylene membrane (pore size = 0.45 μm). The filtered mixture was pushed through a UV-transparent capillary under a pressure of 3 bar by nitrogen. Prior to filling, the capillary was silanized to ensure the covalent attachment of the monolithic structure onto the inner capillary wall as previously reported (52). Both ends of the filled capillary were sealed with rubber septa. Then the capillary was exposed UV irradiation (365 nm, 6×15W) for 800 s to initiate the polymerization step. The monolith-containing capillary then was flushed extensively with acetonitrile (ACN) (2 μL/min, 1h) to remove the porogenic solvent. A fused silica capillary with a UV-transparent external coating (75 μm id × 325 μm od) from InnovaQuartz (Phoenix, ZA, USA) was used for capillary electrochromatography (CEC) experiments and polyimide-coated fused silica capillaries (50 μm id × 363 μm od) from Polymicro Technologies (Phoenix, AZ, USA) were used for capillary zone electrophoresis (CZE) experiments. A glass microfluidic 50 μm depth device was purchased from Trianja Company (USA). The inner wall of the glass chip channel was first flushed with 1.0 M NaOH for 30 min at room temperature. The micro-channel was then successively flushed with deionized water for 15 min and 0.1 M HCl for 30 min, and rinsed with deionized water for 10 min and then with acetone for 15 min. Thereafter, the glass chip channel was purged with dry nitrogen gas for 1 h at a temperature of 120 °C. (Trimethoxysilyl)propyl methacrylate 50% (v/v) solution in acetone was allowed to react overnight with silanols present in the inner wall of the glass chip channel at room temperature. Finally, the chip microchannel was rinsed with acetone for 15 min and dried under a stream of nitrogen for 1 h. The pretreated microchip was then filled under pressure (2 bars) with the polymerization mixture and the same polymerization conditions were used to prepare the monolith inside the glass chip microchannel.

Synthesis of phenyl-like monolithic stationary phase for reversed phase charge transfer CEC P(EDMA-co-NAS)-Phe2.

A solution of 3,3-diphenylpropylamine (1 M in CH2Cl2) was continuously (2 μL/min) pumped through the generic monolith column for 1 h at room temperature to produce monolith-bearing phenyl rings attached via an aliphatic arm (Figure 1). Subsequently, the monolithic column was washed with ACN (1 h, 2 μL/min) and kept at room temperature with sealed ends until used for the CEC.

Synthesis of amino-like monolithic stationary phase for hydrophilic interaction CEC P(EDMA-co-NAS)-NH2.

A solution of ethylenediamine (1 M in CH2Cl2) was pumped continuously (2 μL/min) through the generic monolith column for 1 h at room temperature to functionalize the surface of the monolithic stationary phase with hydrophilic amino groups (Figure 1). After functionalization, the monolith column was washed with acetonitrile (1 h, 2 μL/min) and kept at room temperature with sealed ends until use in the CEC.

Separation of extraterrestrial organic compounds using capillary electrochromatography.

All the electrochromatographic experiments were performed on a P/ACE MDQ (Beckman, Fullerton, CA, USA) equipped with 32 Karat software (version 4.0) for data acquisition. The analyte solutions (0.2 mg/mL) were introduced in the monolith capillary column by electrokinetic injection. The separation runs were carried out in reversed mode while the detection wavelength was set at 214 nm and the temperature of the cassette compartment was kept at 25 °C. A pressure of 3.5 bar was applied to both ends of the capillary to prevent bubble formation during CEC analysis. The columns length was 10 cm to detector, and 31 cm overall. Dimethylformamide (DMF) was selected as un-retained compound for electroosmotic flow (EOF) determination.

Tripeptides and montmorillonite rock sample preparation.

A total 17 tripeptides enantiomers consist of N- and C-terminal L-tyrosine with various canonical and non-canonical amino acid in the center (second residue), were purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA, USA) with >90% purity. Types of the peptides are as follows: L-Tyr-L-Val-L-Tyr, L-Tyr-ABA-L-Tyr, L-Tyr-NorV-L-Tyr, L-Tyr-L-Ala-L-Tyr, L-Tyr-IsoV-L-Tyr, L-Tyr-GABA-

L-Tyr and L-Tyr-βAla-L-Tyr, L-Tyr-L-Ser-L-Tyr, L-Tyr-AIB-L-Tyr, L-Tyr-D-Val-L-Tyr, L-Tyr-Gly-L-Tyr, L-Tyr-D-Ala-L-Tyr, L-Tyr-D-Ser-L-Tyr, L-Tyr-L-Glu-L-Tyr, L-Tyr-D-Glu-L-Tyr, L-Tyr-L-Asp-L-Tyr and L-Tyr-D-Asp-L-Tyr. Lyophilized peptide samples were dissolved in a 1:1 water/acetonitrile solution at a stock concentration of 1 mg/mL. Montmorillonite rock with a mean density of 2.45 g/cm3 (Tantora) was crushed by a hammer into small particles. Particles between 100 to 200 μm in diameter were collected using a nylon mesh filter. Particles were rinsed with MiliQ water and dry-heat sterilized at 150 °C for 2 hrs. The "all tripeptide" solution was prepared by mixing an equal volume of each of the 17 peptide stock solutions. From this solution, 10 μL was added to the 10 mg rock projectiles in a test tube to be fully soaked resulting in 0.59 μg of each peptide per projectile. Likewise, an 11 peptide solution consisting of equal quantities of L-Tyr-(Gly, D-Ala, L-Ala, D-Asp, L-Asp, D-Glu, L-Glu, D-Ser, L-Ser, D-Val, L-Val)-L-Tyr were used to make the "L+D tripeptide" solution, and 10 μL of this solution was used to soak the 10 mg rock particles resulting in 0.91 μg input of each peptides per projectile. In both cases, particles were further dried down using a centrifugal vacuum concentrator to produce a projectile. Based on the mean density, a total of 10 mg of rock projectiles with a diameter distribution from 100 to 200 μm consist of approximately two thousand particles was produced for each of the two peptide solutions.

Hypervelocity Impact Experiment

Hypervelocity impact experiments (HIE) were performed using a two-stage light-gas gun (Figure 2A) at the Institute of Space and Astronautical Science, Japan Aerospace Exploration Agency (ISAS/JAXA). A powder of microparticles containing tripeptides was placed into a spherical cavity inside a double-split cylindrical "sabot" projectile. Shots were set to reach hypervelocity ranging between 5.5-6 km/s, a similar velocity to that experienced during collection on the NASA Stardust mission, and exceeding the estimated encounter velocity of the spacecraft if flying through the plume of Encleadus while orbiting Saturn at ~4 km/s (53). Further, to minimize the thermal alteration of native organic samples and eliminate possible organic contaminants during intact capture, we utilized ultraclean, methylated, two-layered hydrophobic amorphous silica aerogel with a bulk density of 30 mg/cc and 10 mg/cc. This aerogel was originally developed for the Tanpopo mission in a class 1000 clean booth in order to capture micrometeoroids and orbital debris intact at low Earth orbit onboard the International Space Station-Japan Experiment Module (ISS-JEM) exposed facility (EF) (54-56). So far, 10 mg/cc is the lowest density achieved among all space missions utilizing silica aerogels. For the preparation, the aerogel was sliced into a working size of 3 x 2.5 x 4 cm cuboid using a micro-feather blade, and then stabilized with a target holder designed to fit in the vacuum target chamber of the light gas gun. The aerogel holder was fixed inside the vacuum chamber with atmospheric pressure reduced to < 3 torr (Figure 2A). We performed two HIEs, one with the all tripeptide-soaked (shot 1), and other with L+D tripeptide-soaked (shot 2) size-selected montmorillonite rocks as explained in the previous section. After the bulk shots of these peptide-containing microparticles hit the aerogel, the chamber was slowly pressurized to maintain the fragile structure of the aerogel. The target holder with the aerogel was then removed from the vacuum chamber. Impact craters and track images were recorded, and the aerogels stored in safety cases for downstream analysis.

Extraction of peptides from rock and aerogel.

As a control experiment, liquid-based extraction was conducted from the peptide adsorbed 10 mg rock particles prepared in the previous section to explore the recovery rate of each peptides from the particle. Particles were re-soaked in 50 μL of 40% acetonitrile water in a standard 2 mL test tube, and the tube sonicated for 10 min using an ultrasonic bath to enhance the diffusion speed and efficiency of the extraction process. Rock sample with aerogel was prepared with same procedure, but with addition of 1 cm3 aerogel fragment to the test tubes. The tube then was centrifuged to remove aerogel debris. The supernatant was directly analyzed with CZE.

The aerogel shot with rock particles soaked with the all tripeptide solution was carefully cut into small pieces. The pieces were squeezed into a glass 10 mL Hamilton syringe (Hamilton, Reno, NV, USA), and soaked with 2 mL of 40% acetonitrile solution inside the syringe for 5 minutes at room temperature. The solution was then pushed out and subsequently back-aspirated to the syringe to perfuse the rock sample trapped within the aerogel. The repetitive action was performed manually, and after 5 min the piston was pushed fully to crush the aerogel in order to recover the majority of the liquid retained in aerogel. The extract was centrifuged to remove aerogel debris, and the supernatant was transferred to a new tube and subjected to evaporation at 50 °C. The evaporation resulted in about a 10-fold volume reduction to approximately 200 μL and the removal of acetonitrile. Likewise, aerogel shot with particles containing the canonical L-/D- tripeptide solution was soaked with 400 μL of 40% acetonitrile solution in a syringe, repeatedly mixed with solution, and pushed out for evaporation at 50 °C. Evaporation resulted in about a 10-fold volume reduction to approximately 40 μL.

Peptide separation using capillary electrophoresis.

All CZE experiments were conducted using an Agilent 7100 CE System (Agilent Technologies, Santa Clara, CA, USA) equipped with UV diode-array detector. The analysis was monitored at 214 and 280 nm corresponding to the absorbance of the tyrosine aromatic ring. The separation process was performed using bare fused silica capillaries 60 cm long with 50 μm id (Polymicro Technologies, Phoenix, AZ, USA). Hydrodynamic injection of an aqueous standards solution was performed for 8 s at 50 mbar with a separation buffer composed of 40 mM ammonium acetate (pH 3.65) for hydrodynamic injection or 100 mM Tris, 95 mM citric acid (pH 3.65) for the head-column injection field-amplified sample injection (FASI) technique due to the fact that the concentration of peptides in samples was expected to be in ng/mL level (57,58). Tripeptide standard solutions were injected at a concentration of 5 μg/mL for each peptide using FASI technique with a detection limit estimated at 40 ng/mL. In the case of standard hydrodynamic injection, the concentration of peptides used as standards were 50 μg/mL with detection limit estimated at 1 μg/mL. The sample analytical matrix was composed of 20 μL tripeptide sample solution (rock extract, rock + aerogel extract, post HIE aerogel extract) mixed with 5 μL of 5-fold diluted separation buffer adjusted to pH 2.5 using 0.1 M HCl to provide positive ionization of the analytes, and to facilitate the stacking effect (59). A short plug of sample was introduced hydrodynamically (3 s, 50 mbar) followed by electrokinetic sample injection at 10 kV. Injection for as long as 50 s can be performed without any significant band broadening effect. Further extension of injection time resulted in loss of separation efficiency and resolution. At the beginning and at the end of each day, the capillaries were sequentially flushed with 0.1 M NaOH and water (2 bar, 10 min). Additionally, before the first run each day, the capillary was conditioned with a separation buffer for 10 min at 2 bar. Between each analysis, there was a 2 min rinse with 0.1 M NaOH, water and separation buffer.

Results and Discussion

Synthesis of amino-like monolithic stationary phase.

CZE provides for the highly efficient separation with small volumes of samples, and is adaptable to miniaturization, however it is restricted to the separation and detection of ions and compounds that bear charges at the running pH of the analysis. Here, we demonstrate an electrochromatographic approach based on the use of monolithic stationary phases to perform separation under electroosmotic flow. The generic P(EDMA-co-NAS) monolith stationary phase can be chemically grafted by different chromatographic selectors to achieve molecular-level controlled surface properties tailored to the chemical structure of target organic molecules. Figure 1 illustrates the preparation of phenyl-like P(EDMA-co-NAS)-Phe2 and amino-like P(EDMA-co-NAS)-NH2 monolith columns created via grafting of 3,3-diphenylpropylamine or ethylenediamine on the surface of P(EDMA-co-NAS), respectively.

Separation of PAHs, pyrimidines and aromatic acids via monolithic CEC.

The chromatographic selector 3,3-diphenylpropylamine was chosen for the separation of PAHs because it allows combining the hydrophobic interaction because of the presence of aliphatic spacer arm, and charge transfer because of the electron-rich phenyl rings. With this setup, a mixture of nine PAHs composed of toluene, indene, naphthalene, fluorene, anthracene, pyrene, chrysene, 7,12-dimethylbenz[α]anthracene and benzo[α]pyrene, was separated in fewer than four minutes and with high efficiencies (up to 208,000 plates/m) and under a simple isocratic elution mode on the phenyl-bearing monolithic column (Figure 3). The retention of PAHs increased with the size and the number of aromatic rings in the molecules confirming the separation mechanism. Large planar aromatic structures favor interaction with the phenyl ring grafted on the monolith surface via a flexible aliphatic spacer arm. Previously, the direct separation of neutral PAHs was achieved in a CE by implementing negatively charged cyclodextrin as a pseudostationary phase (60) or through micellar electrokinetic chromatography where neutral solutes are separated based on their differential partitioning between micelles and the background electrolyte (61). Such monoliths exhibit pH-dependent electro-osmotic generation ability depending on the charge state of the primary amine. At pH 8, cathodic electroosmotic flow (EOF) is obtained similarly to what was obtained in the case of the phenyl-like monolith. However, the separation mechanism of the amino-like monolith is dictated by the hydrated nature of the stationary phase and such a separation mechanism is well-suited to the separation of polar and hydrophilic solutes. This separation mode has been utilized in hydrophilic interaction liquid-chromatography (HILIC), well known for separating biology relevant organic molecules such as nucleobases and nucleosides (62-64).

Figure 4 shows the electrochromatographic separation of four different nucleobases: thymine, uracil, adenine and cytosine. We observed that the electrochromatographic resolution of thymine and uracil increased with the acetonitrile content in the mobile phase, confirming the proposed separation mechanism. Note that nucleobases possess different pKas and their acid-base properties depends on the solute pH (65). Therefore, it has been shown that nucleobases can be separated by conventional CE at high pH (pH > 10; (66). However, in this study nucleobases were separated in their neutral form at the same pH value (pH 8) as the one used for the PAH separation. To further demonstrate the potential of CEC, we tested the separation of a mixture of benzoic acid derivatives, namely benzoic acid, 2-hydroxy benzoic acid, 4-hydroxy benzoic acid and 3,5 dihydroxy benzoic acid (Figure 5). In a first attempt, the four benzoic acids were separated using a constant voltage (-15 kV) leading to a rather long analysis time of about 15 min. In concordance with the hydrophilic character of the P(EDMA-co-NAS)-NH2 monolithic stationary phase, the dihydroxylated benzoic acid was eluted last, while benzoic acid was eluted first. The monohydroxylated benzoic acids exhibit intermediate retention and their separation can be rationalized by the possibility in the case of 2-hydroxy benzoic acid of the formation of intermolecular hydrogen bonding limiting the interaction with the amino group of the monolithic stationary phase. Thus 4-hydroxy benzoic acid is retained more than 2-hydroxy benzoic acid. With the aim of shortening the analysis time and to improve separation efficiency (narrowing of the peak width), further analyses were performed using a voltage gradient (electropherograms b) and c) in Figure 5). Increasing the separation voltage from -15 kV to -30 kV in a stepwise fashion decreased the analysis time by half and improved efficiency (from 26,500 plates/m in plot a to 40,000 plates/meter in plot c for 4-hydroxybenzoic acid).

Separation of tripeptides including diastereomers using CZE.

Seventeen tripeptides including five pairs of diastereomers (see Materials for detail) served as both a simulant for extraterrestrial organic compounds and for analytes. Separation and identification of such isomeric peptides is challenging, and requires highly efficient chromatographic or electrokinetic separation. Therefore, CZE was chosen as a technique that features extremely high separation efficiency and whose usefulness in stereoisomeric peptide analysis has been proved in a number of studies (67-71). Separation of the compounds in CZE is possible because of the difference in the migration velocities of the ions. Only peptides containing glutamic and aspartic acid in their structure are predicted to feature lower pKa values compare to other analytes. We investigated the influence of the buffer pH in the range from 2.50 to 7.00 on separation. The baseline separation of eight out of eleven tripeptides containing biogenic amino acids was initially achieved with 50 mM ammonium acetate buffer at pH 3.65. However, due to the high electrophoretic mobility of co-migrating ammonium ions (μammonia= 76.2 10-9m2V-1s-1), we observed peak asymmetry and deteriorated separation efficiency and resolution. This was due to the electrophoretic dispersion phenomenon. We therefore used tris(hydroxymethol)aminomethane (Tris) instead of ammonium, as the former ions feature lower electrophoretic mobility (Tris = 29.5 10-9 m2 V-1 s-1). Tris ions have been reported to coat the capillary wall dynamically, and protect peptides and proteins from adsorption to silica in CZE (68). To obtain the proper buffering effect in the desired pH range, citric acid was used to replace volatile acetate. An increase of buffer concentration was found to enhance separation efficiency and detection sensitivity due to the stacking effect (72). Modification of this parameter also had an effect on peak resolution. Dynamic coating of the capillary by Tris ions decreases electroosmotic flow in the capillary simultaneously extending the separation time. Under the optimized condition of 100 mM Tris, 95 mM citric acid (pH 3.65) with field-amplified sample injection (FASI), we were able to distinguish 16 signals originating from 17 tripeptide compounds including diastereomers (Figure 6, red trace). Under this condition most compounds migrated in cationic form while Tyr-Asp-Tyr diastereomers were resolved as anions. While co-migration was observed in the case of the gamma amino acid γ-aminobutyric acid (GABA) and β-alanine-containing tripeptides, separation of the diastereomer was achieved and thus the same separation condition was applied for the samples obtained from the hypervelocity impact experiment.

Extraction and detection of tripeptides resulting from the hypervelocity impact experiment.

The *in situ* analysis of diverse extraterrestrial organic molecules is a challenging goal for astrobiology, given the limitation of size, weight, types of chemicals and energy required for the on-board instrumentation. Also, any flyby space mission would require extra level of difficulty given the expected trace amounts of organic molecules, which can further undergo thermal decomposition and impact alteration during the encounter. Therefore, assessment of HIE exposed organic materials using different capture materials have been discussed. We used two tripeptide-soaked montmorillonite bulk particles (one with all 17 tripeptides and one with 11 L+D tripeptides) as a simulant of solid material with biology relevant molecules and performed independent impact experiments reaching a speed of 5.56 km/s (shot 1 with all tripeptides) and 5.61 km/s (shot 2 with L+D tripeptides), respectively. Microscopic observation of the aerogel impact surface revealed a total 149 (shot 1) and 141 (shot 2) detectable impact craters (Figure 2B). Given the initial total particle numbers to be around two thousand, we estimate approximately 7.5% (shot 1) and 7% (shot 2) of the bulk shot rock particles (Figure 2C) were captured in the aerogel. Longer tracks were obtained from the ultra-low density 10 mg/cc aerogel compared to 30 mg/cc aerogel (Figure 2D and 2E), suggesting less energy and momentum loss per unit length for lower density aerogel. Indeed, clear difference in particle entrance hole area was observed between 10 mg/cc and 30 mg/cc aerogel (Figure 2F). An unbiased logarithmic distribution with only several anomalies (clumped and fragmented particles) indicating that majority of the aerogel encountered particles fall within the diameter size range of 100 to 200 μm.

After the impact, the aerogel targets were perfused with 40% acetonitrile to extract the peptides according to the procedure described in the methods section. As opposed to handling and analysis of individual aerogel captured grains (20), we soaked the entire aerogel to see if bulk liquid extraction and concentration procedure are feasible for detection for captured organic compounds. Due to the methylated surface of silica aerogel, 40% acetonitrile solution in water was selected to provide good solubility of the peptides and sufficient permeability to the crushed aerogel. Prior to this experiment, we had shown that the 40% acetonitrile is capable of dissolving peptides from the montmorillonite particles with a recovery rate ranging from 22.1 to 56.6% (Figure 6, black trace, Table 1). Further, the dissolution of peptides from the montmorillonite projectiles in the presence of blank aerogel was also tested to see if the methylated silica matrix of aerogel possesses affinity toward certain peptides released from montmorillonite surface (Figure 6, blue trace, Table 1). All 16 peaks were detected in the presence of both montmorillonite and aerogel with a diverse recovery rate ranging from 8.5 to 36.8%. However, when we compared the recovery rate of rock projectile alone vs rock projectile in the presence of a blank aerogel sample, results indicated higher recovery of peptides featuring acidic amino acids (Asp and Glu) -bearing tripeptides with lower mobility in electrophoretic experiments (> 62.5%) as compared to faster migrating peptide (< 50.9%; Table 1). This could be due to ionic interactions between silanol moieties of the aerogel and peptides (based on the electrophoretic results, the degree of dissociation of longer migrating species like Tyr-Glu-Tyr and Tyr-Asp-Tyr was expected to be lower as compared to other peptides). The evidence of accretion of compressed aerogel on captured projectiles (23) may also enhance such selective liquid extraction. Furthermore, extraction from blank aerogel showed a clean CZE profile under UV spectra, suggesting no contamination above the detection limit (Figure 6, green trace). Extraction of peptides from aerogel sample with projectiles bearing all 17 tripeptides resulted in several weak signals featuring mobility similar to Tyr-Glu-Tyr diastereomers, but we were unable to resolve the peaks to provide clear answer (denoted with asterisk in Figure 6, violet trace). Because most of the tripeptides were below detection limit (40 ng/mL/peptide = 0.22% recovery), peptide recovery rate to reach the detection limit after the impact was predicted to range from 8.0% to 34.5% under our HIE condition (Figure 7). Thermal alteration is considered to be the main cause of change in organic contents due to the impact shock heating reaching up to few thousand degrees C (73). General track model implies that organic molecules deposited on the impact track will be exposed to high temperature flash heating (74), while HIE with hydrated mineral grain showed that the mineralogical thermal alteration only occur on the projectile surface while interior remain unaltered with just 150 nm below the rim (75). Hence, the recovery rate of the organic molecules depends on their localization within the projectile (surface or interior) as well as the thermal history of the projectile immediately after the impact.

To assess this possibility, we further examined extracts from the second aerogel sample shot with 11 L+D tripeptides. The majority of the tripeptides peaks were undetectable; however, two peaks were detected using hydrodynamic injection (Figure 8, black trace). These peak signals showed identical mobility to that of Tyr-Asp-Tyr diastereomer standards under the same CZE condition (Figure 8, black trace) with recovery rate of 9.6% and 4.7%, respectively (Table 1) indicating extractable peptides survived the impact velocity of 5.61 km/s in montmorillonite particles. Why only the anionic peptides were extracted could be partly explained by the high liquid extraction efficiency of Tyr-Asp-Tyr tripeptides (Table 1), but it does not explain why Tyr-Glu-Tyr tripeptides peaks were not observed. Together, these findings indicate a need for better characterization of sorptive properties of target organic molecules in accordance with the chemical properties of aerogel and presence of other solid materials such as minerals and salts.

Conclusion

We have provided evidence that CEC and CZE are suitable to separate various simple organic compounds including some of which are found in extraterrestrial environments and are also relevant to terrestrial life. Electromigration techniques can be further applied to separate and detect potential biosignatures including extraterrestrial polymers, as well as simpler organics including amino acids, amines, aliphatic and aromatic hydrocarbons, sugars, phenols, alcohols, nucleobase, carboxylic acids, sulphonic acids and more. While analytical detection limit shown in this work is still 100-fold poorer than what would be expected for the actual trace biomarker detection (100 pg/mL), these CZE and CEC approaches have the benefits of miniaturization and versatility to be coupled to high resolution MS or coupled to fluorophore to further lower the detection limit. For example, we show that our generic poly(N-acryloxy-succinimide-co-ethylene glycol dimethacrylate) monolith synthesis protocol can be transferred to a microfluidic chip to further support the application of capability of the monolithic CEC technology for future spaceflight missions (Figure 9).

We have also performed HIE and liquid-based extraction using tripeptides as a biosignature simulant to validate the feasibility of sample capture and analysis for the future astrobiology-oriented flyby mission to the icy moons. We deduced recovery rate of the tripeptides in each step of the HIE (Figure 7) and found that extraction efficiency varies up to 4-fold among peptide samples under the presence of montmorillonite and aerogel. Hence even with a 90% peptide loss after the hypervelocity impact, anionic peptides could be technically detectable due to their efficient liquid extraction, which in part explains the recovery of Y-D-Y diastereomers (Figure 8). Although, it is important to note that the clay mineral projectile used in this study is not relevant to the actual constituents of the plumes of Enceladus (ice particles and salt crystals) and thus results needs to be treated as a reference to the future impact experiment. Hence, clear separation of diastereomeric tripeptides using CZE is promising for further evaluation as a potential biosignature for life detection in space. For future work, both the physical and chemical adsorption effect of peptides and other organics on the aerogel, needs to be evaluated quantitatively to fully assess the practicality of aerogel for future flight missions. Thus, extending the use of aerogel as a capture media for HIE, then soaking the entire aerogel to perform liquid-based extraction, separation and detection of target organic molecules will support future missions involved in the search for extraterrestrial life, which is essential for answering basic astrobiology question: are we alone in the universe?

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Figure Legends

Figure 1. Schematic illustration of the synthetic path applied to the synthesis of monolithic stationary phases for reversed phase-charge transfer (RP-CT) and hydrophilic interaction (HI) capillary electrochromatography. The monolithic stationary phases were obtained via the surface functionalization of a generic monolith P(EDMA-co-NAS) based on N-acryloxysuccinimide (NAS) and ethylene glycol dimethacrylate (EDMA).

Figure 2. Hypervelocity impact experiment using hydrophobic silica aerogel as a target. (A) Schematic representation of the light gas gun setup for at ISAS. Piston is powered through ignition of gunpowder which propel piston to compress the downstream hydrogen gas. Once hydrogen accumulates enough pressure, it bursts the metal disk, accelerating the sabot with projectile (montmorillonite particle with peptides) inside. Sabot rotates and releases the projectile reaching a speed of up to 6 km/sec. Projectiles are captured by the aerogel placed inside the holder within the vacuum chamber. (B) Light microscopy image of post impact aerogel surface. Aerogel targets were shot separately with projectiles containing all tripeptides at velocity of 5.56 km/s (shot 1, left) and L+D tripeptides at velocity of 5.61 km/sec (shot 2, right). Upper half consist of 30 mg/cc bulk density aerogel and lower half consist of 10 mg/cc bulk density aerogel. (C) Microscopy image of size-selected montmorillonite particles (100 to 200 μm in diameter) that were used as a peptide carrier during the hypervelocity impact experiment. (D) and (E) represents aerogel sideview after exposed to HIE (shot 1 and shot 2), respectively. Projectile impacts have resulted on "carrot shaped" tracks by releasing the kinetic energy towards breaking the silica matrix of aerogel. Outermost layers of the montmorillonite particles are fragmented during the impact but large particles remain intact at the end of the track. (F) Cumulative flux plot for two individual HIE. Image J software (76) was used to calculate

the area size for all detectable entrance holes for shot1 (left) and shot 2 (right). The x axis represents entrance hole area in log scale and y axis represents the cumulative flux of the projectile (cumulative number of holes per total impact area). Entrance holes made in aerogel bulk density 10 mg/cc and 30 mg/cc are denoted as open circle and closed circle, respectively.

Figure 3. Electrochromatographic separation of a mixture of nine PAHs: (1) toluene, (2) indene, (3) naphthalene, (4) fluorene, (5) anthracene, (6) pyrene, (7) chrysene, (8) 7,12-dimethylbenz[α]anthracene and (9) benzo[α]pyrene on a phenyl-like monolithic column (CEC conditions: mobile phase 60/40 ACN/PBS (2.5 mM pH 8); injection -5 kV/5 s, voltage -30 kV, detection 214 nm, total and effective lengths of the monolithic column are 31 cm and 10 cm, respectively).

Figure 4. Electrochromatographic separation of four pyrimidines: (1) thymine, (2) uracil, (3) cytosine and (4) adenine on the amino-like monolithic column (CEC conditions: mobile phase 80/20 ACN/PBS (5 mM pH 8), injection -5 kV/5 s.

Figure 5. Electrochromatographic separations of aromatic acids. (1) benzoic acid, (2) 2-Hydroxybenzoic acid, (3) 4-hydroxy benzoic acid and (4) 3,5 dihydroxy benzoic acid on the amino-like monolithic column. The analyses were performed at three different voltage conditions as indicated in the Figure. The electrochromatogram a) was obtained using a constant voltage of -15 kV while electrochromatograms b) and c) were obtained using voltage gradients (stepwise increase to -15 kV and -30 kV at a voltage rate of 30 kV/min) as indicated in the figure (other CEC conditions: mobile phase 80/20 ACN/PBS (5 mM pH 8),

injection -5 kV/5 s, detection 214 nm, total and effective lengths of the monolithic column are 31 cm and 10 cm, respectively).

Figure 6. Capillary zone electrophoresis separation of YNY-tripeptides. Each Peak correspond to (1) L-Tyr-L-Val-L-Tyr, (2) L-Tyr-ABA-L-Tyr, (3) L-Tyr-NorV-L-Tyr, (4) L-Tyr-L-Ala-L-Tyr, (5) L-Tyr-IsoV-L-Tyr, (6) L-Tyr-GABA-L-Tyr, (7) L-Tyr-βAla-L-Tyr, (8) L-Tyr-L-Ser-L-Tyr, (9) L-Tyr-AIB-L-Tyr, (10) L-Tyr-D-Val-L-Tyr, (11) L-Tyr-Gly-L-Tyr, (12) L-Tyr-D-Ala-L-Tyr, (13) L-Tyr-D-Ser-L-Tyr, (14) L-Tyr-L-Glu-L-Tyr, (15) L-Tyr-D-Glu-L-Tyr, (16) L-Tyr-L-Asp-L-Tyr, (17) L-Tyr-D-Asp-L-Tyr. The electrochromatograms are all tripeptide standards (red), canonical L-/D- tripeptides extracted from montmorillonite sample (black), canonical L-/D- tripeptides extracted from montmorillonite rock sample mixed with aerogel (blue), extract of HIE blank sample (green) and extract of HIE sample (violet). Background electrolyte was composed of 100 mM Tris and 95 mM citric acid (pH 3.65). The injection was performed electrokinetically for 20 s using 10 kV except the HIE sample extract analysis for which 10 kV were applied for 50 s. *unidentified peaks.

Figure 7. A schematic representation of organics extraction flowchart for simulated flyby sample capture and analysis. Initial peptide input (10 μg) undergoes stepwise reduction in quantity due to the ratio of particle entry into the aerogel (X), hypervelocity impact alteration/inclusion (Y) and liquid-based extraction efficiency (Z). Particle entry rate X: 7.5% is estimated from the number of impact holes from shot1. Liquid based extraction efficiency under the presence of aerogel Z: 8.5 – 36.8% is estimated based on Table 1. Accordingly, impact alteration/inclusion rate Y: 8.0 – 34.5% was estimated given the FASI detection limit of 40 ng/mL per peptide.

Figure 8. Capillary zone electrophoresis analysis of extract of aerogel sample obtained in HIE (black) and standard mixture of canonical L+D tripeptides (red). Abbreviations:(1) L-Tyr-L-Asp-L-Tyr, (2) L-Tyr-D-Asp-L-Tyr. Background electrolyte was composed of 50 mM ammonium acetate (pH 3.65). The injection was performed hydrodynamically (8 s, 50 mbar). Other conditions can be found in 'Experimental' section.

Figure 9. Scanning electron microscopy images showing cross section views of a) monolith-filled and empty chip channels. Part b) shows a magnification of a monolith-filled chip channel where a macroporous structure consisting of small globuli fused together characteristic of the monolithic morphology can be easily recognized. An intimate contact between the organic monolith and inorganic chip wall is seen confirming the success of the silanization pretreatement of the chip wall providing strong anchoring of the monolith.

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