

Faster Protein and Peptide Liquid Chromatography (FP2LC): Introducing BIOshell™ Fused-Core® U/HPLC Columns

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Abstract

A new and broad product line for the analysis of peptides and proteins by reversed-phase high performance liquid chromatography (RP-HPLC) is introduced under the name BIOshell Fused-Core U/HPLC columns. As the Ascentis® Express column line for the analysis of small molecular mass compounds, BIOshell columns outperform fully porous silica-based columns of the same particle size in terms of one or more important chromatographic attributes; such as efficiency, resolution, analysis time (speed) and back pressure. By providing faster peptide and protein liquid chromatography (FP2LC), the benefits of porous-shell columns are now also available to scientists who are tasked to advance our knowledge about the living world.

Background and Characteristics

The development of Fused-Core U/HPLC columns is based on the pellicular silica technology developed by J. J. Kirkland at E. I. du Pont de Nemours and Company in the late 1960s during the formative years of HPLC, and applied in this century to create very efficient particles in the range of 2.7 to 5 micron.^{1,2} The latest innovations derived from this technology are now available as BIOshell Fused-Core columns for faster protein and peptide liquid chromatography (FP2LC) separations. BIOshell columns for peptide analysis are either packed with 2.7 or 5 micron particles containing 160 Å pores and bonded with a C18 alkyl or alkyl cyano functionality, while protein separations are best performed with 3.4 micron particles containing 400 Å pores bonded with C4 alkyl functional groups. The complete list of available BIOshell columns is provided at the end of this article.

In 2007 Supelco introduced the groundbreaking Ascentis Express Fused-Core column line for the analysis of compounds with low molecular mass. In the succeeding years it has become clear that (a) columns packed with 2.7 micron, 90 Å pore size, porous-shell particles provide a dramatic and unexpected improvement in column efficiency, rivaling the efficiency of columns packed with fully porous

1.7 micron particles, and (b) a paradigm shift had to take place in the minds of theoreticians to adjust the theory of chromatographic band broadening to account for column efficiencies that were, until then, considered outside the realm of possibility.^{3,4}

Like the particles in Ascentis Express columns, the particles in BIOshell columns are composed of a spherical solid glass core surrounded by a thin layer of nano-size silica particles. **Table 1** summarizes the characteristics and maximum operating conditions of the new BIOshell porous-shell columns.

As shown in **Table 1**, to accommodate the larger size of biopolymers, the particles in BIOshell columns either feature 160 Å pores for unhindered access by peptides up to a mass of about 20 kDa, or 400 Å pores for proteins with a molecular mass up to about 500 kDa. Note that the thickness of the porous shell varies as does the percentage of the surface area when compared to a fully porous particle of the same size. Thus, the 0.2 micron shell for the 3.4 micron particles results in a surface area that is 31% of a fully porous particle of that size. Similarly, the 0.5 micron shell for the 2.7 micron particle provides 75% of the maximum surface area, while the 0.6 micron shell for the nominally 5 micron but actually 4.7 micron particles contains 59% of the surface area of a fully porous particle. Although the loss of surface area may seem a cause of concern, Unger et al., first demonstrated that even nonporous matrices provide sufficient retention for larger biomolecules, while Gritti and Guiochon showed that the capacity of core-shell particles is not substantially reduced even for large biopolymers, a conclusion that was recently confirmed in a paper from Guillaume et al., who found that the loading capacity of a wide pore core-shell column was 2–3 fold less than that of a fully porous column.^{5–7}

Table 1 also shows the bonded phase characteristics, recommended pH range, and the highest values for back pressure and temperature at which each particle type was tested. While a maximum pressure of 600 bar or 9,000 psi may seem modest in current day UHPLC systems, a maximum operating temperature of 80–100 °C provides a degree of freedom that can be very beneficial, particularly when working with more hydrophobic proteins, including monoclonal antibodies, as will be illustrated in the following proof statements.

Table 1. Characteristics and Operating Conditions of BIOshell Fused-Core Columns

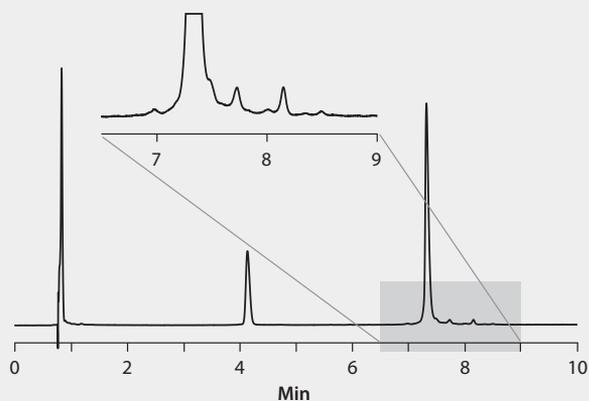
Physical Parameter	Nominal Values						Phase Characteristics and Operating Conditions					
	Dp (µm)	Core (µm)	Shell (µm)	Pore Size (Å)	SBET (m ² /g)	Capacity vs. Porous	Bonded Phase Ligand	End Capped	Tmax °C	pH Range	Pmax (bar)	Frit (µm)
2.7 µm A160 Peptide C18	2.7	1.7	0.5	160	80	75%	di-isobutyl-octadecylsilane	No	100	1–8	600	2
2.7 µm A160 Peptide CN	2.7	1.7	0.5	160	80	75%	di-isopropyl-cyanopropylsilane	Yes	80	1–8	600	2
5 µm A160 Peptide C18	4.7	3.5	0.6	160	60	59%	di-isobutyl-octadecylsilane	No	100	1–8	600	2
5 µm A160 Peptide CN	4.7	3.5	0.6	160	60	59%	di-isopropyl-cyanopropylsilane	Yes	90	1–8	600	2
3.4 µm A400 Protein C4	3.4	3.0	0.2	400	15	31%	dimethylbutylsilane	Yes	90	2–9	600	2

(continued on next page)

The chromatogram in **Figure 1** shows the analysis of IgG2-B subtype monoclonal antibody following solubilization and reduction of the sulfide bonds with 100 mM dithiothreitol (DTT) in 8 M guanidine HCl at 50 °C for 35 minutes. The main components in **Figure 1** are the light (LC) and heavy (HC) chains of IgG2-B. The inset clearly shows several expected variants near the heavy chain peak, although the identity of the minor peaks was not confirmed. The analysis was performed at 80 °C based on the results from Dillon and co-workers at Amgen, which were more recently confirmed by other groups.⁸⁻¹¹

Figure 1. High Temperature Analysis of IgG2-B Antibody Fragments Using a 10 cm x 2.1 mm I.D. BIOshell™ A400 Protein C4 Column

column: BIOshell A400 Protein C4, 10 cm x 2.1 mm I.D., 3.4 μm (66825-U)
 mobile phase: A: water:0.1% TFA, B: acetonitrile:water:0.1% TFA, 80:20
 gradient: 33-40% B in 10 min
 flow rate: 0.25 mL/min
 temp: 80 °C
 detection: 280 nm
 injection: 1 μL
 sample: 0.5 mg/mL IgG2-B treated with 100 mM DTT in 8 M guanidine HCl at 50 °C for 35 min
 instrument: Shimadzu™ Nexera®



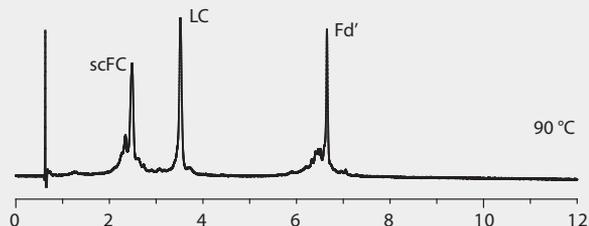
Another example of the need to operate reversed-phase columns at high temperature is provided in **Figure 2**, in which a so-called middle-down, as opposed to a top-down or bottom-up, approach was used to analyze the structure of a monoclonal antibody. In a middle-down approach IgG is digested into a few large fragments prior to further characterization. The novel proteolytic enzyme IdeS, commercially available as FabRICATOR®, cleaves an antibody into F(ab)₂ and scFc fragments, which have approximate molecular masses of 100 kDa and 25 kDa respectively. Using disulfide reduction, the F(ab)₂ fragment can be further resolved into its light-chain (LC) and F(ab) components. Each of the components in the resulting mixture has a molecular mass of about 25 kDa.

Visual inspection of the results shown in **Figure 2** reveals that when run at 90 °C, the BIOshell A400 Protein C4 column (A) provides narrower peak widths and better peak shape than a competitive column packed with fully porous 3.5 micron particles (B). The same conclusion can be drawn from the results for two other competitive columns (C and D) which were operated at 80 °C, the highest temperature recommended by their manufacturer. The ability to operate BIOshell A400 Protein C4 columns at 90 °C provides a beneficial degree of flexibility for biochemists involved in the development and analysis of antibody-based biotherapeutic drugs.

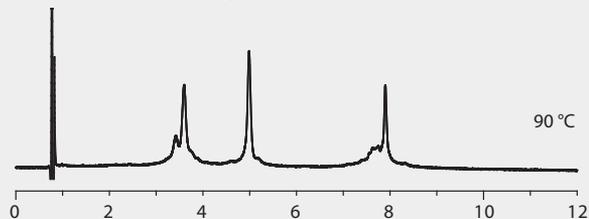
Figure 2. Analysis of Antibody Fragments on Wide Pore Reversed Phase Columns Operated at Maximum Recommended Temperature

column: as indicated, 10 cm x 2.1 mm
 mobile phase A: 80:20, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
 mobile phase B: 50:50, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
 gradient: 30 to 70% B in 12 min
 flow: 0.3 mL/min
 column temp.: as indicated
 detection: UV, 215 nm
 injection: 1 μL, after sample diluted in mobile phase A

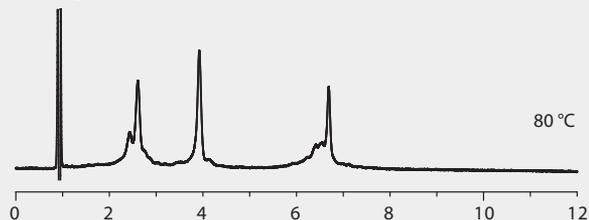
A. BIOshell A400 Protein C4, 3.4 μm



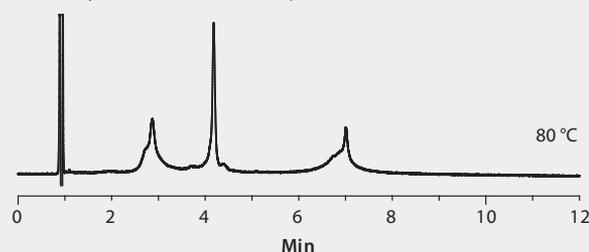
B. Zorbax® 300SB-C18, 3.5 μm



C. XBridge® BEH300 C4, 3.5 μm



D. AcQuity® UPLC® BEH300 C4, 1.7 μm



To determine the stability of columns packed with large pore Fused-Core particles with continued operation at high temperature and flow rate, five globular protein standards were repeatedly injected on a 10 cm x 2.1 mm I.D. BIOshell A400 Protein C4 column. The results are shown in **Figure 3** for the first and last injection of an experiment in which the column was exposed to repeated 10 minute gradients from 25 to 40% acetonitrile/0.1% trifluoroacetic acid (TFA) in water at a flow rate of 0.5 mL/min and a temperature of 90 °C. A noticeable but small loss of retention was observed for all proteins after the column had been in contact with almost 15,000 gradient column

volumes (1 CV ~ 0.2 mL) at 90 °C under acidic (pH ~ 2.0) conditions. Note that the peak shape and peak widths of the test proteins did not change during the experiment, indicating excellent physical stability of BIOshell Fused-Core® columns.

Figure 3. BIOshell A400 Protein C4: Column Stability

column: BIOshell A400, 10 cm x 2.1 mm 3.4 µm (66825-U)
 mobile phase A: water:0.1% TFA
 mobile phase B: acetonitrile:0.1% TFA
 gradient: 25-40% B in 10 min
 flow rate: 0.5 mL/min
 column temp: 90 °C
 detection: 215 nm
 injection: 1.0 µL
 instrument: Shimadzu Nexera

1. Cytochrome c (12.4 kDa)
2. Lysozyme (14.3 kDa)
3. Apomyoglobin (17 kDa)
4. Catalase (250 kDa total; tetramer of ~60 kDa each)
5. Enolase (93 kDa total; dimer of 46.7 kDa each)

Black trace = injection 1

Red trace = injection 385 or after 14,620 column volumes

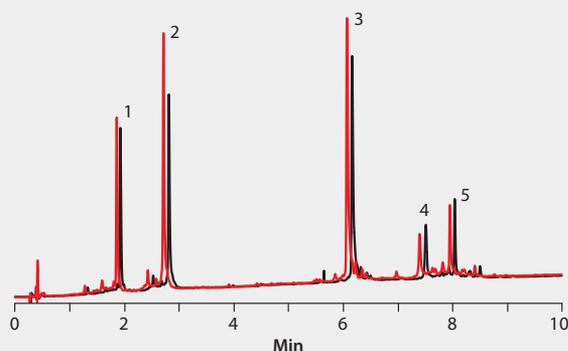


Figure 4. Peak Capacity of a Commercial Fused-Core and Fully Porous Reversed Column for a Peptide Digest Mixture

columns: as indicated, 15 cm x 2.1 mm
 mobile phase A: 0.1 % formic acid
 mobile phase B: 25:75, (0.4 % formic acid):acetonitrile
 gradient: as indicated (in terms of column volumes)
 flow rate: as indicated
 column temp.: 35 °C
 det.: ESI(+)-TOF
 injection: 2 µL
 sample: 10 pmol / µL

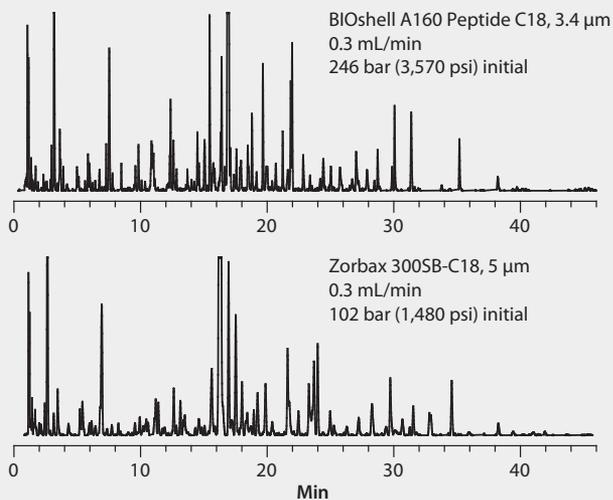


Table 2. Calculated Peak Capacities

Column	t_g^*	w_{ave}	P_c
BIOshell A160 Peptide C18	41.3	0.1213	340
Zorbax® SB300-C18	47.2	0.1951	242

* Recall that the gradient volume is scaled to the same number of column volumes. t_g and w are units of minutes

In a final example, **Figure 4** shows chromatograms of a mixture of 5 different tryptic digests (cytochrome c, beta-lactoglobulin, glutamate dehydrogenase, carbonic anhydrase, myoglobin) run on both a 2.7 micron BIOshell A160 Peptide C18 column and a fully porous 5 micron C18 column of the same dimensions. The analyses were run at the same flow rate of 0.3 mL/min and with the same starting and final gradient mobile phase composition. However, to account for the smaller pore volume of the Fused-Core particles in the BIOshell column, the gradient times were adjusted so that each column was exposed to the same number of gradient column volumes, 46 in this case. About 20 peaks from across the chromatograms (**Figure 4**) were extracted from the MS data to serve as a representative sample in the calculation of the peak capacity (P_c) for each column, using the accepted definition $P_c = \text{gradient time} / \text{average peak width}$. Peak widths were calculated at baseline for each of the 20 selected peptides. **Table 2** lists the experimental values for the gradient time (t_g) and the average peptide peak width (w) as well as the calculated peak capacity for this particular separation on each column.

The 2.7 µm BIOshell column showed a 40% increase in peak capacity over the 5 micron Zorbax 300SB-C18 porous column which is in agreement with the results obtained by Gilar et al. who confirmed results from earlier workers that peak capacity is proportional to the square root of the plate number.¹²

Conclusions

The advances brought about by using porous shell particles with narrow particle size distribution for the analysis of small molecular weight compounds, commercially available as Ascentis® Express columns, have been expanded to the reversed-phase U/HPLC analysis of peptides and proteins. As expected and demonstrated, larger pore size BIOshell Fused-Core columns provide benefits in terms of efficiency per unit pressure drop, which can either be taken advantage of by increasing peak capacity or traded in to reduce analysis time, benefits that are captured in the acronym FP2LC, or Faster Peptide and Protein Liquid Chromatography.

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 Featured Products

Pore Size	Particle Size	I.D. (mm)	L (cm)	C4	C18	CN
BIOshell Fused-Core Peptide and Protein Columns						
400 Å	3.4 µm	2.1	5	66824-U	—	—
400 Å	3.4 µm	2.1	10	66825-U	—	—
400 Å	3.4 µm	2.1	15	66826-U	—	—
400 Å	3.4 µm	4.6	5	66827-U	—	—
400 Å	3.4 µm	4.6	10	66828-U	—	—
400 Å	3.4 µm	4.6	15	66829-U	—	—
160 Å	2.7 µm	2.1	3	—	66901-U	66965-U
160 Å	2.7 µm	2.1	5	—	66902-U	66966-U
160 Å	2.7 µm	2.1	7.5	—	66903-U	66967-U
160 Å	2.7 µm	2.1	10	—	66904-U	66968-U
160 Å	2.7 µm	2.1	15	—	66905-U	66969-U
160 Å	2.7 µm	3.0	3	—	66906-U	66970-U
160 Å	2.7 µm	3.0	5	—	66907-U	66971-U
160 Å	2.7 µm	3.0	10	—	66908-U	66972-U
160 Å	2.7 µm	3.0	15	—	66909-U	66973-U
160 Å	2.7 µm	4.6	5	—	66913-U	66974-U
160 Å	2.7 µm	4.6	10	—	66915-U	66975-U
160 Å	2.7 µm	4.6	15	—	66917-U	66976-U
160 Å	5 µm	2.1	3	—	67001-U	67061-U
160 Å	5 µm	2.1	5	—	67002-U	67062-U
160 Å	5 µm	2.1	7.5	—	67003-U	67063-U
160 Å	5 µm	2.1	10	—	67004-U	67064-U
160 Å	5 µm	2.1	15	—	67006-U	67065-U
160 Å	5 µm	3.0	3	—	67007-U	67066-U
160 Å	5 µm	3.0	5	—	67008-U	67067-U
160 Å	5 µm	3.0	10	—	67011-U	67068-U
160 Å	5 µm	3.0	15	—	67012-U	67069-U
160 Å	5 µm	4.6	5	—	67013-U	67071-U
160 Å	5 µm	4.6	10	—	67014-U	67080-U
160 Å	5 µm	4.6	15	—	67015-U	67081-U
BIOshell Fused-Core Peptide and Protein Guard Columns, pk. of 3						
400 Å	3.4 µm	2.1	0.5	66830-U	—	—
400 Å	3.4 µm	4.6	0.5	66831-U	—	—
160 Å	2.7 µm	2.1	0.5	—	66918-U	66977-U
160 Å	2.7 µm	3.0	0.5	—	66919-U	66978-U
160 Å	2.7 µm	4.6	0.5	—	66921-U	66979-U
160 Å	5 µm	2.1	0.5	—	67016-U	67082-U
160 Å	5 µm	3.0	0.5	—	67017-U	67083-U
160 Å	5 µm	4.6	0.5	—	67018-U	67084-U

 Related Product

Description	Cat. No.
BIOshell Guard Cartridge Holder	66841-U