Published in final edited form as:

Anal Chem. 2015 March 03; 87(5): 2520–2526. doi:10.1021/ac504683d.

Submicrometer Particles and Slip Flow in Liquid Chromatography

Benjamin A. Rogers[†], Zhen Wu[‡], Bingchuan Wei[§], Ximo Zhang, Xiang Cao, Oyeleye Alabi, and Mary J. Wirth^{*}

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, United States

Abstract

Smaller particles have progressively led to higher efficiency in liquid chromatography, particularly for proteins, due to smaller diffusion distances. Particle diameter has recently entered the submicrometer region, with the back-pressure requirements alleviated by slip flow.

Graphical abstract

The efficiency of liquid chromatographic separations has continuously improved over the last 2 decades, giving higher speed and resolution.^{1,2} Two advances have contributed. The first is the use of porous particles as small as 1.7 μm , which reduces the distance over which mass transport occurs. These smaller particles have necessitated higher pressure pumps,³ which ushered in the term “ultra-performance liquid chromatography”, UHPLC. The other advance is the use of core-shell particles, which have a solid core having a thin shell of porous particles on the outside.⁴ The fully porous and the core-shell particles are depicted

^{*}Corresponding Author. mwirth@purdue.edu.

[†]Present Addresses

B.A.R.: Gilead Sciences, 1021 Hayter Rd. NW, Edmonton, AB T6S 1A1, Canada.

[‡]Z.W.: Abbvie Biotherapeutics, 1500 Seaport Boulevard, Redwood City, CA 94063.

[§]B.W.: Genentech, 1 DNA Way, South San Francisco, CA 94080.

The authors declare the following competing financial interest(s): The senior author is part owner of a company that has licensed technology based on this research.

schematically for comparison in Figure 1, showing that each is comprised of uniform, nonporous colloidal silica particles aggregated to achieve the desired morphology. A shell thickness of half of 1.7 μm gives a comparable advantage to that of the 1.7 μm fully porous particles but without the need for the higher pressure pump. Sub-2- μm core-shell particles give even higher efficiency, with thinner shells giving higher speed and better resolution. The core-shell particles have been widely adopted by the pharmaceutical industry for analysis of small-molecule drugs. Figure 1 also depicts a nonporous silica particle, and this article discusses how nonporous particles promise a further advance when they are submicrometer in diameter.

As exciting as the sub-2- μm and core-shell particles have been for separations of small molecules, they do not fully address what is perhaps the most pressing need in separations: proteins.⁵ Protein drugs, particularly therapeutic monoclonal antibodies,⁶ represent the fastest growing segment of the pharmaceutical industry. Liquid chromatography is indispensable for characterizing composition of small-molecule drug substances, and for proteins the task is all the more difficult due to the multiplicity of post-translational modifications, oxidation of methionine groups, disulfide scrambling, fragmentation, aggregation, and other processes that change the protein sample. Top-down proteomics is another area where better separations of intact proteins are needed⁷ because insufficient resolution requires multiple dimensions of separations. This feature article explores why smaller particles and thinner shells have not impacted protein separations more significantly than they have and how the nonporous submicrometer particles of Figure 1 promise to improve protein chromatography.

SMALLER PARTICLE SIZE IMPROVES COLUMN EFFICIENCY

The gradual development of smaller particles and thinner shells has been guided by the role they play in reducing the broadening of peaks. The van Deemter equation is a simple way of describing this role. The plate height, H , is a measure of peak variance normalized for separation length. Smaller H is thus better. The van Deemter equation is expressed as H vs v , where v is the mobile phase velocity.

$$H = A d_p + \frac{2\gamma D}{v} + \frac{C_{\text{pores}} d_p^2}{2D} v \quad (1)$$

The equation illustrates that smaller particle diameter, d_p , gives a smaller plate height by affecting both the first and third terms. Diffusion is described by the diffusion coefficient, D . The term A is a measure of the radial heterogeneity of the packing density. The term $2\gamma D$ is referred to as the B term, where γ is the factor by which the diffusion along the separation axis is obstructed by the solid particles, and D is the diffusion coefficient. The term C_{pores} is the factor by which the diffusion in and out of the porous medium is obstructed by the solid particles. There are also extra-column contributions, e.g., the nonzero injection and detection volumes, the parabolic flow profile through the tubing, and any broadening from the frits and the geometric effects of the column itself. Two other C terms can contribute but are neglected.⁸ One is C_m , which results from the parabolic flow profile between particles. It

plays the same role with respect to particle size as C_{pores} . The other is C_s , which is related to the desorption time of the analyte from the stationary phase. These terms are left out because they usually give smaller effects that would needlessly complicate the discussion of why smaller particle diameter reduces broadening in liquid chromatography.

SMALLER PARTICLE SIZE EVENTUALLY GIVES DIMINISHING RETURNS

To illustrate how much can be gained from smaller particle diameter, or thinner shell of a core-shell particle, van Deemter plots for varying particle sizes are provided in Figure 2a for the case of small-molecule separations. The curves were generated by fitting published van Deemter plots for 5 μm particles to recover A , B , and C .⁹ The synthetic curves show that the minimum plate heights are on the order of twice the particle diameter, hence the advantage of smaller particles. The magenta line is the projected curve for the hypothetical case of fully porous particles of 0.5 μm in diameter, calculated using the Kozeny–Carman equation.¹⁰ While it reduces plate height by another factor of 2, the pressure is unreachable. Current UHPLC instruments would give a mobile phase velocity of less 0.2 cm/s for the 0.5 μm particles in a column of these dimensions, thus giving no advantage over the 1.7 μm particles currently used. These curves also depict the general behavior of core-shell particles having shell thicknesses of half the particle diameter. In other words, the diffusion distance for a 0.5 μm shell thickness is equivalent to a 1 μm fully porous particle. The shell can be arbitrarily thin without adding to the pump pressure, but ultimately, the surface area of the stationary phase would give insufficient retention. It should be noted again that equation to generate these curves does not include any broadening from the instrument, H_{ec} , in eq 1, and it does not include slow kinetics of desorption that might occur with some samples. The curves suggest that current technology is approaching the optimum for both porous and core-shell particles in separations of small molecules; little is to be gained in going with smaller diffusion distances.

For proteins, the picture is changed markedly because of the much larger molecular size of the protein, slowing the diffusion by an order of magnitude. Synthetic van Deemter curves for a protein with a 10-fold slower diffusion coefficient than the small-molecule are shown in Figure 2b. The optimal flow rate is now much lower, giving a potentially huge advantage in using 0.5 μm particles. The achievable pressure of modern instruments, which is 17 500 psi, would give a velocity of 0.05 cm/s for 0.5 μm particles and these column dimensions, resulting in about a 5-fold smaller plate height. For core-shell particles, a shell thickness of 0.25 μm is projected to give this same advantage, and longer column lengths could be used because back-pressure is much less of an issue.

ACCESSIBLE SURFACE AREA IS AN ISSUE

Before rushing to the lab to make 0.5 μm porous or core-shell particles, there is a second factor that needs to be considered: pore size. The pore size that has traditionally been used for protein separations is 300 Å. This might seem adequate since the diameter of a 100 kDa protein is about 100 Å, but entropically, a 100 Å sphere does not readily diffuse into a 300 Å pore. This is well-known from size-exclusion chromatography, where 300 Å is a common pore size that significantly excludes 100 kDa proteins. We studied the entropic exclusion of

molecules as a function of pore size experimentally by packing capillaries with the colloidal silica, which is the same material that is used to make fully porous particles and porous shells. We measured obstructed diffusion coefficients for various sizes of proteins to measure the accessible pore volume as a function of pore size and molecular size. The tabulated results from the published work have been prepared in graphical format in Figure 3.¹¹ The percent of accessible pore volume is plotted vs molecular radius for molecules ranging in size from a small dye to a monoclonal antibody. The solid curves represent theoretical curves we derived based on the theory behind size-exclusion chromatography.¹² The curves fit the data well and extrapolate to 100% accessibility for infinitely small molecules. The message from this figure is that the 300 Å pore size excludes half of the pore volume for large proteins, such as monoclonal antibodies. For every loss of free volume, there is a disproportionate loss of surface area because much of the surface area is in the narrow apex regions where particles meet. Since pore size is controlled by the diameters of the colloidal particles used to form porous particles, the data in Figure 3 suggest that larger colloidal particles, e.g., 0.5 μm , need to be used to form the fully porous or core-shell particles for these important proteins.

Forming small porous particles with such large colloidal particles would mean that the porous particles or core-shell particles would be made of fewer colloidal particles. One might ask, since the 0.5 μm particles were shown in the van Deemter plot of Figure 2b to be so advantageous and the predicted pressure was not extreme: why not just pack the nonporous 0.5 μm colloidal silica particles themselves rather than using them to form particles? This would not only save the trouble of forming the porous or core-shell particles, it would also minimize the distance across the mobile phase between particles to reduce the C_m terms. It is certainly worth a try, and this is exactly what we did.

Submicrometer particles of uniform size spontaneously form face-centered cubic crystals on glass slides, giving beautiful opalescent coatings.¹³ Opals in geology are formed from natural colloidal silica of uniform size, hence the opalescence is not a mere coincidence. In separations, the uniformity of the face-centered cubic crystals would drastically reduce the A term in the van Deemter equation because the medium is almost perfectly ordered. We found that silica colloidal crystals could be coaxed into forming in capillaries, albeit with small crystalline domains, and these indeed gave an immeasurably small A term of 5 nm or less.¹⁴ We used these in electrochromatography at that time to prepare a van Deemter plot which fit to just the second term of eq 1, with broadening entirely due to diffusion. We also reported a preliminary result indicating that pressure-driven flow was feasible,¹⁵ although this result was initially met with skepticism because the flow rates were higher than chromatographic theory predicted.¹⁶

SLIP FLOW REDUCES THE NEEDED PRESSURE

Nonetheless, we studied pressure-driven protein chromatography in capillaries packed with silica colloidal crystals. The results¹⁷ surprised us in two ways: the back-pressure was much lower than is predicted,¹⁸ and the plate height was very much lower than is predicted.¹⁹ Both were pleasant but confounding surprises. We now understand why the back-pressure is reduced: the phenomenon known as slip flow is occurring.^{20,21} The chromatography

textbooks had taught us that the velocity of the mobile phase goes to zero at the wall, whereas in fluid dynamics this would be presented as the “no-slip” boundary condition. In reality, the velocity would be zero at the wall only if the attractive interactions between the mobile and stationary phase molecules were exactly as strong as those between the mobile phase molecules themselves. This is far from the case for RPLC since the functional groups on the surface have been chosen to be hydrophobic, giving little interaction with the mobile phase in reversed-phase liquid chromatography. Consequently, the velocity of the mobile phase at the wall is nonzero.

With slip flow, the average velocity, $\langle v_{\text{slip}} \rangle$, is increased from the conventionally expected velocity, $\langle v_{\text{no-slip}} \rangle$, by an additive amount from the nonzero velocity at the wall, v_{wall} .

$$\langle v_{\text{slip}} \rangle = \langle v_{\text{no-slip}} \rangle + v_{\text{wall}} \quad (2)$$

This additive relation is illustrated in Figure 4a. Note that the velocity profile is parabolic profile for both the slip and no-slip cases and that there is simply an additive term in the slip case due to the velocity at the wall. For the case depicted in Figure 4a, the average velocity is a factor of 4 higher than that for the no-slip case. Because the parabolic profiles are the same for the slip and no-slip cases, the relative velocity distribution is narrower for the slip case.

Submicrometer particles can give high flow enhancements because of the nonzero wall velocity. Figure 4b shows the experimentally measured flow enhancements for a wide variety of particle diameters, ranging from 0.12 to 1.3 μm , all for nonporous particles with C_4 bonded phases and a mobile phase of water.¹⁰ The data show that the flow rate enhancement depends on particle diameter, and the enhancement becomes small for particle diameters about a micrometer. This may seem perplexing at first because the wall velocity is related to the fluid–surface interactions, not the particle size. In fact, the wall velocity is the same regardless of particle size, but wall velocity has a bigger impact on the overall flow rate when the velocity in the middle of the stream is smaller. Consequently, slip flow is only noticed when the walls are very close together. The relation between interstitial dimension and flow enhancement is described mathematically by the geometry depicted in Figure 4a, where a tangent is drawn to the parabola at the fluid–surface boundary. This tangent is $(dv/dr)_{\text{wall}}$, and the red triangle in the figure shows that this is the ratio of the velocity at the wall to the so-called slip length, L_s .

$$\left(\frac{dv}{dr} \right)_{\text{wall}} = \frac{v_{\text{wall}}}{L_s} \quad (3)$$

The slip length is what is fixed by the fluid surface interactions, with longer slip lengths giving higher flow enhancements. It was shown by Navier in 1823 that the volume flow rate (or the average velocity) is enhanced for fluid flowing through a capillary according to the slip length and the capillary radius, r .²²

$$\langle v_{\text{slip}} \rangle = \langle v_{\text{no-slip}} \rangle \left\{ 1 + \frac{4L_s}{r} \right\} \quad (4)$$

The solid curve in Figure 4b is a plot of this equation, using the effective hydrodynamic radius of the packed bed calculated from fluid dynamics,²³ and the best fit reports a slip length of $L_s = 63 \pm 3$ nm. This value is in agreement with the range of values estimated from previous studies of water–hydrocarbon surfaces,¹⁰ albeit much more precisely determined. The enhancement of 5-fold in flow rate for the 0.5 μm particles enables use of the same 5–10 cm column lengths widely used protein chromatography.

SMALLER PARTICLES GIVE INORDINATE IMPROVEMENTS IN PLATE HEIGHT

The second surprise mentioned earlier is that the improvement in plate height is about an order of magnitude better than was predicted from theory.¹⁹ Figure 5 shows a series of raw images of two protein peaks imaged inside a 75 μm i.d. capillary as they approach the end of a nearly 2 cm separation length. For a capillary packed with particles of 0.47 μm , we measured plate heights that were as small as 0.015 μm for BSA. This means that the plate height can be nearly 30-fold smaller than the particle diameter. Theory at the time suggested that the plate height could not be less than the particle diameter, so we were perplexed as to why the plate height was as much as 30 times smaller than expected. This exciting but perplexing news posed a challenge in publishing the work. For monoclonal antibodies, which are not pure, the plate height was measured to be 0.07 μm , which means that these are quite promising in biopharmaceutical analysis. Much of the discrepancy between theory and experiment has been explained by revised theoretical predictions in this region of unprecedentedly small plate heights.²⁴ Much of the earlier discrepancy is also due to a lowering of the plate height from the smaller distribution of velocities from slip flow, and the plate height is further decreased due to the inaccessibility of the regions where particles meet, which would contribute the most to plate height.¹¹

What is most important is a parameter that people rarely think about, which is the physical size of the peaks. Figure 5 shows a series of images from fluorescence microscopy in which two proteins have been well separated through a capillary length of just over 17 mm and are moving through the detector region. The peaks are extraordinarily narrow; narrower than the 75 μm width of the capillary. For comparison, a capillary packed with core–shell particles of 5 μm in overall diameter and shell thickness of only 0.25 μm gave a base width of only 5 s for one especially sharp peak.²⁵ Converting this to distance units gives a base width of 3.7 mm. This is vastly larger than the capillary using 0.5 μm particles. One normally thinks about peak width in the time domain, and a 5 s base width seems quite narrow for a protein, but it is the high flow rate that makes it intuitively seem sharp. The much narrower physical width afforded by submicrometer particles will not only give better resolution, but will also give better sensitivity because the peak is less diluted by mobile phase. The small physical broadening promises increased sensitivity and dynamic range.

The comparison of plate heights from the earlier van Deemter plots did not predict such a large improvement over conventional capillaries. As mentioned earlier, factors that make the capillary packed with 0.5 μm particles more efficient include the homogeneous packing, the lack of a pores, the obstructed diffusion, and the smaller distribution of mobile phase velocities. A factor that needs to be considered is the instrument, which we discuss in the next section.

INSTRUMENT CONTRIBUTES SIGNIFICANTLY TO BROADENING

So far we have presented results where we laboriously dipped capillaries into protein solutions to accomplish injection, put these into pressure fittings, and then performed on-column detection by fluorescence imaging. This avoided any instrument contribution, revealing that the column itself contributes very little broadening. Despite its extreme efficiency, it is not a commercially viable solution. For translating this work to practice, it is essential to use these capillaries with a commercial instrument enabling autosampling and injection, a programmable gradient for separation with integrated detection. The question now is whether broadening by the instrument becomes a significant factor.

We have studied protein separations using a commercial, fully automated, high-pressure nanoLC for autosampling and injection, along with gradient elution coupled with nanospray and detection by a commercial mass spectrometer.²⁶ Figure 6a shows a photograph of a capillary packed with 0.5 μm particles. The pressure fitting and pulled tip are visible in the photograph. As expected, the tip-pulling process burned off much of the polyimide coating for this capillary. Figure 6b shows a photograph of the tip area, more clearly revealing the opalescence of the packing material. A drop of mobile phase is visible on the end of the capillary, demonstrating that flow of mobile phase is achieved.

Using nanoLC–MS, the narrowest peak we have ever observed is shown in Figure 6c. On the basis of the mass of 7323 Da, this is a large peptide impurity, possibly a degradation product of one of the proteins. The fit to a Gaussian shows that the standard deviation in time units is 0.2 s. The peak widths for proteins were widely varying, which is likely due to protein heterogeneity, e.g., overlapping post-translationally modified forms. For this discussion, we will compare the smallest observed peak widths. To convert the narrow peptide peak width in Figure 6c to distance units, the 200 nL/min volume flow rate and 100 μm i.d. give a linear flow rate of 0.042 cm/s to give a base width of 340 μm . For the case of laborious injection without using a nanoLC, the 17 mm long capillary gave a base width of 64 μm . Correcting for separation length and for the fact that the peak width was measured inside of the packing material, which stretches the peak more than 3-fold due to the low porosity, the commercial nanoLC–MS combination broadens the peak about 10-fold. We are currently investigating how much of this order of magnitude in broadening from the nanoLC–MS can be reduced to enable full automation without sacrificing column efficiency.

Figure 6d shows the chromatogram for the entire model protein mixture. A fast acetonitrile water gradient and a C₄ bonded phase were used for the room-temperature separation. There is high resolution for the early eluting peaks and congestion for the late-eluting peaks. We showed that a slower gradient and a C₁₈ bonded phase begins to resolve the late eluting

peaks, which are contaminated by impurities and multiple proteoforms.²⁶ An interesting point is how to compare columns. People usually use peak capacity, taking the useful separation time range over the average peak width in the chromatogram. However, this is not accurate because none of the peak widths are attributable to a single species. In fact, better resolution from a higher accessible surface area will begin to pull proteoform peaks apart from one another and make apparent peak capacity look worse when the resolution is actually better. You need a delta function, i.e., a pure sample, to compare two different columns accurately. A perfectly pure protein will not necessarily give as narrow of a peak as a peptide because slow desorption from the stationary phase can occur, depending on conditions such as mobile and stationary phase as well as temperature. Reversed-phase chromatography columns could be more accurately compared if the proteins were preliminary purified to reduce the heterogeneity with respect to hydrophobicity.

Figure 6d also displays the chromatogram on expanded scales, where the *y*-axis was simply expanded by 10× in the middle panel and then 100× in the bottom panel. Peaks barely visible as bumps on the baseline have high S/N on the 100× expanded scale. The peak shapes are generally symmetric for nonoverlapped peaks. The sharpest peaks are the peptide peaks, which again illustrates that the protein peaks likely broaden by heterogeneity. This set of chromatograms illustrates the high dynamic range of the separations with submicrometer particles, which is due to the small physical volumes of the peaks.

FUTURE PROSPECTS

Most chromatographic columns are commercialized in stainless steel format for UV–visible detection. This format is widely used for quality control in the pharmaceutical industry because most organic drugs impurities will give absorbance at 210 nm. Further, not all impurities are detectable by mass spectrometry, and UV detection gives better quantitative reproducibility. Capillaries have short path lengths, limiting the sensitivity of UV detection. This raises the question of whether one can gain any advantage with submicrometer particles in stainless steel columns. The best UHPLC instruments contribute approximately 10 μL in dispersion volume, giving a 5.6 mm base width for a 2.1 mm i.d. column. This is enormous compared to the broadening from capillaries. The comparison is summarized visually in Figure 7 to illustrate how much is lost in going from capillaries to stainless steel columns. Contributions from the instrument include the parabolic flow profile through the tubing and the detector volume. Since columns do contribute broadening beyond the instrument contribution due to packing heterogeneity, the homogeneity of packed submicrometer particles could still have an impact. NanoLC instruments might begin competing in this arena since sensitivity is greatly enhanced from less peak broadening. The short path length across a capillary could be fully offset by the narrower peak width to give comparable sensitivity. Given the potential for such higher resolution without sacrificing sensitivity of UV absorbance, nanoLC might have a bright future in UHPLC.

A good question is whether submicrometer particles will impact small-molecule separations. The van Deemter plot of Figure 2a shouts no, but this was generated in the absence of slip flow. If you move that magenta dot to a 5-fold higher flow rate, there is now a 2-fold advantage in plate height for the 0.5 μm particle. The advantage is actually larger because

the model for the van Deemter plots did not consider that the A and C terms become negligible, so it is an open question. The surface area of $0.5\ \mu\text{m}$ particles is much lower than that of small-pore silica gel, so the selectivity would suffer. Using smaller particles would offset this, but other factors, such as the injected width and the diffusion rate on the stationary phase, become important. Instruments with higher pressure would shift the balance toward submicrometer particles. On the intermediate scale of molecular weights, peptide separations require wider pore silica, so it is possible that the submicrometer particles would already be advantageous in bottom-up proteomics.

The discussion has largely involved reversed-phase liquid chromatography because the phenomenon of slip flow enables use of $0.5\ \mu\text{m}$ particles with reasonable column lengths and flow rates. Normal-phase chromatography, e.g., chiral chromatography, would have slip flow when a nonpolar mobile phase is combined with a polar stationary phase, although these are typically small-molecule separations. Protein separations in which there is no slip flow can benefit from the use of submicrometer particles when the diffusion is obstructed, as the van Deemter plot of Figure 2b illustrates. For example, we obtained high resolution of intact glycoproteins of ribonuclease B by hydrophilic interaction liquid chromatography, commonly called HILIC.²⁷ This uses polar mobile and stationary phases. Currently, the slow diffusion of proteins, along with the smaller A and C terms, make virtually any protein separation potentially advantageous for submicrometer particles.

Acknowledgments

This work was supported by the National Institutes of Health under Grant R01GM101464.

Biographies

Benjamin Rogers earned his B.Sc. in Chemistry from Queens University in Kingston, Ontario, Canada, and his Ph.D. in Chemistry from Purdue University where he studied the theory and applications of slip flow chromatography. He is currently working for Gilead Sciences in Edmonton, Alberta, Canada, where he is working on developing and commercializing small-molecule active pharmaceutical ingredients.

Zhen Wu graduated from Tsinghua University with a B.S. and an M.S. in Chemistry. She worked for Johnson & Johnson Consumer China for 2 years and then earned her Ph.D. at Purdue University, where her research was on intact protein separations using slip-flow with nanoliquid chromatography–mass spectrometry.

Bingchuan Wei obtained his B.S. in Chemistry from Peking University. He graduated from Purdue University with a Ph.D in Chemistry, investigating the use of submicrometer particles for protein separations. He is now an associate scientist in the Protein Analytical Chemistry department in Genentech Inc.

Ximo Zhang graduated from University of Science and Technology of China with a B.S. in Chemistry. Currently she is a Ph.D. candidate in the Wirth group at Purdue University, working on LC–MS of peptide and intact proteins using submicrometer nonporous silica particles.

Xiang Cao graduated from the University of Science and Technology of China with a B.A. in Chemical Physics. As a Ph.D. candidate in the Wirth group, he has been developing highly efficient chromatographic columns for monoclonal antibody characterization.

Oyeleye A. Alabi earned his B.S in Chemical Engineering from Obafemi Awolowo University in Ile-Ife, Nigeria, and his M.S. in chemistry from Emporia State University. Currently, he is a Ph.D. candidate in the Wirth group, focusing on the development of nonporous silica reversed phase columns for the separation of impurities in intact monoclonal antibodies.

Mary J. Wirth graduated from Northern Illinois University with a B.S. in Chemistry and from Purdue University with a Ph.D. in Chemistry. Her research blends separation science and materials science. She is currently the W. Brooks Fortune Professor of Chemistry at Purdue University.

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Figure 1.
Schematic depiction of the three common particle morphologies used in chromatography.

Figure 2.

Calculated van Deemter plots are shown as a function of particle diameter, d_p , for two different molecular size scales: (a) small-molecule and (b) protein. The magenta dot in each panel for the 0.5 μm particle diameter is at the velocity currently achievable, without slip flow, by commercial instruments for a 5 cm column length and random packing. The entire velocity range is achievable with all of the larger particle sizes. These graphs help to explain that conventional chromatographic theory predicts an advantage with submicrometer particles only for protein separations.

Figure 3.

Plot of experimentally measured accessible pore volume vs molecular radius for four different nonporous particle sizes, giving four different pore diameters ranging from 280 to 4 200 Å. The solid curves are theoretical functions derived using size exclusion theory. The dashed black curve is the theoretical function for the commonly used 300 Å pore diameter for commercial silica. The dotted red line indicates the radius of a monoclonal antibody drug, showing that this class of drugs is significantly excluded by typical wide pore LC columns of 300 Å in pore diameter.

Figure 4.

(a) Illustration of slip flow concept for the case of reversed-phase liquid chromatography, where hydrophilic walls give zero velocity of fluid at the wall, and hydrophobic walls give a nonzero velocity. (b) Plot of experimentally measured flow enhancement for water relative to toluene in capillaries packed with various diameters of silica particles bearing a C4 bonded phase. The fit to the theory is shown as the solid curve, revealing a slip length of 63 ± 3 nm. The figure is taken from a paper by our group. Reprinted from ref 10. Copyright 2013 American Chemical Society.

Figure 5.

Plot of a time series of images showing the progression of two BSA peaks as each passed through the 1 mm detection region after traveling more than 17 mm through the capillary. Plate heights were calculated by fitting the spatial intensity distributions to Gaussians, then dividing the variance of the Gaussian to the separation length. Each image is separated by 0.3 s in time. The red arrow points to a defect that happened to be in the middle of the capillary in this instance, allowing one to see its perturbation on the zone shape.

Figure 6.

Capillaries and data for LC-MS with 0.5 μm particles. (a) Photograph of capillary in pressure fitting, showing pulled tip and mobile phase dripping from the end, (b) photograph of the capillary tip taken through a zoom microscope, showing opalescence of the packing material, (c) chromatogram of the narrowest peak, for which the circles are the data points and solid curve is a Gaussian (s.d. = 0.20 s), (d) chromatogram of a set of commercial proteins and their impurities at three different intensity scales to illustrate the dynamic range. Panels a and b are taken from a previous publication of ours. Reprinted from ref 26. Copyright 2014 American Chemical Society.

Figure 7.

Illustration of how much broadening is currently imparted by commercial instruments.

These are plots of Gaussians to help visualize (1) the 64 μm base width for no instrument contribution vs (2) the $\sim 400 \mu\text{m}$ base width in typically images observed using a nanoLC for injection with MS for detection vs (3) the 2.5 mm base width calculated for the current best-performing UHPLC instrument with a 5.5 μL dispersion volume, considering no contribution from the column. All base widths are for peaks inside the medium.