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Salt Segregation and Sample Cleanup on Perfluoro-Coated Nanostructured Surfaces for Laser Desorption Ionization Mass Spectrometry of Biofluid Samples

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S Supporting Information

ABSTRACT: We present a surface assisted laser desorption ionization (SALDI) technique, coupled with fluorocarbon coating, to achieve selective segregation of ionic and/or hydrophilic analytes from background biofluid electrolytes for quantitative mass spectrometric analysis. By controlling the contact angle of (1H,1H,2H,2H-perfluorooctyl) trichlorosilane or (1H,1H,2H,2H-perfluorooctyl) dimethylchlorosilane to a specific range (105–120°), background electrolytes can be made to segregate from hydrophilic analytes during a drying step on the surface of a highly nanoporous thin film. Nanoporous silicon films were prepared using glancing angle deposition (GLAD) thin film technology, then coated with fluorocarbon. This desalting method directly separates highly polar, ionic metabolites, such as amino acids, from salty biofluids such as artificial cerebrospinal fluid (aCSF) and serum. Derivatization, extraction and rinsing steps are not required to separate the analytes from the bioelectrolytes. With on-chip desalting, the limit of quantitation for histidine spiked in aCSF is $\sim 1 \mu\text{M}$, and calibration curves with internal standards can achieve a precision of 1–9% within a 1 to 50 μM range. Five highly polar organic acids in serum were successfully quantified, and the SALDI-MS results obtained on the desalted serum sample spots show both good reproducibility and compare well to results from NMR and liquid chromatography–mass spectrometry. Putative identification of a total of 32 metabolites was accomplished in blood using time-of-flight MS with perfluoro coated Si-GLAD SALDI, by comparison to tabulated data.

Liquid chromatography and electrospray ionization mass spectrometry (LC–MS) is a standard and powerful tool in metabolite fingerprinting,^{1–8} partially due to convenient online sample preparation with LC. As an alternative, perfluoro coated porous silicon laser desorption ionization MS (pSi-LDI) offers remarkably high sensitivity in detecting small molecules, and applications in biofluid metabolite analysis have been explored.^{9–11} While LC–MS methods are clearly the most powerful and effective for broad, untargeted metabolomics analysis and biomarker discovery, the batch processed, spot analyses offered by pSi-LDI^{12,13} are an attractive approach to routine assay methodology once specific biomarkers are known. Nevertheless, the challenges associated with using pSi-LDI, often performed as desorption ionization on silicon (DIOS), and other nanostructured surfaces with complex sample matrixes have been widely reported.^{9,14–16} There are few reports of quantitative analysis of such samples with nanostructured surfaces.

For less polar and higher mass analytes, perfluoro-coated pSi-LDI takes advantage of hydrophobic interactions between metabolites and the fluorocarbon.^{13,16,17} Several differential adsorption and extraction methods allow measurements of less

polar analytes such as codeine, alprazolam, and morphine spiked in human serum.¹³ However, for low mass ionic metabolites such as amino acids in serum, derivatization of the analytes is needed to enhance extraction and retention onto the fluoro-coated LDI substrate,^{9,18} and quantitative results with this approach are not available. Adding derivatization steps reduces the advantages of pSi-LDI as an easy-to-operate tool for biofluid metabolite analysis. In this report, we demonstrate a novel technique for improving quantitative analysis by segregating the background electrolytes of biological samples from ionic metabolites of interest. The method uses a simple spot development process on a form of pSi chip, effected with fluorocarbon coatings adjusted to give a contact angle for aqueous samples of $\sim 120^\circ$, which indicates these are coatings with imperfect surface coverage.

We have previously demonstrated that glancing angle deposition (GLAD) films¹⁹ provide a highly sensitive, easily fabricated, engineered, and controlled nanoporous surface for

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Figure 1. Workflow for biofluid sample analysis by SALDI-MS. (A) Cleaving silicon GLAD film wafer into small pieces for SALDI chips. (B) Modifying Si GLAD film with 1.2% pFMe₂SiCl solution to obtain a perfluoro coated surface with $\sim 120^\circ$ contact angle. (C) Human serum sample deproteinized through ultrafiltration. The filtrate was collected and acidified to 0.2 M HCl, before depositing on the modified SALDI surface in 1.5 μ L aliquot. (D) On-chip desalting step through segregated salt crystallization during drying. (E) Mounting the SALDI chips on a custom MALDI plate for MS analysis.

performing surface assisted LDI (SALDI).^{20,21} In this report we evaluate the challenges from background chemical noise below 250 Da and explore the use of perfluoro coatings on Si-GLAD films to reduce the background, as first shown by Siuzdak et al.⁹ The salt crystallite deposition patterns of dried spots depend in complex ways upon solubility, surface tension of the salt crystal, wetting angle of the substrate, and location of the nucleation sites.^{22–26} We utilize these effects to segregate the crystallization of salts and remove the significant interference of background electrolytes in biofluids. The method involves adjusting the contact angle of perfluoro-surface coatings and controlling the rate of droplet evaporation.

As a vehicle to motivate the application of SALDI to biological samples, we have targeted free amino acids (FAA), which are important in neurotransmission and are implicated in neurotoxicity.^{27,28} Fonteh et al. determined concentration changes in FAA of cerebrospinal fluid (CSF), plasma, and urine samples in probable Alzheimer's disease (pAD) subjects compared with control subjects using LC–MS in tandem MS format (LC–MS²).²⁹ LC–MS² is a powerful tool to identify target metabolites in a complex mixture, and it is suitable for online sample analysis. In this study, we focus on developing a more rapid, direct-insertion MS technique, which, without any chromatographic separation, can achieve detection of FAA concentration changes for metabolite quantitation in batches in serum and artificial CSF.

EXPERIMENTAL SECTION

SALDI Chip Preparation. Vertical nanocolumns 500 nm tall were deposited on piranha-cleaned silicon wafer substrates (Silicon Materials, prime grade, 500 μ m thick) using GLAD. Substrates were maintained at a fixed deposition angle of 86° from the substrate normal, and substrate rotation was employed throughout the deposition (2.4 nm deposited/rotation). Electron-beam evaporation of silicon (Kurt J. Lesker, p-type, 99.999% purity), held in a Ta crucible and heated under vacuum, was employed. The base pressure was $\sim 5 \times 10^{-7}$ Torr, and the Si deposition rate (as measured with a quartz crystal thickness monitor oriented normal to the crucible) was ~ 1.6 Å/s, leading to a column growth rate of ~ 5.5 nm/min. The films gave SALDI detection of des-Arg⁹-bradykinin (904 Da) with a 0.6 fmol limit of quantitation (LOQ, defined as a signal-

to-noise ratio (S/N) of 10) in positive ion mode, comparable to the results we reported previously for Si-GLAD films.²⁰

After deposition, the silicon GLAD films were handled in ambient air, resulting in a native oxide on the silicon nanocolumns. The silanol-rich surface was covalently fluorinated by soaking chips in dilute solutions of (1H,1H,2H,2H-perfluorooctyl) trichlorosilane (pFSiCl₃, Gelest) or (1H,1H,2H,2H-perfluorooctyl) dimethylchlorosilane (pFMe₂SiCl, Gelest) for 30 min at ambient temperature. The silane solutions were prepared by adding 10–200 μ L of pFSiCl₃ or pFMe₂SiCl to 5 mL of distilled methanol, followed by 30 s of vortex mixing. The chips were then stored in a Petri dish overnight for polymerization, followed by rinsing in a critical point drier (Tousimis Research Corporation) to remove trace methanol and excess silane reagent.

Sample Preparation. Analyte standards of histidine, glutamine, asparagine, and des-Arg⁹-bradykinin were from Sigma-Aldrich and prepared as stock aqueous solutions, diluted with water/methanol (50/50, v/v) to obtain individual analyte or mixed concentrations from 0.05 to 100 μ M. Des-Arg⁹-bradykinin solutions were diluted with 0.1% TFA/methanol (70/30, v/v). Artificial cerebrospinal fluid (aCSF) solution was prepared following the protocol from ALZET (www.alzet.com); components are listed in Table S-1. To prepare the aCSF samples, analyte stock solutions were diluted with aCSF to obtain individual analyte or mixed concentrations from 0.5 to 100 μ M.

Human serum (Innovative Research, pooled normal) was thawed on ice, then deproteinized through ultrafiltration. Centrifugal filter units (EMD Millipore, Amicon Ultra-4, 3K molecular weight cutoff) were prerinsed to remove glycerol bound to the ultrafiltration membranes, with 4 mL of deionized water in each unit, followed by a 10 min spin at 4 000g with a swinging bucket rotor (Beckman Coulter, Allegra X-22). Serum samples were then transferred into the rinsed centrifugal filter units and spun at 4 000g and 4 $^\circ$ C for 30 min. The downstream filtrate was collected and acidified with 2 M HCl to achieve 0.18 M HCl in the sample. When quantitation was not performed, no further dilution was made.

Pure samples in 0.8 μ L aliquots were spotted on 1% concentration pFSiCl₃ SALDI chips and open-air-dried at room temperature. For biofluid samples (aCSF or deproteinized

human serum), 1.5 μL aliquots were spotted on 0.3% pFSiCl₃ or 1% pFMe₂SiCl fluorinated SALDI chips with contact angles of 105–120°. The typical work flow for biofluid samples is indicated in Figure 1. For internal standard (IS) calibration curves of aCSF samples on 1% concentration pFSiCl₃ coated surfaces, samples were prepared by mixing 1 aliquot of aCSF sample (10–100 μM histidine or glutamine), 1 aliquot of 10 μM asparagine in water as IS, and 4 aliquots of methanol. On 1.2% concentration pSiFMe₂Cl coated surfaces, salt dilution was minimized, in order to mark the chips' high salt tolerance. A 200 μL aliquot of aCSF sample was spiked with 2 μL of IS in water. The original "analytical" concentrations in the aCSF samples are stated throughout. To achieve desalting, the aCSF spot was usually dried in a small Petri dish (Fisherbrand, 35 mm $\times$ 10 mm) at 4 °C for several hours.

The metabolites in serum were quantified using the standard addition method. Six centrifugal filter units were prerinsed and each was filled with 0.5 mL of serum. Then various volumes of standard solutions of metabolite (taurine, histidine, aspartic acid, glutamic acid, and malic acid) were spiked in serum samples. The mixture was diluted with water to a total volume of 1 mL and thoroughly mixed before ultrafiltration. The filtrate was acidified with 2 M HCl to achieve 0.18 M HCl in the sample. Various spot drying procedures were used, as presented where relevant below. As a comparison, the concentrations of the serum metabolites quantified by SALDI-MS were determined by ¹H NMR spectroscopy or reverse phase LC-MS; protocols are detailed in the Supporting Information. The ultrafiltration step was used in NMR sample preparation as well.

Mass Spectrometry. SALDI chips were attached to a modified matrix assisted laser desorption ionization (MALDI) target plate (Figure 1) with conductive double-side carbon tape (Electron Microscopy Sciences). MS measurements were performed on a Voyager Elite MALDI-TOF mass spectrometer (AB Sciex) equipped with a pulsed nitrogen laser (337 nm, 3 ns pulse). Mass spectra were acquired in reflector, delayed extraction mode, and each spectrum is the accumulation of 100 laser shots as the laser is rastered over the surface in regions free of large salt crystals. Other instrument settings (Table S-2, Supporting Information) were applied for optimal resolution and S/N in the mass range below 400 Da. The S/N of peaks were calculated from raw spectral data by Data Explorer 4.0 (AB Sciex) without any data preprocessing. The metabolites in serum were identified based on exact mass (m/z) using the HMDB database. The LOQ was estimated from plots of S/N versus analyte concentration. The IS calibration curves were constructed by plotting the peak height ratios of analyte to IS versus the concentrations of analyte in aCSF, and error bars represent the standard deviation from 10 samples. The statistical analysis for detection limits and interpolation of concentrations followed standard practice, outlined in the Supporting Information.

RESULTS AND DISCUSSION

Perfluoro Coating Improvements for Low Mass Analytes. A series of LDI-studies were performed on Si-GLAD films. These films, nominally 500 nm thick, were prepared at a fixed 86° deposition angle and continuously rotated during deposition to produce vertical posts with a 20–50 nm columnar spacing. Compared to masses above 400 Da, background chemical noise and ion suppression due to the background result in far poorer limits of detection on the same

films for low mass compounds such as histidine (155 Da), even with common cleaning methods.^{20,21,30} In negative ion mode with Si-GLAD films, 20 μM histidine was detectable with a high background signal, comparable to performance reported for histidine on etched porous silicon.³¹ Since many small molecule biomarkers are present in the 20 μM range or lower and quantitative work requires better precision than found near the LOQ, it is important to reduce the background chemical noise in the sub-400 Da mass range.

Siuzdak and co-workers showed that perfluoro-chlorosilane coatings could be used with etched porous silicon and a high-quality mass spectrometer to give a zeptomole detection limit for bradykinin.⁹ It was proposed that the coating facilitates adsorption of analytes on the surface for preconcentration and that the hydrophobic character of the surface prevents droplet spreading, thus concentrating the dried sample in a smaller spot. It is clear from their data that the coating greatly reduces background noise, making perfluoro coatings an excellent candidate to improve low mass detection limits on GLAD films.

Modification of Si-GLAD films with a 1% concentration of perfluorooctyltrichlorosilane (pFSiCl₃) yields a superhydrophobic surface, with a contact angle in the range of 135–150° from batch to batch. Aqueous samples need to be mixed with methanol to spot them on a superhydrophobic surface from a pipet tip, otherwise the aqueous droplet clings to the pipet tip and is not released; a 50/50 mixture gives a contact angle of $\sim$ 112° and a droplet size of $\sim$ 2 mm. High-quality mass spectra can be obtained in negative ion mode from 1 to 200 μM analytical concentrations for histidine with these coatings, as shown for 1, 2, and 10 μM in Figure S-1. (Analytical concentration is the concentration of sample before dilution with any additional reagents such as methanol.) The LOQ for pure samples of asparagine, glutamine, and histidine were 470, 340, and 340 fmol or 0.6, 0.4, and 0.4 μM , correspondingly, extrapolated from S/N versus concentration plots that show very high linearity ($R^2 > 0.99$). Coated surfaces showed no change in LOQ or background chemical noise in the low mass range over 7 days of atmospheric exposure. Direct calibration curves are subject to considerable ionization suppression at higher concentrations, so that use of an internal standard is required. Selecting 10 μM asparagine as an internal standard, linear calibration curves using peak height ratios were obtained for glutamine ($R^2 = 0.99$) and histidine ($R^2 = 0.98$) samples in the range of 0.5–50 μM . Quantitation limits (LOQ) were $\sim$ 0.1 μM with 0.8 μL sample spot delivery.

The results obtained with SALDI on pFSiCl₃ coatings show that a very high-performance and good shelf life can be achieved for small molecules using the perfluoro coating when the samples are relatively pure, such as they may be in the study of synthetic samples or drug purity evaluations.

Segregation of Interfering Salts on Perfluoro Surfaces. Cerebrospinal fluid has been shown to provide a fingerprint of metabolites, including several amino acids,^{29,32} whose concentration changes indicate pAD. A common artificial CSF (aCSF) composition was used in this study, consisting of a mixture of salts (Table S-1) with 150 mM NaCl dominant. Salt reduces the quality of the SALDI data notably on the pFSiCl₃ coated surface (prepared at 1% concentration to give $\sim$ 150° contact angle), as illustrated in Figure 2. Depositing a 50/50 methanol/sample mixture yielded salts crystallized over the entire spot surface, significantly masking the GLAD film underneath and greatly reducing or eliminating histidine signal. For 20 μM histidine, the S/N ratio observed was 18 ± 4 , arising

Figure 2. (a) SEM image of 500 nm thick nanostructured Si thin films fabricated using GLAD. In parts b and c, artificial cerebral spinal fluid (aCSF) was deposited on a pFSiCl₃ coated SALDI chip (1% pFSiCl₃ coating concentration for 150° contact angle surface). Salt in the aCSF dried to a thick layer, masking the nanoporous structure (see Figure S-2 for an SEM image showing the entire sample spot.) 10 μM histidine spiked in aCSF was not ionized and detected by SALDI-MS. In parts d–f, the same sample was deposited on a pFSiCl₃ surface (pFSiCl₃ coated at 0.3% concentration to give a 120° contact angle) and slowly dried at 4 °C in a closed chamber. After slow air-drying, the background electrolyte formed large crystals. Many of the nanopores are salt-free, in contrast to the salt deposits in part b. The MS measurements were taken in regions without visible crystals. The interference from salt was greatly reduced. The [His – H][–] peak at *m/z* 154.0 was detected. The asterisks mark background peaks at *m/z* 112.9 and 199.9.

from a dramatic reduction in signal, and even at 50 μM ~30% of the laser shots gave zero signal. The negative impact of salt on LDI from Si substrates has been frequently reported.^{9,14,33}

By using internal standards (IS) and diluting the original aCSF sample to 16.7% of its analytical concentration, as described in the Experimental Section, the salt problem was partially alleviated. The analytical concentration that could be reliably detected was in the range of 10–100 μM, yet calibration curve error ($R^2 = 0.96$) was high compared to salt-free aqueous/methanol samples. More directly, the standard error in using these calibration curves to interpolate the concentration of an unknown in the central region of the curves is ~24%, obviously increasing at the extremes of the calibration range. The concentration change of histidine in pAD is about 22%, and other FAA have smaller percentage concentration changes.²⁹

Rinsing these surfaces following drying of the sample spot showed the amino acids were as poorly retained as the background electrolyte, so there was no advantage in rinsing. Desalting the aCSF samples with strong cation exchange ZipTips was not effective; only >1 mM histidine in aCSF was detectable by SALDI-MS, and glutamine was not observed at any concentration. This result is consistent with the assessment of Trauger et al., who sought alternatives to ZipTips for desalting of hydrophilic analytes analyzed by DIOS-MS.⁹ The similarity of retention of electrolytes and amino acids mean that chromatographic methods are the most effective method of sample preparation, in general.

We found that by reducing the deposition concentration of pFSiCl₃ to 0.3%, the surface contact angle was reduced to ~120°, eliminating the need to dilute with methanol to get the droplet to stay on the surface, and that higher quality SALDI data could be obtained for aCSF samples. Further improvement was achieved by drying the sample droplets slowly (~5 h) in a closed chamber at 4 °C. Figure 2d shows that on a surface with

a 120° aqueous contact angle, several large, localized salt crystals form, leaving much of the SALDI surface with no visible salt deposit, Figure 2e. High-quality MS measurements were obtained from regions without visible crystals, as shown by Figure 2f.

On the basis of optimization studies discussed below, SALDI chips stored in ambient conditions for a week or longer, then coated with a 1.2% concentration of pFMe₂SiCl were selected for on-chip sample desalting and mass analysis of small metabolites in biofluids. aCSF samples were slowly dried in a closed chamber at 4 °C to maximize the salt segregation effect. Using this procedure, and avoiding large salt crystals that form on the spotted region, the linear response of a S/N versus concentration plot for histidine in aCSF extends from 1 to 100 μM ($R^2 = 0.99$). The S/N at 1 μM is 29 ± 16 , considerably better performance than seen on pFSiCl₃ coated surfaces with ~150° contact angle that do not result in salt segregation. The improved S/N comes from a major increase in signal, even though noise also rises. Figure S-3 shows that a mix of 20 μM amino acids (Leu, Gln, His, and Tyr) can be readily determined simultaneously in aCSF matrix, when using the salt segregation process on a surface with ~120° contact angle. Automation of the method is feasible, as the signals drop to near zero over large salt crystals, giving silent spots as observed in all MALDI methods. Thus, scanning across a spot without visually identifying the crystals to avoid them yields the same pattern of signal response as in automated MALDI.

Using asparagine spiked at 20 μM as IS, the calibration curve for histidine in aCSF shows good linear response from 2 to 50 μM, with improved R^2 values of 0.99, as shown in Figure S-4. Using 1-methylhistidine spiked at 50 μM as IS, the calibration curve for histidine gave a linear range from 1 to 100 μM. Using glutamine for the IS gave a good curve with R^2 of 0.98 from 2 to 50 μM. The error for interpolation of an unknown across the full range of these calibration curves varies from 14% at the

Figure 3. SALDI-MS for the analysis of metabolites in human serum. The deproteinated serum was spotted on a pFMe_2SiCl SALDI coated chip (1.2% coating concentration to give a 120° contact angle) and slowly (~ 5 h) air-dried. The salt in the sample aggregated in a small region of the dried sample spot, as observed under (a) optical ($10\times$) and (b) helium ion microscope imaging. Part c shows the nanopores are salt free after the sample deposition, but there is some nanocolumn clumping. The MS measurements in parts d and e were taken in regions without visible crystals, obtained in negative and positive ion modes, respectively. The mass spectra of serum metabolites in the low mass range are assigned in accompanying tables.

extremes to 1% in the center of the curve, making desalting segregation on SALDI a good tool to detect FAA concentration changes for early diagnosis of pAD. Clearly, a variety of IS choices may be made, giving flexibility in quantitative measurement of multiple components.

Serum Sample Analysis. The desalting method was also applied to commercial human serum samples. The serum samples were deproteinated by ultrafiltration, then acidified to denature any remaining smaller proteins and precipitate lipids, before spotting $1.5\ \mu\text{L}$ on a perfluoro-pSi SALDI chip for desalting and MS analysis. Similar to the results observed for aCSF samples, background electrolyte in the serum segregated as the sample was dried, and a large fraction of the nanopores were left free of the dominant salts (Figure 3a,b). However, as discussed below, slow drying was not a requirement for good performance. Quantitation of each serum metabolite was performed on the same batch of SALDI chips. Figure 3d,e show the mass spectra of the human serum in the low mass range. Peaks with m/z over 300 in negative ion mode and peaks with m/z over 1000 in positive mode were not observed. We note that in absence of a perfluoro coating, no signals were observed from serum spots, while the addition of 50/50 v/v methanol to serum to allow spotting on a 150° contact angle surface coated the entire surface in salt (similar to Figures 2b and S-2), resulting in no meaningful signals for analytes.

Five putative metabolites in the serum samples, taurine, aspartic acid, malic acid, glutamic acid, and histidine, ionized in the form of $[\text{M} - \text{H}]^-$, were identified and quantified using a standard addition method, as shown in Figure 4. The coefficients of variation (CVs) of all five metabolites were below 30%. The results are summarized in Table 1 and compared with the normally observed concentrations in blood, along with results from NMR spectroscopy and LC-MS on these commercial serum samples. The concentrations of taurine, malic acid, and histidine observed by SALDI are

Figure 4. Standard addition curves of (a) malic acid, (b) aspartic acid, (c) glutamic acid, and (d) taurine in human serum. SALDI chips were coated with a 1.2% concentration of pFMe_2SiCl , 120° contact angle. Before deproteination by ultrafiltration, 0.5 mL of serum was mixed with standard solutions and subsequently diluted to 1 mL. The x-axis in parts a–d represents the final concentration of the standard in the 1 mL mixture. Therefore, the readings at $y = 0$ represents half of the analytical sample concentrations in the serum. The error bar is the SD of 9 individual measurements.

within the physiological ranges. The concentrations of aspartic acid and glutamic acid are out of the normal range but consistent with the NMR results, indicating the two metabolites are abundant in the commercial sample. Malic acid and taurine are not quantifiable by NMR, as discussed in the Supporting Information, so taurine was analyzed by a reverse phase LC-MS method.

Combined with on chip desalting, the SALDI chip works in a similar concentration range as NMR for quantitative analysis of serum metabolites but detects and quantifies a complementary

Table 1. Concentrations of Metabolites in Human Serum Quantified by SALDI-MS and NMR, Compared with the Normal Concentrations in Blood

metabolite	mass (Da)	SALDI-TOF-MS (μM)	database ^a (μM)	NMR or LC-MS (μM)
taurine	125.01	72	45–130	80.0 ^b
aspartic acid	133.04	55.3	<25	62.7 ^c
malic acid	134.02	17.3	12.0 (0.0–21.0)	N/A
glutamic acid	147.05	353.2	<100	261.5 ^c
histidine	155.07	85.7	26–120	114.0 ^c

^aData are from Human Metabolome database (HMDB, www.hmdb.ca). ^bQuantified by AbsoluteIDQ kit. ^cQuantified by NMR.

list of metabolites. The method is easy to apply in terms of sample preparation and offers rapid batch analysis of multiple metabolites.

The TOF mass analyzer we employed offers a mass accuracy of ± 0.2 Da below m/z 3500, so using m/z alone makes unequivocal identification of the many compounds observed difficult. Nevertheless, we evaluated the reproducibility of the MS results obtained on 8 desalted serum sample spots. Figure 5

Figure 5. Coefficients of variation (CVs) of the peak heights at m/z 100–250 (negative ion mode). The CVs were calculated from mass spectra of 8 serum sample spots on SALDI chips. Excluding the peaks with S/N less than 20, 36 peaks were detected in negative ion mode. The Xs represent the five quantified metabolites in Table 1.

shows the coefficients of variation (CV) of signal intensities for the strong peaks at m/z 100–250 detected in negative ion mode and for the strong peaks at m/z 110–300 detected in positive ion mode. Excluding peaks with S/N less than 20, there were 36 and 34 peaks detected in negative and positive ion modes, respectively. The CVs of the peaks are mainly in the 20–40% range, comparable to the range seen using other MS techniques in metabolomic studies.^{34,35} Negative ion peaks were assigned to metabolites using the Human Metabolome Database (HMDB, www.hmdb.ca), assuming the adduct is $[\text{M} - \text{H}]^-$. The molecular weight tolerance was ± 0.1 Da, and only metabolites known to be found in blood were considered. Table S-3 in the Supporting Information shows that 32 of 35 peaks observed could be assigned, and most of these putative species were compounds with carboxyl functionality. The good reproducibility of the SALDI spot tests demonstrates their potential in clinical applications and suggests ready coupling with MS/MS for identification of serum metabolites.

Optimization and Evaluation of Salt Segregation.

While optimizing the deposition concentration of the pFSiCl_3 coating, we found that the primary indicator of success was obtaining an average contact angle in the range of ~ 105 – 120° . These contact angles are indicative of a lower quality film, with defects in the coverage of the silica surface functionality on the columnar structure. Shifting to the monochloro derivative (per-

fluorooctyl)dimethylchlorosilane (pFMe_2SiCl) from pFSiCl_3 made it far easier to obtain contact angles of 120° as a function of coating concentration and reaction time, when testing with a $2\ \mu\text{L}$ aCSF droplet. The results for both coating materials are shown in Figure 6, evaluated for aCSF samples.

Figure 6. (a) S/N of $10\ \mu\text{M}$ histidine spiked in aCSF on perfluoro coated SALDI surfaces with different contact angles. (b) The contact angles on fluorinated SALDI surfaces, controlled by varying the silane, the silane coating concentration, and the storage times of GLAD films (1 day–6 months). The leftmost line is for pFSiCl_3 coatings on a 3 month old surface; the other lines are for pFMe_2SiCl coatings, differentiated by GLAD film storage times before surface modification. The error bar in part b is the SD of 9 individual measurements.

Figure 6a shows the S/N is highest, with the least variance in S/N, for coatings in the range of 105 – 120° contact angle, while S/N is very poor at the highest contact angles. Contact angles in the range of 120 – 135° are difficult to predictably achieve and tend to either work effectively or not work at all, resulting in the large variance in S/N for this contact angle range. Contact angles were manipulated by varying the concentration of either pFMe_2SiCl or pFSiCl_3 , as indicated in Figure 6b. Concentration values that gave large standard deviations in contact angle represent the critical concentrations at which the state of the surface between hydrophilic, hydrophobic, or superhydrophobic is difficult to control. Concentrations giving low standard deviation in the contact angle are far more attractive to use, since the intended contact angle is much more readily achieved at these concentrations. Figure S-5 in the Supporting Information presents the data in an alternate format that emphasizes the relationship between signal, noise, and coating concentration.

We also noted that the age of the GLAD film prior to coating was a significant factor. Surfaces that were coated on the day the film was fabricated showed higher contact angles for a given coating concentration. It was far easier to achieve the apparently optimal contact angle (105 – 120°) across a broad range of coating concentrations for surfaces at least briefly stored under ambient conditions. The shaded box in Figure 6b highlights conditions that provide the optimal performance for the various surfaces tested. We conclude that SALDI chips stored in ambient conditions for a week or longer, then coated with a 1.2% concentration of pFMe_2SiCl will routinely give highly reproducible desalting performance, as determined by the S/N ratio for histidine in aCSF samples.

The influence of the drying rate was evaluated on the pFMe₂SiCl coated surfaces for aCSF samples, as described in the [Experimental Section](#). For aCSF samples, high S/N values were achieved only with ~5 h drying time in a humidity chamber at 4° and could consistently be associated with the appearance of a few large salt crystals. In contrast, serum samples, which contain many biochemical components, gave good quality performance for various drying conditions: rapidly at ambient conditions, for ~1 h in a humidity chamber at RT, or for ~5 h in a humidity chamber at 4°. The S/N observed for the five compounds analyzed in [Figure 4](#) was independent of drying conditions, within experimental error, as illustrated in [Supporting Information Figure S-6](#). Rapid drying of serum spots allowed observation of 27 putative analytes, while drying under controlled humidity at RT or 4° increased the analytes observed to 34 and 36, respectively.

The drying of droplets and the pattern of colloid or salt deposition is surprisingly complex.^{22–26} While coffee ring-like deposition is often seen, many other deposition patterns are also known to occur. The perfluoro-coated nanoporous films studied here do not show coffee ring-like deposition. Instead large salt crystals are observed toward the center of the droplet, with very little spreading of salt dendrites, and a heterogeneous but random distribution of sample across regions not coated in major background electrolyte crystals. Shahidzadeh-Bonn and colleagues evaluated NaCl, CaSO₄, and Na₂SO₄ crystallization from droplets on hydrophilic and hydrophobic surfaces.^{23,24} For contact angles above 90°, NaCl crystals were found to migrate from the perimeter toward the center, driven by surface tension effects when the crystal is exposed to air, which causes unique flow patterns that may distribute deposits more evenly.²⁴ Using their measured 10 M super saturation limit, a serum or aCSF sample must drop from ~1 µL to ~15 nL to initiate NaCl crystallization. For a 1 µL droplet reduced to ~15 nL, a 50 µM concentration of metabolite would rise to ~3 mM, still far from the precipitation point of most metabolite salts. Segregation of the salts thus commences as a result of the significant concentration difference, so long as the dominant salt forms only a few large crystals. Slower evaporation rates and lower temperatures will tend to encourage nucleation of fewer, larger crystals that are also more likely to disturb the conventional coffee ring-flow profile,²⁴ though the reason this is more critical for aCSF than serum is not clear.

The observed difference between a ~120° contact angle surface and a ~150° contact angle surface may depend upon a number of elements, but an experimental evaluation of the potential mechanism is well beyond the scope of this work. Nonetheless, we note that a solid silica or glass surface treated with perfluor coatings will give a contact angle of 90–110°, whereas the superhydrophobic angle of 150° arises from surface tension that greatly reduces wicking of water into the underlying nanoporous structure.^{24,36–38} Consequently, a contact angle of 120° on a nanoporous surface indicates substantive defects in the surface coating, with sufficient surface hydroxyl exposed, either in small patches or homogeneously distributed, that solvent can enter the porous GLAD layer. The concentration of metabolites is low enough that they will not start to deposit until the evaporating droplet volume is less than the internal void volume of the GLAD film, ~0.6 nL. This means that wicking phenomena, capillary forces, and viscoelastic effects on solvent transport will all play a role on the crucial stages of analyte deposition.^{25,26} Any differences in penetration of the porous layer due to contact angle differences

can be expected to have significant impact on the last stages of drying and deposition of low concentration analytes. Finally, other electrolytes such as Ca²⁺ and phosphate are also present in reasonable abundance compared to the metabolites of interest, and their response to changing hydrophobic conditions may be important as well.

The salt desegregation step described in this work should be of value where a large number of samples is to be processed and the target analyte response to disease or diet is well-known, such as in clinical diagnostics. The work here extends the application of SALDI methods to ionic and hydrophilic compounds in complex biofluids for quantitative analysis. The dominant methods for untargeted metabolomic analysis are nuclear magnetic resonance (NMR)^{39,40} and LC–MS.² Compared to MS, NMR requires minimal sample preparation and is quite versatile, but is limited by relatively poor sensitivity (LOD > 10 µM).³⁹ LC–MS in the form of reverse phase for lipophilic compounds and hydrophilic interaction chromatography (HILIC) offers the potential for more universal metabolite profiling, due to high sensitivity and chromatographic sample separation.^{4–8,41–44} The power of these methods is not questioned, although they do face their own complexities,^{5,8} and they are perhaps ultimately less well suited to routine clinical assays than the SALDI approach developed here.

■ CONCLUSION

Perfluoro-coatings on nanoporous GLAD films provide low background chemical noise in the low mass range relevant to small biomarkers, allowing accurate measurements to be made of relatively clean samples. Through the generation of a coated, nanostructured surface with a controlled extent of defects, as evidenced by contact angle measurements, we have developed a method to effect quantitative analysis of amino acids and other ionic metabolites in complex biological fluid samples. Coating defects generated in the surface modification encourage segregated crystallization of the dominant salt content from the analyte ions, effectively desalting the sample matrix. While further refinement may be required for salty fluids without other contaminants in order to reduce drying time, the rapid drying time found for serum indicates the method has considerable promise in clinical assays. This desalting method facilitates the detection and quantification of highly polar metabolites in serum with a simple spot test on a laser desorption ionization chip.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](#) at DOI: [10.1021/acs.analchem.6b03934](https://doi.org/10.1021/acs.analchem.6b03934).

Experimental section: NMR spectroscopy of three amino acids and reverse phase liquid chromatography–mass spectrometry analysis of taurine; statistical analysis; table of artificial cerebrospinal fluid composition (aCSF); and table of mass spectrometry conditions for SALDI. Results and discussion: mass spectra of several pure amino acids on 150° contact angle surface; SEM image of aCSF spot dried on 150° contact angle surface; mass spectrum of amino acids in aCSF on 120° contact angle surface; internal standard calibration curves for amino acids in aCSF on 120° contact angle surface; perfluoro

silane coating concentration impact on signal-to-noise ratio, noise, and contact angle; effect of drying rate on signal-to-noise performance for 5 analytes in serum (PDF)

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Notes

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