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Superficially Porous Particles with 1000 Å Pores for Large Biomolecule High Performance Liquid Chromatography and Polymer Size Exclusion Chromatography

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Abstract

To facilitate mass transport and column efficiency, solutes must have free access to particle pores to facilitate interactions with the stationary phase. To ensure this feature, particles should be used for HPLC separations which have pores sufficiently large to accommodate the solute without restricted diffusion. This paper describes the design and properties of superficially porous (also called Fused-Core®, core shell or porous shell) particles with very large (1000 Å) pores specifically developed for separating very large biomolecules and polymers. Separations of DNA fragments, monoclonal antibodies, large proteins and large polystyrene standards are used to illustrate the utility of these particles for efficient, high-resolution applications.

Keywords

HPLC; superficially porous particles; very large pores; DNA fragments; monoclonal antibodies; size exclusion chromatography (SEC)

1. Introduction

Theory and experiments have clearly established that particles used for the HPLC separation of molecules should have pores that are sufficiently large to allow free movement of such molecules within the pore structure and not restrict diffusion [1, 2]. If the pores are not sufficiently large, diffusion can be restricted and the efficiency of the separation compromised. It has been claimed that the average pore size of the particles should be at least four to ten times the hydrodynamic diameter of the solute so that restricted diffusion does not occur [1, 3]. The optimum pore size dimension clearly is dependent on solute configuration in the mobile phase used for the separation.

In packed-bed liquid chromatography there are two main sources of zone broadening which limit the resolution and speed of a chemical separation [1, 4]. The first of these sources includes fluid-phase Taylor dispersion [4, 5] and various perturbations due to the flow-field being modified by the presence of the particle packing [4-6]. The second of these is the resistance to mass transport which is due to the solute(s) being delayed in the stationary packing material [4, 6, 7]. Included in the resistance to mass transport in the solid packing material (also known as solid-phase mass transport) are four important components generally lumped together under the term “pore diffusion” [8]:

1. mass transport in the fluid surrounding the particle into the pore system.
2. pore diffusion in the liquid-filled pores within the particle.
3. resistance to binding at the liquid-adsorbent interface.
4. diffusion in the adsorbed phase.

The mathematical formulation of diffusion in restricted pore spaces has been explicitly stated for chromatographic particles [8] and that terminology will be utilized here. A review covering fundamental fluid mechanics of convection and diffusion in restricted pore space is available [9].

The diffusion coefficient in a cylindrical pore, D_e , is highly dependent on the ratio of the solute average radius, r_m , to pore radius r_{pore} such that $\lambda_m = r_m / r_{pore}$. The hindered pore diffusion can be expressed as the non-dimensional ratio of D_e to the free solution diffusion coefficient, D_0 such that:

$$\frac{D_e}{D_0} = \frac{\varepsilon_p}{\tau} \psi_p \quad (1)$$

The hindrance coefficient ψ_p is scaled by the particle porosity ε_p and divided by a tortuosity factor τ which is unity for cylindrical pores. Hence, the effective “slowing” of the pore diffusion process, which expresses the hydrodynamic resistance of molecules near walls, can be calculated through simple interpolation formulas [8] for ψ_p .

Figure 1 shows the result from the theory of Brenner [8, 10] for ψ_p , as a function of λ_m , given by the following equation:

$$\psi_p = 0.865(1 - \lambda_m)^2(1 - 2.1044\lambda_m + 2.089\lambda_m^3 - 0.984\lambda_m^5) \text{ for } \lambda_m > 0.2 \quad (2)$$

This plot suggests that pore size is critical to the chromatographic process. Larger pores minimize the effect of the reduction in diffusion coefficient, helping prevent the loss of chromatographic efficiency. Smaller pores increase the surface area of the adsorbent which increases the capacity for preparative and analytical chromatography. However, smaller pores reduce efficiency, except for very small molecules, due to the reduction in diffusion coefficient within the particle. The slowing of solute diffusion within a pore space as λ_m increases has been observed through simulation [11] by varying solute size relative to the pore.

Thus, there is a compromise between efficiency and capacity (surface area). Larger molecules of biological interest and larger industrial polymer molecules need large pore sizes or else the mass transport rate into and out of the pore will be exceedingly slow and lead to excessive zone broadening. For preparatory separations under (ideal) thermodynamic control, maximum adsorption capacity is vital, while the kinetics are of secondary interest. However, for analytical separations, where kinetic-based efficiency is crucial, matching the pore size to molecule size is extremely important, especially for cases where molecules of similar size, shape, and structure are to be separated.

When the pore size is optimum for particular solutes, maximum column performance can be expected. Optimum can be defined here as the largest pore size having sufficient surface area for desired solute retention and mass capacity for a given solute. As a result, the goal has been to develop superficially porous particles (SPPs) with different pore sizes for best results for different size solutes; 90 Å pores for small molecules [12], 160 Å pores for peptides, small proteins and related samples [13], and 400 Å pores for proteins and larger molecules

[14, 15]. This paper describes fused core silica particles with an average pore size of 1000 Å, which are specifically designed for separating very large biomolecules and polymers.

2. Experimental

2.1 Particles and columns

The superficially porous particles described in this investigation are synthesized using layer-by-layer techniques [16] to create high-purity Type B silica with a narrow particle size distribution. Figure 2A is an image obtained using scanning electron microscopy (SEM) which shows a group of these 1000 Å particles, verifying uniformity and spherical shape, despite the very large pores in the outer porous shell. Figure 2B is a focused ion beam (FIB) SEM cross-section [17] of one of the 1000 Å particles, showing the solid fused core and the porous shell comprised of large pores. In this case, the thickness of the porous shell is about 0.5 µm. SEM images were obtained using a Zeiss (Jena, Germany) Auriga 60 High Resolution Focused Ion Beam & Scanning Electron Microscope at the University of Delaware (Newark, DE).

Columns of HALO[®] Fused-core silica particles were made within or obtained from Advanced Materials Technology, Inc. (Wilmington, DE). All SPP columns lasted for a minimum of 100 injections and were run at back pressures up to 600 bar. Columns of fully porous particles (FPPs) were from Waters Corporation (Milford, MA). All columns used were C4 bonded phase except for the polystyrene separations which were conducted on bare silica.

2.1.1 Particle characterization—In this study, superficially porous particles with 1000 Å pores were synthesized with different particle sizes and different shell thicknesses to allow evaluation of different configurations (Table 1). The particle letters will be used to help identify various particles described in this paper. Multisizer 3 and Multisizer 4e Coulter Counters (Indianapolis, IN) were both used to measure particle size in addition to SEM. For SEM, a minimum of 30 particles of each sample were imaged in both the x and y directions creating a total of at least 60 measurements, which were then averaged. It was found that the particle sizes measured by SEM were larger than the particle sizes measured by the Coulter Counter. This is caused by the presence of large pores which are speculated to disrupt the electrical measurements used to calculate the particle size by the Coulter technique. For the purposes of this paper, the sizes measured by SEM are the ones that are referred to in the text, unless otherwise noted. The nitrogen adsorption surface area of these particles vary from 4 - 26 m²/g, depending on particle size and porous shell thickness. Since surface area is reported on a per gram basis, it is difficult to compare absolute surface areas, given different particle configurations with varying core to particle size ratios. For this reason, the retention factor of a small molecule (1-chloro-4-nitrobenzene) is provided in Table 1 for unbiased comparisons. Evaluation of the different configurations continues in this laboratory, and this presentation gives results of some of the studies to date.

2.2 Chemicals

Mobile phase modifiers and proteins were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. DNA fragment samples were from Sigma-Aldrich and Thermo Scientific (Waltham, MA). SILuTMLite SigmaMAb was obtained from Sigma-Aldrich. Myosin was from Cytoskeleton, Inc. (Denver, CO). Trifluoroacetic acid (TFA) was from Pierce Chemicals (Rockford, IL) and acetonitrile (ACN) from EMD (Gibbstown, NJ). Difluoroacetic acid (DFA) was purchased from SynQuest Laboratories (Alachua, FL). Polystyrene standards were from both low and high molecular weight polystyrene standards kit (Supelco, Bellefonte, PA). Unstabilized HPLC grade Tetrahydrofuran (THF) was from J. T. Baker (Center Valley, PA).

2.3 HPLC

HPLC data were collected with an Agilent Model 1200 SL liquid chromatograph (Palo Alto, CA) or a Shimadzu Nexera liquid chromatography instrument (Tokyo, Japan). Peak widths (full width, half max) were used for measurements. A 2 μ L UV flow cell was used with the Agilent 1200, and 1 μ L semi-micro cell was used for the Shimadzu Nexera. All of the capillary tubing connections and needle seats were of 0.12 mm ID for the Agilent instrument. No corrections for instrumental extra-column band broadening effects were applied to any of the chromatographic data. Since the SigmaMAb was run under gradient conditions, corrected retention times were calculated (same equation as retention factor under isocratic conditions) and were normalized the same way that was done for the small molecule data.

3. Results

3.1 Pore and Particle Size

Figure 3 shows a pore size distribution plot of one of the 1000 Å SPPs (Particle I), measured by nitrogen adsorption on a Micromeritics Tristar II (Norcross, GA). For reference, the pore size distribution of both 400 Å SPPs and 300 Å FPPs (sometimes called totally porous particles or TPPs) are included in Figure 3. A larger number of smaller pores are observed for the fully porous particles, while these are absent from the 1000 Å particles distribution. Notice that the pore volume on a per gram basis is considerably larger on the 1000 Å SPPs compared to the 400 Å SPPs (shell thickness of 0.2 μ m) due to the presence of a significantly thicker 0.5 μ m shell on the 1000 Å SPPs.

The 1000 Å particles were prepared with a narrow particle size distribution, as shown in Figure 4. The graph shows examples of the 2.7 (Particle I) and 3.2 μ m (Particle F) 1000 Å particles. For comparative purposes, a SPP 3.4 μ m 400 Å particle size distribution is included, as well as a 1.7 μ m 300 Å fully porous particle size distribution. The polydispersities for all of the SPPs are less than 5%, while the polydispersity for the FPPs is 17%. A smaller polydispersity corresponds to a narrower particle size distribution. The broad particle size distribution of the 1.7 μ m particles is starkly contrasted by the narrow particle size distributions of the SPPs. The relationship between narrow particle size distributions and column performance has been extensively investigated and is debatable

[18-21]. Our preference has been to favor narrow distributions since this property has usually led to columns with high reproducibility and superior efficiency.

Figure 5 shows a comparison of separations for a series of proteins performed with two 1000 Å particles with similar size, but with different shell thicknesses (Particles F and G). Only minor variations in results are noted, with Particle G having a thinner outer shell (bottom trace) showing slightly lower retention and similar peak widths in most instances. Within the limits of the experiment, shell thickness (shorter diffusion path) does not appear to make a significant difference for these protein sizes. Table 2 lists the proteins with corresponding Protein Databank (PDB) number, molecular weight, and the length of each protein in the x, y, and z directions. These lengths are calculated for non-denaturing conditions, but may be used as approximations of the size of each protein.

3.2 DNA Fragments

To estimate the limit of solute size that could be used with the 1000 Å particles before restricted diffusion can occur, a series of double stranded DNA (dsDNA) fragments with known numbers of base pairs (bps) were separated. This experiment was intended as a way to characterize the pore size and not as an application. The long term stability of the columns has not been evaluated under the conditions used. dsDNA is a relatively stiff polymer with a persistence length of ≈ 500 Å [22]. The persistence length is the length by which a worm-like chain polymer can be constructed between hinged segments. This does not mean that dsDNA is a rod; lengths longer than a few persistence lengths will start forming a random coil. The distance between 10 base pairs per turn is known to be 34.6 Å by x-ray determination [23]. Using this value and the persistence length of 500 Å, we show in Table 3 the total molecular lengths of some of these dsDNA fragments and the corresponding number of persistence lengths. These numbers indicate the extent with which the dsDNA will fit in the pore. In most cases shown in the table, the DNA fits into the pore laterally, but in some cases of large length, such as the 1000 bp dsDNA, which has a non-dimensional persistence length of ≈ 7 , the occupancy may occur as a random coil or has partial occupancy.

Figure 6 shows gradient elution separations of a pUC 18 DNA Hae III Digest with nine of the eleven components resolved using two different columns: a column of a commercial HALO Protein 400 Å silica and a column of 1000 Å particles (Particle F) both with a C4 stationary phase. The two smallest fragment base pairs (11 and 18) elute at the void volume for both columns and have not been included in Tables 3 and 4. Neither of these columns appear to exhibit any diffusion restrictions for any of the DNA fragments as peak widths are relatively constant throughout the gradients (Table 4), even for the largest fragment (587 base pairs). Early-eluting peaks in the gradient typically are sharper for small DNA fragments.

One feature of the separation using the 1000 Å pores is that there is increased resolution between the 257 and 267 bp fragments and between the 434 and 458 bp fragments as denoted above the peaks in Figure 6. In all cases, effects from restricted diffusion appear absent for these columns, indicating that both the 400 Å and 1000 Å pores are well suited for solutes up to more than 350,000 Da molecular weight (for solutes of this configuration).

This molecular weight is estimated by multiplying the highest number of base pairs in the digest (587) by 660 (average molecular weight of dsDNA base pair). Similar results were reported by Close et al. when larger pore size SPP columns were used for the same DNA restriction digest [2].

To test the limit of solute access, a DNA fragment sample containing sixteen different DNA base pair fragments ranging from 100 base pairs to 2642 base pairs (about 1,700,000 molecular weight) were separated by gradient elution with the 400 Å and 1000 Å particle columns, as shown in Figure 7. The 500 and 1000 base pair fragments are two to three times more concentrated than the other base pairs, which explains their larger absorbance relative to the other base pairs. The peaks for the larger DNA fragments are clearly narrower for the 1000 Å column (Particle F), as shown in Table 5. The 1000 base pair peak (about 660,000 Da) for this column gave little evidence of zone broadening, possibly due to restricted diffusion. The 2642 base pair peak showed larger peak widths compared to the smaller fragments for the 400 Å and 1000 Å columns, but the increase in peak width was more severe with the 400 Å column. This suggests that a large DNA fragment of this size may be beginning to experience crowding in the pores even with the very large pores of the 1000 Å SPP.

With the increased complexity of this sample, a very shallow gradient was needed in order to improve the separation. The 1000 Å particle (Particle F) column shows improved resolution of the DNA base pairs that elute between 1000 and 2642 base pairs (five components consisting of 1100, 1200, 1300, 1400, and 1500 base pair fragments) compared to the 400 Å pore size column. Since all of the peaks were not resolved under the conditions used, individual 1000 and 3000 DNA base pair standards were also run on the 400 and 1000 Å pore size columns. These results (not shown) confirm that the peak widths are smaller for both the 1000 and 3000 base pair DNA standards with the 1000 Å pore size SPPs.

3.3 Separation of Large Proteins

As noted previously, the 1000 Å pore particles are uniquely suited for separating very large molecules without loss of resolution. Figure 8 shows the comparative separation of the myosin heavy chain, a highly complex motor protein with a molecular weight of about 480,000 Da, using a 1.7 µm 300 Å FPP commercial column with surface area of about 90 m²/g and a 2.7 µm 1000 Å SPP column (Particle I) with surface area of 23 m²/g, both with C4 stationary phases. For brevity, only the comparative data for 1000 Å and 300 Å are shown for the myosin heavy chain, but peak widths and retention times similar to 1000 Å were observed with the 400 Å column. The advantage of the larger pores is clearly shown by the narrower peaks and higher resolution with the 1000 Å SPPs for the two myosin heavy chain peaks later in the gradient. This is the case in spite of the larger particle size of the 1000 Å SPPs, demonstrating the importance of pore size in the separations of larger molecules.

Figure 9 shows another example of the advantage of SPPs with larger pores for biomolecules in separating a monoclonal antibody, in this case SigmaMAb. Using the same columns as in Figure 8 and a 400 Å HALO Protein C4 column, both the 2.7 µm particle column of 1000 Å pores (Particle I) and the HALO Protein C4 column show narrower peaks and better

resolution than a commercial column of 1.7 μm 300 $\text{\AA}$ fully porous particles. See Figure 9 for the peak width values in minutes. One might speculate that the 300 $\text{\AA}$ column has the beginning of mass transport limitations for this large protein molecule of about 150,000 Da molecular weight.

Another interesting observation is the increased retention for both myosin and SigmaMAb that is observed for both the 400 $\text{\AA}$ and 1000 $\text{\AA}$ pore size SPP columns compared to the 1.7 μm FPP column. Under identical conditions, an isocratic separation of small molecules (Figure 10) demonstrates that the 1.7 μm FPP has greater surface area than both the 400 $\text{\AA}$ and 1000 $\text{\AA}$ pore size SPP columns as judged by retention measurements. Figure 11 further illustrates the retention anomalies observed between columns containing 1000 $\text{\AA}$ and 300 $\text{\AA}$ particles for small and large molecule separations. Surprisingly, the increased surface area of the FPP did not correlate to increased retention for larger molecules.

This retention anomaly may be explained by the pore size distribution graph in Figure 3. The pore size distribution of the 1.7 μm 300 $\text{\AA}$ FPPs contains a significantly greater amount of pores which are smaller than 200 $\text{\AA}$ compared to the 3.4 μm 400 $\text{\AA}$ and 2.7 μm 1000 $\text{\AA}$ SPPs. The reduced retention for myosin and SigmaMAb suggest that there may be regions of inaccessible pores in the 1.7 μm FPP pore structure. Despite their lower surface area, the large pore SPPs demonstrated increased retention and narrower peak shapes of myosin and SigmaMAb indicating an improved accessibility to the particle surfaces of the wider-pore particles.

3.4 Size exclusion chromatography (SEC) of polymers

The interest in biomolecular separations has driven the development of many innovations in HPLC. However, industrial polymer characterization has also benefitted from these innovations. The separation of specific polystyrene standards on a 400 $\text{\AA}$ SPP column and 1000 $\text{\AA}$ SPP column (Particle A) is shown in Figure 12. As anticipated for SEC, the elution order is from largest solute to the smallest solute. A detailed experimental comparison of SEC between FPPs and SPPs has been conducted [24] and shows that SEC with SPPs offers advantages in speed, efficiency and plate production per unit time. The range of molecular weights used in Figure 12 spans the radius of gyration range [24] of 43 $\text{\AA}$ to 662 $\text{\AA}$ showing that the lowest MW standard of 17.5 kDa will fit into the smallest pore material in Figure 12. The largest MW standard is excluded by both pore systems with the caveat that the pore size distribution shown in Figure 3 demonstrates that a limited number of pores exist larger than 1000 $\text{\AA}$.

As shown in Figure 12, the 1000 $\text{\AA}$ pore material shows abundant resolution in a short amount of time. Certainly for the size range of the solutes shown here, the 1000 $\text{\AA}$ particle is more appropriate than the 400 $\text{\AA}$ particle. More of the separation space is used with the 1000 $\text{\AA}$ particle compared to the 400 $\text{\AA}$ particle due to the larger excluded volume (0.19 mL compared to 0.15 mL) of the 1000 $\text{\AA}$ particle. Also note that the peaks here are quite narrow; typically, about a tenth of a minute (6 seconds) across the baseline. This high performance can be increased by optimizing the shell and core dimensions. The highest molecular weight solute, at 1.8 MDa, may be partially excluded from the largest pores. The plate counts observed here are ≈ 3000 plates for the higher molecular weight solutes and ≈ 5000 plates for

the lower molecular weight solutes. On a plates per meter basis this is 60,000 to 100,000 plates per meter or using plate heights, this is 17 to 10 μm , respectively.

4. Discussion

SPPs with very large pores appear to be the particle morphology of choice for many biomolecule and polymer separations. This discussion will continue with simple scaling arguments as to why we believe this is the case. The plate height term, H_s , for the resistance to mass transport in the stationary (particle) phase with pores of depth d was derived by Giddings [6, 25] noting the “s” subscript is for the particle (stationary) phase:

$$H_s \sim \frac{d^2}{D_s} \quad (3)$$

The symbol $\sim$ is read as “is proportional to” or “scales as” which is commonly used in the physics literature. In Equation 3 D_s is the effective diffusion coefficient of solute in the particle. From Equation 3 it can be seen that this plate height contribution scales as the square of the pore depth. SPPs can reduce this contribution to zone broadening by making d smaller; reducing d by a factor of 2 will reduce H_s by a factor 4. Derivations of H_s with more detail specifically for SPPs are available [26, 27] and are starting points for rigorous derivations of this stationary phase mass transport term. However, restricted diffusion also affects the B-term in the plate height expression [28]. The B-term has been determined to be lower in SPPs relative to FPPs [28], and this term is significant in explaining the total plate height advantage of SPPs over FPPs.

The experimentally observed plate height includes contributions from the mobile phase plate height, stationary (particle) phase plate height and extracolumn and instrumental effects [1, 4, 6]. For biomolecules, additional sources of zone broadening include sample heterogeneity effects due to conformational shifts and multiple conformational states even when denatured, may be present. For example, denatured biomolecules can produce multiple conformations which are then separated, but rarely are resolved. Hence, using reduced shell thickness particles as opposed to FPPs can aid in faster and more efficient separations especially for biomolecules.

We can expand the D_s term in Equation 3 by substitution of the diffusion coefficient with that in Equation 1 so that

$$H_s \sim \frac{d^2}{D_0 \psi_p} \quad (4)$$

Equation 4 shows that restricted diffusion can contribute greatly to zone broadening. A simple example suffices to show this importance. Catalase has two principal axes lengths on the order of 100 Å and when combined with 400 Å pores, λ_m is calculated to be 0.25.

Subsequently through Figure 1, $\psi_p \approx 0.3$ and the particle plate height increases by roughly a factor of 3.33. When a 1000 Å pore particle is used for catalase, λ_m is calculated to be 0.1 which corresponds to $\psi_p \approx 0.6$. This results in a plate height increase of only 1.66, which is roughly half that of the 400 Å pore particle, and would result in a more efficient separation. Specifically, the particle phase mass transport resistance is less with the 1000 Å pore particle compared to the 400 Å pore particle. This simple calculation further illustrates the potential performance advantages of the 1000 Å material as compared to smaller pore material.

Large pore materials of 1000 Å diameter pores have been described previously [29] and are typically based on organic materials such as cross-linked polystyrene-divinyl benzene. Pore sizes as large as 6000 Å allow flow in many cases through these FPPs [30-32] and this has been described as “perfusion chromatography” (PC). Many of these materials have a bimodal pore structure with the large pores (≈ 6000 Å) delivering flow into somewhat smaller pores (≈ 1000 Å). Convective flow in pores aids mass transport and unlike restrictive diffusion, where the effective diffusion coefficient is a fraction of the free-solution diffusion coefficient, PC increases the effective diffusion coefficient relative to the free-solution diffusion coefficient [30-32]. The theoretical treatment of convective flow in pores has been developed [33], and some recent insights into PC have recently been published [34].

In studying the high resolution fluid mechanics around individual model SPPs with pore diameter of ≈ 1000 Å [35], we have discovered that there is a small amount of flow within the porous shell region. This is interesting because this flow is found in the immediate outer layers of a wide pore FPP, but quickly diminishes as one moves toward the particle center. Due to the thin shell thickness in a wide-pore SPP, significant flow is found in the shell region; the mass flux in the shell region has been estimated to be 10% of the total mass flow [35]. This may greatly aid chromatographic performance for this type of particle and further study is under way to categorize this flow and understand it more using experimental and advanced simulation techniques. Nonetheless, this internal flow, along with reduced pore distance and wide pore diameter, help counteract the deleterious effects of restricted diffusion and tend to produce a high performance chromatographic particle.

5. Conclusions

Superficially porous Type B highly-purified silica particles with 1000 Å pores designed specifically for separating large biomolecules and industrial polymers are described in this publication. Separations of large biomolecules confirm the advantages of particles with very large pores in minimizing mass transport effects for better resolution. Studies with known DNA fragments indicate that the 1000 Å particles separate 1000 base pairs of about 660,000 Da with minimal effects of restricted diffusion. Comparative separations of large biomolecules (myosin and SigmaMAb) using 2.7 μm 1000 Å SPPs and 1.7 μm 300 Å FPPs clearly show the advantage of the SPPs with wider 1000 Å pores in providing higher resolution and increased retention. This trend is also demonstrated in the high performance separations of polystyrene standards shown with 1000 Å SPPs.

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Highlights

- Separation of biomolecules is enhanced using 1000 Å pores in core-shell particles.
- Rapid mass transport in 1000 Å pores provide high resolution for large molecules.
- DNA fragments, mAbs, large proteins, and polystyrene separations are demonstrated.
- Wide pore core-shells are compared with other particle morphologies and pore sizes.

Figure 1. The hindered diffusion factor ψ_p as a function of the ratio of molecule radius to pore radius λ_m

Figure 2.

A. SEM of 1000 Å superficially porous particles.

B. FIB SEM of 1000 Å superficially porous particles with a porous shell thickness of about 0.5 μm.

Figure 3.

Pore size distribution measurement of 1.7 μm 300 Å FPP, 3.4 μm 400 Å SPP, and 2.7 μm 1000 Å SPP by nitrogen adsorption. For convenience, primary and secondary y-axes are used due to the much smaller pore volume per gram of the SPPs.

Figure 4.

Particle size distribution of 1.7 μm 300 $\text{\AA}$, Particle I and Particle F 1000 $\text{\AA}$ SPP, and 3.4 μm 400 $\text{\AA}$ SPP measured by Coulter Counter. The polydispersity is calculated as the ratio of the square root of the second moment to the first moment of the Coulter Counter data. These moments are obtained by curve fitting an exponentially modified Gaussian peak shape to the raw Coulter Counter data using a nonlinear least-squares program written in MATLAB.

Figure 5. Effect of shell thickness for 1000 $\text{\AA}$ particles

Conditions: Columns: 2.1×100 mm; Mobile phase A: water/0.1% TFA; Mobile phase B: ACN/0.1% TFA; Gradient: 25-52% B in 15 min; Flow rate: 0.35 mL/min; Temperature: 60 $^{\circ}\text{C}$; Injection volume: 0.7 μL ; Instrument: Shimadzu Nexera; Detection: 220 nm; Peak identities: 1) Ribonuclease A 2) Cytochrome c 3) Lysozyme 4) α -Lactalbumin 5) Catalase. Peak widths in minutes above each peak.

Figure 6. Ion pair-Reversed Phase Separation of pUC 18 DNA *Hae* III Digest

Conditions: Columns: 2.1×100 mm; Mobile phase A: 0.1 M triethylammonium acetate, pH 7; Mobile phase B: 50/50 acetonitrile/0.1 M triethylammonium acetate, pH 7; Gradient: 24-36% B in 20 min; Flow rate: 0.25 mL/min; Temperature: 50 °C; Instrument: Agilent 1200 SL; Detection: 254 nm; Sample: pUC 18 DNA *Hae* III digest; Injection volume: 2 μ L of 522 μ g/mL DNA fragment base pairs are indicated above each peak.

Figure 7. Ion pair-Reversed Phase Separation DNA Molecular Weight Marker XIV
Conditions: Same as for Figure 6 except Gradient: 24-36% B in 50 min. Sample: DNA Molecular Weight Marker XIV, 16 fragments. 250 μg/mL; DNA fragment base pairs as indicated on graph.

Figure 8. Comparison separation of large protein, myosin heavy chain

Conditions: Columns: 2.1×150 mm; Mobile phase A: water/0.1% DFA; Mobile phase B: ACN/0.1% DFA; Gradient: 32-52% B in 28 min; Flow rate: 0.35 mL/min; Temperature: 80 °C; Sample: myosin; Injection volume: 5 μL of 0.5 mg/mL; Instrument: Shimadzu Nexera; Detection: 280 nm; Peak widths in minutes above each peak.

Figure 9. Comparison separation of monoclonal antibody, SigmaMAb

Conditions: Columns: 2.1×150 mm; Mobile phase A: water/0.1% DFA; Mobile phase B: ACN/0.1% DFA; Gradient: 29-35% B in 12 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Sample: SigmaMAb; Injection volume: 2 μL of 0.5 mg/mL; Instrument: Shimadzu Nexera; Detection: 280 nm; Peak widths in minutes above the major peak.

Figure 10. Small Molecule QC Results

Conditions: Columns: 2.1×150 mm; Mobile phase A: water; Mobile phase B: ACN; Isocratic: 25/75 A/B; Flow rate: 0.3 mL/min; Temperature: 25 °C; Sample: as indicated; Injection volume: 0.2 μL ; Instrument: Shimadzu Nexera; Detection: 254 nm; Peak identities: 1) Uracil 2) Phenol 3) Propiophenone 4) 1-Chloro-4-nitrobenzene; Peak statistics listed for the last peak.

Figure 11. Relative Retention for 300 Å FPP vs. 1000 Å SPP for Small and Large Molecules
Relative retention factor was calculated by normalizing to the higher retention factor value.

Figure 12. Separation of polystyrene standards

Conditions: Columns: 4.6 × 50 mm; Mobile phase: 100 % THF; Flow rate: 0.5 mL/min; Temperature: 25 °C; Sample: as indicated; Injection volume: 1 μL; Instrument: Shimadzu Nexera; Detection: 254 nm for (top) 1000 Å silica particles and (bottom) 400 Å silica particles. Molecular weights utilized are shown on both figures.

Table 1

1000 Å Superficially Porous Particles Investigated

Particle	Particle Size Mode via Coulter Counter (µm)	Particle Size Average via SEM (µm)	Shell Thickness via SEM (µm)	Core/Particle Size Ratio via SEM	Surface Area (m ² /g)	Retention Factor
A	4.2	4.4	0.6	0.73	15.5	2.09
B	3.6	3.9	0.4	0.79	13.6	1.97
C	3.5	3.6	0.2	0.89	4.3	0.98
D	3.5	3.6	0.3	0.83	10.8	1.55
E	3.5	3.6	0.2	0.89	6.1	1.02
F	3.0	3.2	0.3	0.81	13.3	1.83
G	3.0	3.0	0.1	0.93	6.4	1.17
H	2.7	2.9	0.6	0.59	25.6	2.58
I	2.5	2.7	0.5	0.63	22.7	2.43

Retention factor is measured for 1-chloro-4-nitrobenzene using uracil as the unretained solute and the following conditions: Columns: 2.1 × 100 mm; Mobile phase A: water; Mobile phase B: ACN; Isocratic: 25/75 A/B; Flow rate: 0.3 mL/min; Temperature: 25 °C; Injection volume: 0.2 µL; Instrument: Shimadzu Nexera; Detection: 254 nm

Table 2

Proteins with Corresponding PDB number, MW and lengths in the x, y, and z direction under non-denaturing conditions. Lengths are calculated from the crystal structure with principal axes rotation.

Protein	PDB number	Molecular weight (Da)	length x (Å)	length y (Å)	length z (Å)
Ribonuclease A	5D97	13768	50.7	35.0	29.0
Cytochrome c	2B4Z	12212	42.2	40.3	40.7
Lysozyme	4Z98	14390	140.8	97.2	40.4
α -Lactalbumin	1A4V	14173	49.7	33.8	33.5
Catalase	3J7U	245452	107.6	104	84.4

Table 3
dsDNA Base Pairs with corresponding total length and non-dimensional persistence length

Number of dsDNA Base Pairs	Total Length (Å)	Non-dimensional Persistence Length (total length/500 Å)
80	277	NA
102	353	NA
174	602	1.2
257	889	1.8
267	924	1.8
298	1031	2.1
434	1502	3.0
458	1585	3.2
500	1730	3.5
587	2031	4.1
1000	3460	6.9
2642	9141	18.3
3000	10380	20.8

Table 4
pUC 18 DNA *Hae* III Digest Fragments with Corresponding Retention Times and Peak Widths

Base Pairs	400 Å C4		1000 Å C4	
	Retention (min)	Peak Width (min)	Retention (min)	Peak Width (min)
80	2.82	0.09	2.14	0.07
102	5.24	0.11	4.15	0.11
174	10.00	0.10	9.59	0.12
257	12.59	0.11	12.61	0.12
267	12.87	0.12	12.95	0.12
298	13.52	0.11	13.69	0.12
434	15.38	0.12	15.96	0.13
458	15.66	0.13	16.37	0.13
587	16.57	0.13	17.55	0.14

Table 5
Selected DNA Molecular Weight Marker XIV Base Pairs with Corresponding Retention
Times and Peak Widths

Base Pairs	400 Å C4		1000 Å C4	
	Retention (min)	Peak Width (min)	Retention (min)	Peak Width (min)
500	33.0	0.24	34.1	0.28
1000	38.5	0.33	41.4	0.26
2642	40.4	0.78	44.3	0.54