Confocal Raman Microscopy for in Situ Measurement of Octanol–Water Partitioning within the Pores of Individual C_{18}–Functionalized Chromatographic Particles

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Supporting Information

Abstract: Octanol–water partitioning is one of the most widely used predictors of hydrophobicity and lipophilicity. Traditional methods for measuring octanol–water partition coefficients ($K_{ow}$), including shake-flasks and generator columns, require hours for equilibration and milliliter quantities of sample solution. These challenges have led to development of smaller-scale methods for measuring $K_{ow}$. Recent advances in microfluidics have produced faster and smaller-volume approaches to measuring $K_{ow}$. As flowing volumes are reduced, however, separation of water and octanol prior to measurement and detection in small volumes of octanol phase are especially challenging. In this work, we reduce the receiver volume of octanol–water partitioning measurements from current practice by six-orders-of-magnitude, to the femtoliter scale, by using a single octanol-filled reversed-phase, octadecylsilane-modified (C_{18}-silica) chromatographic particle as a collector. The fluid-handling challenges of working in such small volumes are circumvented by eliminating postequilibration phase separation. Partitioning is measured in situ within the pore-confined octanol phase using confocal Raman microscopy, which is capable of detecting and quantifying a wide variety of molecular structures. Equilibration times are fast (less than a minute) because molecular diffusion is efficient over distance scales of micrometers. The demonstrated amount of analyte needed to carry out a measurement is very small, less than 50 fmol, which would be a useful attribute for drug screening applications or testing of small quantities of environmentally sensitive compounds. The method is tested for measurements of pH-dependent octanol–water partitioning of naphthoic acid, and the results are compared to both traditional shake-flask measurements and sorption onto C_{18}-modified silica without octanol present within the pores.

The octanol–water partition coefficient ($K_{ow}$), defined as the ratio of the concentration of a molecule in an octanol–water partition coefficient to its concentration in an aqueous-phase in a two-part octanol–water extraction, is a widely used predictor of lipophilicity. $K_{ow}$ is commonly used to predict potential pharmacological activity or toxicity of a compound in medicinal chemistry research, as well as fate, and ecotoxicity of a compound in the environment. There are numerous methods available for determining $K_{ow}$; however, the most common conventional techniques are slow-stirring and shake-flask methods. Here an analyte is allowed to equilibrate across the boundary between octanol and water phases, where the sample is stirred or shaken to increase the rate of transport between the two phases. Each phase is then analyzed to determine $K_{ow}$. While these methods are simple, they require large (milliliter) quantities of analyte solution and are slow, often requiring hours to reach equilibrium. For screening new drug candidates, the large sample quantities needed for this measurement are costly to produce and screening of many structures is time-consuming.

To address the need for a rapid, small-volume method of determining octanol–water partition coefficients, recent research has adapted microfluidic sample handling to the measurement of $K_{ow}$. Experiments are generally of two types, slug flow or side-by-side flow. In slug flow, droplets of octanol and aqueous solution are transported in contact with one another through a microfluidic channel. Equilibration between aqueous and octanol phases occurs quickly due to a high surface-area-to-volume ratio and constant mixing of the interface as the droplets flow through the channel. Side-by-side flow experiments maintain a parallel flow profile between water and octanol in a microfluidic channel with analyte equilibration occurring as the two phases traverse the channel. These experiments have lowered the octanol-volume requirements to the nanoliter scale and allow determination of $K_{ow}$ in minutes. Further reduction in octanol–water partition volumes has been challenged by detection and fluid manipulation in small volumes. In side-by-side flow channels, equilibrium occurs at a distance down the channel where the two-phases have remained in contact for a sufficient time for diffusion and partitioning to equilibrate the analyte concentrations in the two phases. The analyte concentrations in each of the phases as well

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as the distance to reach equilibrium have been determined using fluorescence measurements. To expand the scope of measurements beyond fluorescent compounds, slug-flow studies have been carried out with UV–vis absorption detection. UV–vis monitoring limits further miniaturization, however, due to the path length requirements for sensitive detection, necessitating sample volumes of hundreds of nanoliters or more.

Another approach to measuring octanol–water partitioning is through the use of reversed-phase liquid chromatography. This method allows smaller source-phase sample volumes (typically microliter) and experiments generally require less than an hour. In this case, however, K_{ow} is inferred from retention data by comparison to the behavior of similar compounds, where K_{ow} has been measured by traditional extraction methods. This empirical determination of K_{ow} is not always straightforward because chromatographic retention depends on competing interactions between an analyte and the stationary phase and mobile phase. This interpretation is further complicated for charged molecules where retention of the analyte at the interface between the stationary- and mobile-phases is driven by a lowering of the surface tension between the two phases, which is not present in bulk octanol–water partitioning. In addition, small differences in structure of solutes, such as planarity, in otherwise similar molecules can result in drastic changes in the affinity of a molecule for a bonded stationary-phase, which can further complicate the prediction of octanol–water partitioning from chromatographic retention data.

Chromatographic support materials can be used to measure octanol–water partitioning, while avoiding interfacial interactions governing reversed-phase retention, through the use of a generator column. Here, a packed chromatography column is filled with octanol containing the dissolved analyte. The bulk octanol between the particles is then displaced by flowing water through the column while intraparticle octanol remains confined within the pores of the particles. Octanol-saturated water is then flowed at a sufficiently slow rate to allow equilibration with analyte in the intraparticle octanol phase, and the analyte that elutes is collected by solid-phase extraction. The analyte that was washed from octanol column is then quantified ex situ (usually by gas chromatography (GC) or high-pressure liquid chromatography (HPLC)) and used to determine K_{ow}. The generator column method is procedurally similar to HPLC or GC (carrying out a measurement is simple) while advantageously measuring K_{ow} directly. However, generator columns are similarly challenged by large sample requirements and long measurement times. Measuring K_{ow} of charged compounds also remains a challenge due to limited solubility in octanol leading to depletion of the analyte from the generator column.

In the present work, we employ the concept developed in generator column measurements to measure partitioning of an analyte into an octanol phase confined in the pores of individual C_{18}-chromatographic silica particles. The overwhelming challenge of displacing and manipulating the 100 fl volume of octanol trapped in a single particle is avoided by determining the concentration of octanol-partitioned analyte inside the particle using confocal Raman microscopy. This approach reduces the volume of the octanol receiver phase by more than a factor of 10° compared to microfluidic techniques and decreases the amount of analyte required to make an octanol–water partition measurement to less than 3 pmol. Confocal Raman microscopy has been previously utilized to probe the interior chemistry of chromatographic silica particles, including studies of the functionalization of the silica surface, the interfacial solvation environment of C_{18}-stationary phases, the mechanism of surfactant-mediated ion-pair retention, and femtoliter-scale solid-phase extraction of PAH compounds. In the present work, within-particle confocal Raman microscopy is extended to liquid–liquid (octanol–water) partitioning. Collection of Raman scattering from analytes within octanol-filled pores of C_{18} silica particles is demonstrated, where quantitative determination of the octanol–water partition coefficient is achieved by using the Raman scattering signal from the n-octanol and surface-bound C_{18} alkyl chains as an internal standard. The method is tested for measurements of the pH-dependent octanol–water partitioning of naphthoic acid, and results are compared to traditional shake-flask measurements and to sorption onto C_{18}-modified silica without octanol present within the pores.

### EXPERIMENTAL SECTION

#### Reagents and Materials.
Spherical chromatographic silica particles were obtained from YMC America (YMC-Pack ODS-A, Allentown, PA). The particles were monofunctional, C_{18} derivitized, and end-capped with trimethylchlorosilane. The specific surface area (93 m^2/g), mean particle diameter (10 μm), carbon content (6.9%), pore diameter (319 Å), and pore volume (0.80 mL/g) of the silica were reported by YMC America. The carbon content and specific surface area were used to determine the C_{18} surface coverage of 3.4 μmol/m^2. The density of the silica skeletal structure (1.85 g/cm^3) used in the single-particle pore volume calculation was determined by methanol displacement of a known mass of silica particles. The volume occupied by the C_{18} phase of 0.17 mL/g was determined by measuring methanol–water displacement before and after removing the C_{18}-phase by oxidation in a muffle furnace at 800 °C. This value was also used to determine the pore volume of the bare silica, 0.97 mL/g.

Octanol (Reagent, 99%) was obtained from MP Biomedicals, LLC (Solon, OH). Methanol (Photrex, ≥99.8%) and hydrochloric acid (Macron, 36.5–38%) were obtained from Avantor (Center Valley, PA). 1-Naphthoic acid (Aldrich, 96%) and sodium hydroxide (Aldrich, ≥98%) were obtained from Sigma-Aldrich (St. Louis, MO). Water used for this experiment was filtered using a Barnstead GenPure UV filtration system (ThermoFisher Scientific, Waltham, MA) and had a minimum resistivity of 18.0 MΩ cm.

A microfluidic flow cell was constructed for in situ Raman microscopy. A diagram of the cell has been published previously briefly; briefly, two 4 mm diameter holes were drilled 11 mm apart in a 5 mm thick, Pyrex glass cover plate (VWR, Radnor, PA) to which two 2.4 mm i.d. Luer adapters (Value Plastics, Inc., Fort Collins, CO) were connected with Devcon 5 min epoxy (ITW Devcon, Danvers, MA). A glass coverslip (Gold Seal, Erie Scientific Co., Portsmouth, NH) was attached to the coverplate using 3M, 140-μm thick double sided tape (TapeCase Ltd., Elk Grove Village, IL), where a 2.5 mm wide × 11 mm long channel was cut between the holes in the cover plate creating a 3.9 μL flow channel. Tubing used in flow experiments was 1.6 mm o.d. × 2.4 mm i.d. Viton elastomer (Cole-Parmer, Vernon Hills, IL). A microscopy well cell was constructed for measuring naphthoic acid/octanol standards in solution. The cell consisted of a 12 mm length of 10 mm i.d. glass tubing fixed to a glass coverslip (Gold Seal, Erie Scientific Co.) and had a minimum resistivity of 18.0 MΩ cm.

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Confocal Raman Microscopy. A detailed description of the confocal Raman microscope used in this work has been described previously. Briefly, a 638 nm diode laser beam (Innovative Photonic Solutions No. 1063SH0035B-TH-L) is directed into a spatial filter (Thorlabs, KT310) where the beam is focused through a 5-μm pinhole and then collimated using a pair of antireflective, aspheric lenses (Thorlabs, C560TME-A). The collimated beam is then passed into a filter cube carrying an excitation filter (Semrock, FF01-640/14) and dichroic beam splitter (Semrock, Di02-R635) which reflects the beam into a 100X, 1.4 N.A. oil immersion objective (Nikon, Plan APO VC) mounted on an inverted fluorescence microscope frame (Nikon Eclipse TE-200), slightly overfilling the back aperture. Scattered light is collected using the same objective and passed back through the dichroic beam splitter. The light leaving the microscope is collected and collimated before passing through a final, high-pass filter (Semrock, BLP01-635R). The filtered, Raman scattered light is then focused onto the entrance slit of a Chromex 250IS spectrograph which is set to 50 μm, defining the confocal aperture in the horizontal-dimension. Spectra are dispersed using a diffraction grating with 600 lines/mm blazed at 750 nm and collected on a charge couple device (CCD) detector (Andor, iDus DU401A) where the vertical dimension of the confocal aperture is defined by limiting acquisition to 3-pixel rows (78 μm).

To collect Raman scattering from the interior pores of single chromatographic particles, the laser beam was brought to focus at the coverslip–solution interface where a visible reflection of the focused spot is produced. The microscope stage was translated in the x and y dimensions, until the focused spot was positioned directly below the center of an isolated particle on the coverslip surface. The microscope objective was then translated upward 5 μm in the z dimension, centering the confocal volume inside the particle. Raman spectra were collected using 60-s acquisitions. Raman spectra of the octanol–naphthoic acid standards were collected from ~0.5 mL aliquots in a microscope well cell. The laser was brought to focus at the coverslip–solution interface and then translated 5–15 μm into solution. A spectrum was acquired with 60-s exposure time from each of three standards. The ratio of the areas of the naphthoic acid ring-breathing mode and the octanol CH2-twisting mode were averaged for the three samples to provide relative intensities for quantitative analysis. All spectra were baseline corrected using a custom Matlab (Mathworks, Natik, MA) program which fits the baseline portions of the spectrum to a seventh-order polynomial. Following correction, Raman scattering bands were fit with Gaussian shapes to determine peak areas.

Sample Preparation and Characterization. Naphthoic acid samples were prepared in 0.5 mM citrate buffer adjusted to a final pH by addition of small amounts of sodium hydroxide or hydrochloric acid. Mutually saturated solvents were used in all experiments to avoid changes in solvent composition. For measuring octanol–water partitioning, the pores of C18 chromatographic particles were filled with octanol by dispersing particles into octanol; the dispersion was centrifuged to collect particles, the supernatant removed, and particles suspended in octanol saturated water by sonication for a short (~10 s) time. Octanol-filled particles in water were injected into a micro-fluidic flow cell and allowed to settle and adhere to the glass surface for ~2 h. A syringe pump (Harvard Pump 11 Plus, Harvard Apparatus, Holliston, MA) was used to flow octanol-saturated water into the flow cell at a rate of 0.5 mL/min for ~5 min while monitoring the flow cell using bright-field microscopy to confirm particles adhered to the surface. Following identification of stationary particles, naphthoic acid samples were introduced at a flow rate of 0.5 mL/min to supply a constant concentration of naphthoic acid to the particle. The CH2-twisting mode at 1303 cm−1 from C18 and octanol n-alkyl chains as well as the octanol C=O stretch at 182 cm−1 were monitored throughout the experiment to ensure constant presence of octanol in the pores. Accumulation of naphthoic acid was monitored by measuring the increase in Raman scattering from the naphthoic acid ring-breathing mode at 1382 cm−1 using sequential 15 s acquisitions; equilibrium was confirmed when Raman scattering remained constant across subsequent acquisitions. At this point, Raman spectra were collected from the interior of three different particles for quantitative analysis.

For measurements of partitioning onto the C18 surface, C18-silica particles were first wetted by dispersing in methanol. Methanol/particle solution was injected into a flow cell and particles were allowed to settle before the cell was placed in a 120 °C oven for 5 min allowing methanol to evaporate which causes the particles to adhere to the glass surface. Particles were rewetted with methanol and rinsed with water prior to introduction of a naphthoic acid sample into the cell. A syringe pump (Harvard Pump 11 Plus, Harvard Apparatus, Holliston, MA) was then used to flow water into the cell at a rate of 0.5 mL/min while monitoring Raman scattering from the particle interior until no methanol Raman bands were observed in the spectrum. At this point, a naphthoic acid sample was introduced. Accumulation of naphthoic acid was monitored by measuring the increase in Raman scattering from the naphthoic acid ring-breathing mode at 1382 cm−1 using consecutive 15 s acquisitions, where equilibrium was confirmed when the Raman scattering intensity was no longer changing. Raman spectra for quantitative analysis were then collected from the interior of three different particles.

In order to compare the results to traditional measurements of octanol–water partitioning, the distribution of naphthoic acid between bulk octanol and water phases was determined using a traditional shake-flask approach. Stock solutions of 50 mM and 5 mM naphthoic acid were prepared in water-saturated octanol for pH 2.0 and pH 6.0 measurements, respectively. Aliquots (1 mL) of the stock solution were transferred to scintillation vials and 19 mL of octanol-saturated 10 mM citrate buffer at pH 2 or pH 6 were added. Vials were shaken until turbid and mixed overnight (~15 h) to ensure equilibrium concentrations of naphthoic acid in each phase. Equilibrated samples were centrifuged to separate phases and the absorbance of the water phase measured. UV–vis spectra of naphthoic acid standards were measured using a Hitachi U-3310 spectrophotometer at pH 2 and pH 6 to determine the naphthoic acid absorption maxima and a calibration curve was prepared at each pH (see the Supporting Information). Naphthoic acid concentrations in the aqueous phases were determined by comparing the measured absorbances to the calibration curves; the concentration in the octanol phase was determined by the difference.

RESULTS AND DISCUSSION

Confining Octanol within the Pores of C18-Functionalyzed Silica Particles. In this work, the goal is to measure the
partitioning of analytes from aqueous solution into an octanol phase that is confined within the porous network of a chromatographic silica particle. To this end, maintaining the octanol within the pores of the particle is essential to the measurement. Octanol confinement within bare silica particles has been demonstrated previously in generator-column experiments; however, longer-term exposure of the octanol to an aqueous mobile phase caused octanol to leach out of the particles.17,26 To address this issue, the pores of bare silica particles were filled with octanol, and then the flow was switched to octanol-saturated water while monitoring Raman scattering from within the particle. Within several minutes of switching the flow water, octanol leached from the particle interior despite the water being octanol-saturated. To solve this problem, hydrophobic (octadecylsilane) functionalized silica was tested for octanol confinement. Hydrophobic surfaces have been used previously to control fluid flow of “wetting” versus “nonwetting” solvents in microfluidic circuits,9,27 and has specifically been used to separate octanol from water in 50-μm channels.9 A larger (32 nm diameter) pore, C18-functionalized silica was chosen so that the octanol-filled pore volume would dominate the solvation environment. When these particles were exposed to octanol and the solution switched to octanol-saturated water, octanol was retained indefinitely within the pores.

To demonstrate this result, a Raman spectrum from the interior of a C18-silica particle in contact with water is compared with an octanol-filled particle after exposure to octanol-saturated water (Figure 1). Upon filling with octanol, the C–H stretching modes at 1064 and 1077 cm\(^{-1}\), the CH\(_2\)-twisting mode at 1303 cm\(^{-1}\), and the C–H bending mode at 1438 cm\(^{-1}\) and C–H stretching modes in the 2800 cm\(^{-1}\) to 3000 cm\(^{-1}\) region all increase relative to the empty particle, due to the incorporation of the C\(_8\) acyl chain from octanol. In addition, the octanol C–O stretch at 963 cm\(^{-1}\) appears, further indicating that the pores have been filled with octanol. Retention of octanol is confirmed by the Raman scattering from octanol remaining constant as experiments are conducted over hours.

**Quantitative Measurement of Octanol–water Partitioning within Octanol-Filled C\(_{18}\) Silica.** The octanol–water partition coefficient is defined as the ratio of the concentration of a molecule, \(M\), dissolved in octanol relative to its concentration in water in a two-phase partition equilibrium:

\[
K_{ow} = \frac{[M_{oct}]}{[M_{H_2O}]} \tag{1}
\]

Because the magnitude of \(K_{ow}\) varies widely from one molecular structure to another, this partition coefficient is often expressed logarithmically as \(\log K_{ow}\). As an example compound to test the proposed methodology, the partitioning of naphthoic acid into octanol confined within the pores of a single C18-derivitized chromatographic particle was measured by collecting Raman scattering from within the particle interior after equilibration; see Figure 2. Quantification of naphthoic acid was achieved by measuring Raman scattering from the n-alkyl chains of octanol and surface-bound C18 as an internal standard. It has been previously demonstrated that the area of the CH\(_2\)-twisting mode (1303 cm\(^{-1}\)) from n-alkyl chains responds linearly to the concentration of methylene groups, with a slope that is independent of chain length.23 Thus, by comparing the scattering from the naphthoic acid ring-breathing mode (1382 cm\(^{-1}\)) to scattering from the CH\(_2\)-twisting mode in a free-solution sample of known hydrocarbon concentration, it is possible to generate a response factor that allows the concentration of naphthoic acid to be quantified within the octanol-filled pores of the particle.

Calibration of the naphthoic acid response was achieved by measuring Raman scattering from naphthoic acid/octanol standards in methanol (see the Supporting Information). A response factor was determined from the peak areas, \(A\), of the naphthoic acid ring-breathing mode and of the octanol CH\(_2\)-twisting mode (eq 2), ratioed to the molar concentrations (in brackets) of naphthoic acid and octanol, and the corresponding concentration of CH\(_2\) groups, in the solution:

\[
F = \frac{A_{CH_2}[naphthoic \text{ acid}]}{A_{Naphthoic}[\text{octanol} \times 7CH_2]} = \frac{A_{CH_2}[naphthoic \text{ acid}]}{A_{naphthoic}[CH_2]} \tag{2}
\]

![Figure 1. Raman spectra from the interior of a single C\(_{18}\) particle before (black) and after (red) filling with octanol.](image)

![Figure 2. Normalized Raman spectra from the interior of a single octanol-filled chromatographic particle after equilibration with 500 μM naphthoic acid in pH 2 buffer.](image)
Determining the naphthoic acid concentration within the pores of the particle was then accomplished by multiplying the measured Raman scattering from naphthoic acid relative to the CH$_2$ scattering by the response factor and by concentration of CH$_2$ in the pores. The total concentration of CH$_2$ groups in the pores derives from the concentration of octanol (from the density of octanol times the pore volume of the C$_{18}$-derivatized silica divided by the pore volume of the bare silica, [octanol] 7 [C$_{18}$] = 5.22 M) and the concentration of C$_{18}$ chains in the pores (from carbon analysis of the stationary phase and the pore volume of the bare silica, [C$_{18}$] = 0.33 M):

$$\text{[naphthoic acid]} = f \frac{A_{\text{naphthoic}}[\text{CH}_2]}{A_{\text{octanol}}}$$

$$= f \frac{A_{\text{naphthoic}}([\text{octanol}] \times \chi_{\text{octanol}} + [\text{C}_{18}] \times \chi_{\text{C}_{18}})}{A_{\text{octanol}}}$$

(3)

Dividing the within-pore concentration of naphthoic acid in octanol by its source-phase (aqueous) concentration gives the partition coefficient. From the results in Figure 2, the octanol—water partition coefficient of naphthoic acid at pH 2.0 (98% protonated based on its pK$_a$ of 3.7) is $K_{ow} = 840 \pm 40$ or log $K_{ow} = 2.9 \pm 0.1$. The uncertainty derives from the reproducibility of measurements made in several different particles (see the Supporting Information).

Solvation of the naphthoic acid within the wide (32 nm) octyl-porous chromatographic silica should be dominated by octanol, having a mole fraction in the pores of 94% compared to 6% C$_{18}$ alkyl chains. However, the longer C$_{18}$ alkyl chains represent 13% of the methylene groups within the pores. To test whether the C$_{18}$ chains significantly change the solvation of naphthoic acid in the pores compared to a bulk octanol phase, the partitioning of naphthoic acid between octanol and water at pH = 2 was measured using a traditional EPA shake-flask approach with UV–vis detection (see the Supporting Information). The resulting log partition coefficient, log $K_{ow} = 3.04 \pm 0.06$ is indistinguishable from the Raman microscopy result, log $K_{ow} = 2.9 \pm 0.1$. These results indicate that the C$_{18}$ chains within the pores do not produce a significantly different solvation environment for naphthoic acid compared to bulk octanol.

Measuring pH Dependent Octanol–Water Distribution of Naphthoic Acid. For acidic or basic compounds, the protonation state of the molecule plays a major role in governing octanol–water partitioning. The changes in molecular charge, as protons are added or removed from the molecule, leads to pH dependent distribution of the molecule between the two phases. To provide a measure of lipophilicity of an acidic or basic compound near its pK$_a$, where both protonated and deprotonated forms of the molecule can exist, the distribution coefficient $D$ or its log expresses the ratio of sum of concentrations of protonated and deprotonated forms in octanol, relative to the sum of their concentrations in water:

$$D = \frac{[\text{HA}]_o + [\text{A}^{-}_o]}{[\text{HA}]_w + [\text{A}^{-}_w]}$$

(4)

The pH-dependent distribution of a compound can be modeled by combining simple acid–base equilibrium (eq 5) with the definition of the distribution coefficient (eq 4):

$$K_d = \frac{[\text{A}^{-}_w][\text{H}^+_w]}{[\text{HA}_w]}$$

(5)

$$D = \frac{K_{HA}^{\text{ow}} + K_{A^{-}_w}^{\text{ow}}(10^{\text{pH} - pK_a^{\text{ow}}})}{(1 + 10^{\text{pH} - pK_a^{\text{ow}}})}$$

(6)

where $K_{HA}^{\text{ow}}$ and $K_{A^{-}_w}^{\text{ow}}$ are the octanol–water partition coefficients of the protonated and deprotonated forms of the compound.

To test the measurement of a pH-dependent octanol–water distribution, Raman scattering from naphthoic-acid partitioned into the octanol-filled pores was measured as a function of pH at intervals of 0.5-pH units around the pK$_a$ of naphthoic acid (Figure 3A). Raman spectra show the intensity of the naphthoic acid ring-breathing mode decreasing as the pH of the surrounding aqueous solution is increased, while scattering from the CH$_2$-twisting modes of octanol and C$_{18}$ chains remains constant. This is the expected result, as the deprotonated naphthoate anion should be less soluble than neutral naphthoic acid in the nonpolar octanol phase. Converting the Raman scattering intensity from the ring-breathing mode of naphthoic acid and naphthoate to the within-pore concentrations (see above) yields distribution coefficient data (Figure 3B), which can be fit to eq 6. The pH dependence of the distribution coefficient is well fit by the model allowing determination of the partition coefficients of both naphthoic acid and naphthoate and the pK$_a$ of the proton transfer reaction. The log partition coefficients of the two species at pH 2 and pH 6 are log $K_{HA}^{\text{ow}} = 2.9 \pm 0.1$ and log $K_{A^{-}_w}^{\text{ow}} = 1.5 \pm 0.2$ and are in agreement with shake-flask results, 3.04 ±
0.06 and 1.505 ± 0.009 (see the Supporting Information); the fitted value of the pK<sub>a</sub> = 3.6 ± 0.1 is within its uncertainty of the literature value, pK<sub>a</sub> = 3.69.  

**Comparing Octanol–Water Partitioning to Sorption to a C<sub>18</sub> Surface.** Retention of a compound on a reversed-phase chromatographic column is often used to predict octanol–water partitioning. It is relevant, therefore, to examine how sorption of naphthoic acid at the C<sub>18</sub>–water interface compares to its distribution into octanol-filled pores in the same C<sub>18</sub> stationary phase material. When naphthoic acid was removed by oxidation in a furnace (see the Experimental Section), to ensure that the interfacial population of naphthoate ions at a C<sub>18</sub>–water interface would differ significantly from its partitioning from water into octanol. To test this hypothesis, Raman scattering from naphthoic-acid accumulating at the C<sub>18</sub> interface from the aqueous solution was measured across the same pH range as octanol–water partitioning, and the results compared in Figure 4 to partitioning into octanol. Quantification of naphthoic acid in the C<sub>18</sub> chains was based on scattering from the ring-breathing mode relative to scattering from the CH<sub>2</sub>-twisting mode of the surface-bound C<sub>18</sub> chains (eq 7).

\[
\text{naphthoic acid} = F \frac{A_{\text{naphthoic}} \left( C_{\text{18}} \right) \times 17 C_{\text{H}_2}}{A_{\text{CH}_2}} 
\]

The concentration of the C<sub>18</sub> chains in the interphase, [C<sub>18</sub>], was determined by dividing the moles of C<sub>18</sub> (from carbon analysis) by the volume of the C<sub>18</sub>-phase determined from the difference in volume displacements between the C<sub>18</sub>-derivitized silica, and the same silica where the C<sub>18</sub> phase was removed by oxidation in a furnace (see the Experimental Section). To ensure that the interfacial population of naphthoate did not saturate the C<sub>18</sub> surface, an accumulation isotherm was measured over a range of naphthoic acid concentrations; 50-μM source-phase was used for pH values at or below pH 3.5 to ensure results remained within the linear response region of the isotherm (see the Supporting Information). Beyond pH 3.5, naphthoic acid concentrations were increased 10-fold to maintain sensitivity for detection; at pH values greater than 3.5, sorption of naphthoic acid is greatly decreased and surface concentrations remain within the linear isotherm region at the higher solution concentration.

In agreement with the expectations, the results in Figure 4 show that naphthoate anion is indeed surface-active and accumulates at the C<sub>18</sub>–water interface at a 2.0-fold greater concentration than in octanol. It is interesting to note that an even greater discrepancy exists with naphthoic acid at the low-pH limit, where naphthoic acid accumulates in the C<sub>18</sub> phase at a 5.2-fold greater concentration than it partitions into octanol. This is likely due to a combination of both interfacial activity of naphthoic acid and strong hydrophobic interactions where naphthoic acid can avoid contact with water in the less polar C<sub>18</sub> phase compared to octanol. These results demonstrate the difficulty in predicting partition coefficients based on reversed-phase chromatographic retention, where a single correction factor would not accurately predict partitioning across the pH range of an acid–base equilibrium. The results further support the concept of measuring accumulation of solutes into octanol-filled pores in a reversed-phase chromatographic support material as a realistic model for octanol–water partitioning.

**Sample Size and Throughput.** One goal of this work was decreasing the amount of sample required to measure octanol–water partition coefficients. This could be especially important for characterizing new drug candidates or small quantities of potential environmental threats. From the concentration of naphthoic acid in the octanol/C<sub>18</sub>-filled pores in the low pH limit (0.42 M) and the pore volume of a single 10-μm diameter particle (0.34 pL), it is possible to determine the amount or analyte required to satisfy the single-particle collector, 0.14 pmol. The source phase would need to supply a ∼20-fold excess of this amount to avoid depletion of the analyte concentration or 2.8 pmol. The volume of a 500-μM solution required to supply this amount analyte is 5.6 nL, which is a ∼20-fold decrease compared to microfluidic approaches. The sample amount and source phase volume required for this measurement scales linearly the octanol volume, which is proportional to the particle volume. While 10-μm particles are convenient for handling, the quality of the laser beam focus, which has a radial size (1/e<sup>2</sup>) of w = 0.59 μm, allows detection within particles as small as 2-μm diameter with no loss in sensitivity, where the probe volume can reside entirely within a particle of this size. This is apparent in the quality of the laser focus produced in the center of a 2-μm particle (see Supporting Information). Since particle volume varies with the cube of the particle diameter, a 125-fold reduction in the required sample volume can be achieved with a 2-μm diameter particle with no sensitivity loss, as demonstrated in Supporting Information. The amount of naphthoic acid needed to equilibrate this particle (with a 20-fold molar excess) is 22 fmol corresponding to 45 pL of a 500-μM source-phase solution.

Traditional methods of measuring octanol–water partition coefficients are tedious and slow. With both generator column and shake-flask methods, equilibration times range from tens of minutes to hours. This is because the large volumes of source and receiver phases, where the achievement of equilibrium is slow due to molecular transport over large distances. In the present work, equilibration is fast as a consequence of the efficiency of diffusive transport over small distances that characterize the receiver and source phases. The time required for accumulation of 500 μM naphthoic acid (pH 2.0) into a 10-
μm octanol-filled particle is ~40 s (see Figure 5). Because the quantification by confocal-Raman detection is in situ within the receiver phase, separation of the two phases and ex situ measurement are avoided, which means that the measurement can be achieved within the partitioning equilibration time.

Octanol–water partitioning is commonly used in screening applications to determine the therapeutic potential of drug candidates or the environmental risk of toxic compounds. Using traditional methods, screening is costly in both time and reagents. In situ detection within a single chromatographic particle eliminates the need for large quantities of sample and reagents. With C18-functionalized particles and octanol-saturated water, the within-particle octanol phase remains confined to the particle pores. Because of the small collection volume, transport out of the octanol phase is fast, as shown in Figure 5. This means that multiple partitioning measurements could be made using a single particle by exchanging the analyte-containing source phase with buffer which quickly prepares the particle for evaluation of a new sample. This approach to the measurement should lead to greater reproducibility, which is presently ±10% particle-to-particle (see the Supporting Information). While this level of reproducibility is acceptable for many sample-limited applications, the variability is presently greater than results obtained using traditional shake-flask methods. The precision of the within-particle measurement should benefit from improvements in sample handling to deliver analyte to a single-particle collector.

**ASSOCIATED CONTENT**

Supporting Information

Raman scattering from a standard solution of octanol and naphthoic acid; UV–Vis calibration of protonated and deprotonated naphthoic acid; repeatability of Raman scattering measurements from octanol-filled particles; naphthoic acid to C18 adsorption isotherm; Raman spectra of naphthoic acid partitioned into octanol within 2-μm particles; and references. This material is available free of charge via the Internet at http://pubs.acs.org. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00634.

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**Notes**

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