Fast Analysis of Biomolecules by Using Smaller and Innovative Particles

Daniel Eßer, Ann Marie Rojahn, Mathias Hehn, YMC Europe GmbH, Dinslaken, Germany

Abstract

Modern stationary phases for BioLC have to offer high resolutions, fast methods, high robustness and high lot-to-lot reproducibility for reliable results. Furthermore, the separation modes typically used in BioLC present different challenges. These requirements can be fulfilled by state-of-the-art particles of optimised sizes and types. Selected solutions for different biological substances are described in this article.

Introduction

Requirements in BioLC differ from those encountered in small molecule analyses. For example, analytes usually have a higher molecular weight (MW) and a greater hydrodynamic volume. Due to their increased size, these substances such as proteins or antibodies also are extremely hydrophobic. Furthermore, closely related compounds have to be separated from each other including monomers from aggregates or fragments, native compounds from post translational modified ones, or target compounds from side products such as those with additional/missing amino acids or nucleotides.

The demand for BioLC products is increasing thanks to ever-expanding research activities with all types of biomolecules as well as the plethora of biological compounds entering the commercial market which must be analysed in quality control labs (QC) [1]. Consequently, faster analytical solutions to enhance the sample throughput are desired. As encountered in small molecule QC labs, reliable, robust and reproducible (ultra) high performance liquid chromatography ((U)HPLC) products are required to ensure high-quality standards for these products entering the commercial space.

Over the past several years the UHPLC column market for BioLC has grown according to the needs of users in both the R&D and QC environments [2]. Solutions for the different types of biomolecules are now available: peptides and proteins, monoclonal antibodies (MAbs) and antibody-drug-conjugates (ADCs) as well as oligonucleotides and nucleic acids. To analyse these compounds, products for the various separation modes are available: reversed phase (RP), size exclusion (SEC), ion exchange (IEX) and hydrophobic interaction (HIC). Small particles with different base materials ensure quick runs and different detection options, such as UV, MS or MALLS (multi-angle laser light scattering).

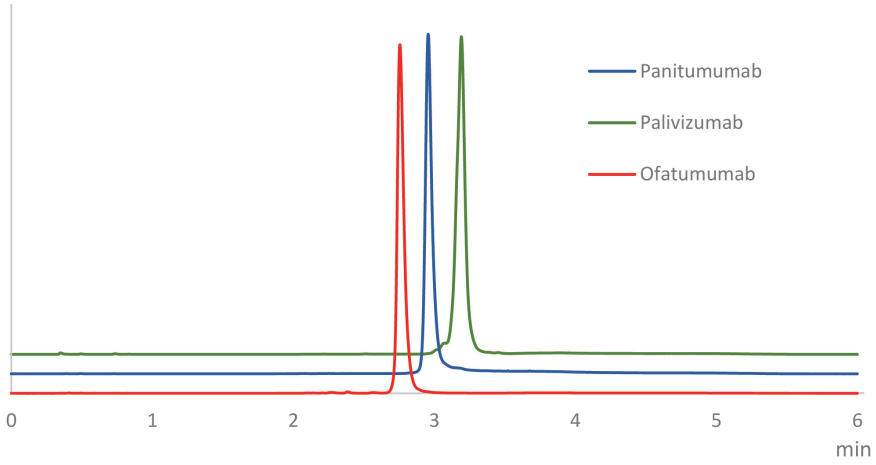

Figure 1: Symmetrical peaks for challenging MAbs using UHPLC column YMC-Triart Bio C4 (1.9 µm, 300 Å, 50 x 2.1 mm ID) at an elevated temperature of 90 °C. Eluent: A) 0.1 % TFA in water, B) 0.1 % TFA in acetonitrile; gradient: 25–50 % B (0-4 min); flow rate: 0.4 mL/min; detection: fluorescence ex 280 nm; em 350 nm; injection: 0.5 µL. Courtesy of University of Geneva, School of Pharmaceutical Sciences, Department of Analytical Pharmaceutical Chemistry.

UHPLC columns for RP based on hybrid particles

In RP, sub-2 µm particles for UHPLC applications have been a standard for several years. As biomolecules have different requirements than small molecules, larger pore sizes and higher temperature tolerances are crucial. This permits the analysis of large proteins, antibodies, and strongly hydrophobic compounds, which require the lower hydrophobicity provided by wide pore phases with short chain modifications. In addition, particle robustness and mechanical stability are mandatory, which is very challenging in UHPLC due to the high pressures encountered.

In general, employing higher temperatures for analysis often has a beneficial effect on the chromatographic results; such as

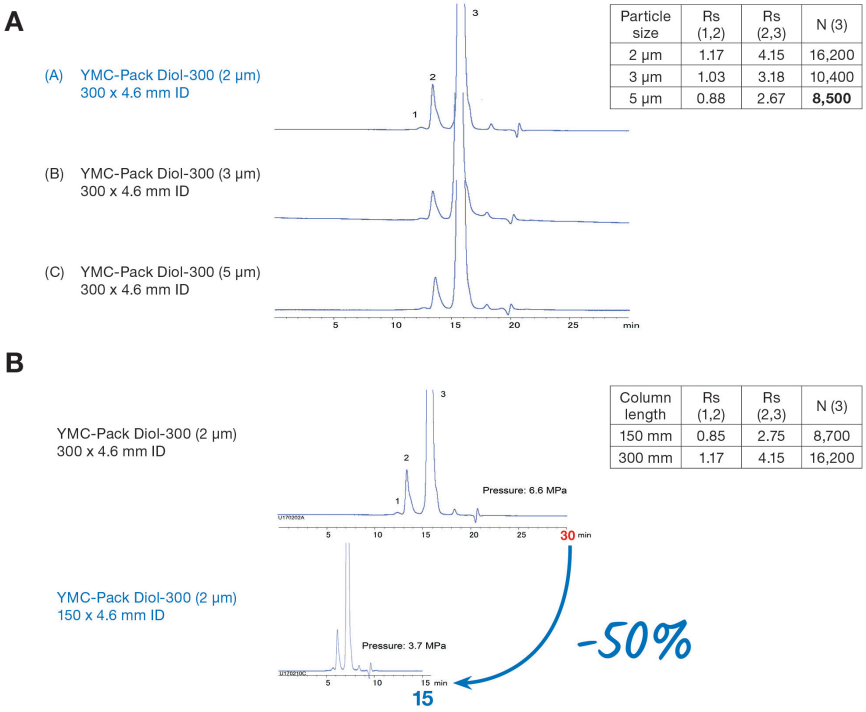

Figure 2: All three particle sizes show identical separation patterns for the analysis of IgG1. Using UHPLC particle size greatly improves the resolution (A). Use of 2 µm particles further allows higher throughput of analyses by using shorter columns (B). Eluent: 0.1 M KH_2PO_4-K_2HPO_4 (pH 7.0) containing 0.2 M NaCl; flow rate: 0.2 mL/min; temperature: ambient; detection: UV at 280 nm.

improved resolution or increased peak capacity. The higher column temperature reduces the viscosity of the mobile phase allowing compounds to enter the pores and interact with the particle surface more easily and reduces adsorption onto the particle surface. Temperature can affect configuration of a protein as well as the diffusion rate and therefore effectively increases the performance.

Sub-2 µm UHPLC particles are difficult to produce, since the particles encounter higher backpressures and consequently have to be more rugged than their larger particle counterparts. One possible approach to ensure particles can tolerate this high-pressure is to reduce the porosity of the silica particles, which in turn results in very low surface areas and which comes at the cost of lower resolution [3]. In addition to the pressure related particle strength challenge, the thermal stability of the stationary phase also must be addressed.

A different solution is offered here. Instead of conventional silica-based particles, organic/inorganic hybrid silica particles are produced that contain ethylene bridges in their silica backbone. This provides high mechanical strength with greater chemical stability, resulting in high temperature tolerance (up to 90 °C) and a working pH range from 1 to 12. These high surface area particles (standard ≥300 m²/g for 120 Å) are produced with standard pore sizes of 120 Å for peptides or oligonucleotides and with large pores of 300 Å for larger peptides or antibodies. Additionally, the particle surface possesses increased inertness due to the multi-step endcapping process employed.

Currently four different modifications for BioLC are available in these organic/inorganic hybrid silica columns (YMC-Triart/YMC-Triart Bio, YMC Co., Ltd., Kyoto, Japan). These stationary phases are available for UHPLC use with the 1.9 µm particle size and are fully scalable to 3 or 5 µm as well. C18 with 120 Å pores is the ideal choice for peptides, peptide mapping or oligonucleotide analysis. C8 columns (120 Å) allow shorter run times especially in oligonucleotide separations. Widepore Bio C18 is dedicated to larger peptides or proteins, while Bio C4 (both 300 Å) not only allows separations of large proteins but also intact MAbs (Figure 1).

In addition to the very inert base particles which don't contain any metal impurities, inertness of the column housing can be optimised as well. For that purpose, a bioinert, metal-free hardware is available. By using a PEEK-lined stainless steel column body together with PEEK frits, any interaction with the hardware is eliminated while simultaneously maintaining the pressure stability of the column.

Columns for SEC with special requirements towards inertness

The separation principle in SEC is purely based on MW, more specifically, hydrodynamic volume, and the accessibility of the pores with no actual interaction with the stationary phase. Any secondary interaction has to be avoided so as not to influence the SEC separation. Consequently, the more inert the SEC stationary phase, the more reproducible the corresponding results will be. Due to the separation mechanism, larger column volumes and lower flow rates are required which results in longer analysis times. Reducing the particle size allows shorter columns and smaller column internal diameters resulting in reduced run times while maintaining or even improving resolution.

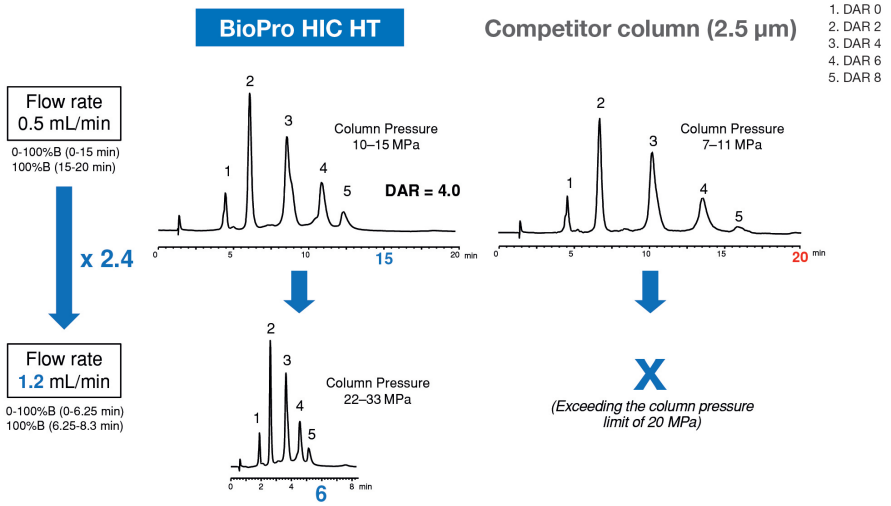

Figure 3: DAR determination of the ADC Brentuximab vedotin using BioPro HIC HT (2.3 µm, 100 x 4.6 mm ID) and a competitor column. BioPro HIC HT allows higher flow rates, while the competitor column can only be used at standard flow rates to operate below its pressure limit. Eluents: A) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 1.0 M $(NH_4)_2SO_4$, B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (85/15); temperature: 25 °C; detection: UV at 280 nm; injection: 10 µL (2.5 mg/mL).

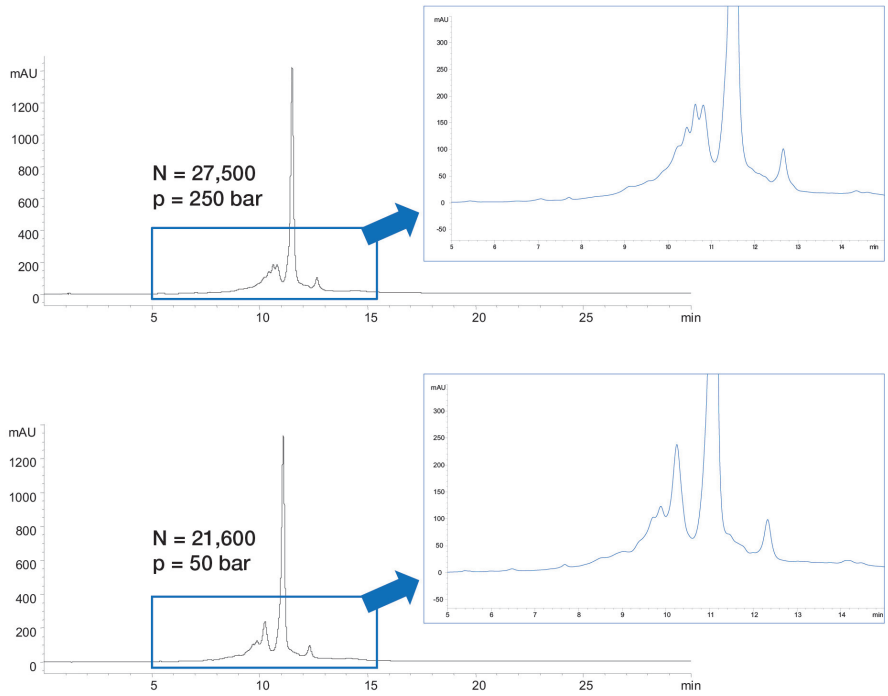

Figure 4: Separation of Bevacizumab (Avastin®) using non-porous BioPro IEX SF with 3 µm (top) and 5 µm (bottom). Eluent: A) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8), B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8) containing 0.2 M NaCl; gradient: 0-50 %B (0-30 min) 0 %B (30-45 min); flow rate: 0.5 mL/min; temperature: 25 °C; detection: UV at 215 nm; injection: 10 µL (0.25 mL/min).

SEC particle sizes of 2 µm or smaller for UHPLC are very challenging to produce. Similar to RP, typical approaches to provide higher pressure resistance are either to reduce the porosity or to use different base materials such as hybrid materials as described above for RP. In addition to the hurdles described previously, the second option has the disadvantage of reduced inertness, as the organic components of the base particles can provide undesirable secondary interactions [4]. An additional aspect of using small particle sizes of sub-2 µm also must be considered since elevated pressures can lead to an increased formation of aggregates during the chromatographic process, which alters the actual rate of aggregation in the sample [5].

Silica-based SEC phases typically with diol modification (e.g. YMC-Pack Diol or YMC-SEC MAB) provide the highest inertness. Different pore size and particle size combinations cover different analyte types from small peptides to antibodies and their aggregates. Particles are available in 3 and 5 µm for HPLC separations. The two most common pore size particles are 200 and 300 Å, Diol-200 and Diol-300, which are used e.g. MAbs, are also produced as 2 µm particles for UHPLC purposes. These 2 µm particles provide the same porosities and properties as their larger counterparts (Figure 2). Therefore, they not only allow for the development of new and improved methods, but also a linear down scaling of existing HPLC methods to UHPLC due to the same particle properties being used over different particle sizes.

In order to further improve the column inertness, it is ideal to suppress any option of secondary interaction. Even if the stationary phase itself is inert, the hardware used might also have an influence. Therefore, using bioinert hardware such as PEEK-lined stainless steel column bodies can be a possible approach and is currently under evaluation.

HIC columns designed for high throughput

Hydrophobic interaction chromatography (HIC) is used as a standard technique to determine the drug-to-antibody ratio (DAR) of antibody-drug-conjugates (ADCs) [6]. Therefore, HIC methods are routinely used for quality control purposes of ADCs. Because quality control departments demand a high throughput, and shorter runtimes which can be achieved by the use of smaller particle sizes are required.

In HIC, polymer-based stationary phases separate the substances according to hydrophobicity, typically using a reverse salt gradient to re-hydrate proteins salted out by a high concentration of chaotropic salt. Even though more stable non-porous particles can be used, pressure specifications of most products on the market are limited to typically 200 bar to avoid particle deformation and the resulting loss of performance. Therefore, lower flow rates have to be used, which result in longer retention times which is counterproductive to higher throughput.

In order to overcome this drawback, a new stationary phase, (BioPro HIC HT, YMC) was developed which is based on a non-porous polymethacrylate particle. The butyl bonded particle is available with 2.3 µm particles and is designed for the analyses of biomolecules, especially ADCs. This very rigid phase is different to other phases as it allows much higher flow rates and therefore shorter run times thanks to its pressure stability of up to 400 bar (twice that of other commercially available phases).

Using BioPro HIC HT, analysis times for DAR determinations of ADCs such as Brentuximab vedotin, which are already quite fast, can be further reduced to 6 min by applying a 2.4 times higher flow rate, while maintaining the high resolution (Figure 3). This allows a 2-3 times higher sample throughput, which would not be possible with the competitor column due to the much lower pressure limit of only 200 bar.

Are smaller particles in IEX an ideal solution?

In general, polymer-based particles are also used in IEX, especially for charge variant analyses of MAbs. The same particle technology is used in this case as for the HIC columns, though optimised for IEX chromatography. The strong exchangers with either quaternary ammonium or sulfonic acid groups are available as porous (BioPro IEX QA/SP, YMC) or non-porous (BioPro IEX QF/SF, YMC) types. The porous type (5 µm) allows high sample loadings and high resolutions, while the non-porous phases provide outstanding efficiencies at low sample loads typically used in QC separations. The non-porous exchangers are available in 5 or 3 µm particles.

Although less pronounced, compared to the separation modes described above, a smaller particle size still improves resolution in IEX. On the other hand, the backpressure is much higher (Figure 4). Consequently, not all applications benefit from the use of smaller particles. Depending on the mobile phase, the backpressure can be close to the 3 µm column's pressure limit and the separation cannot be improved much further [7].

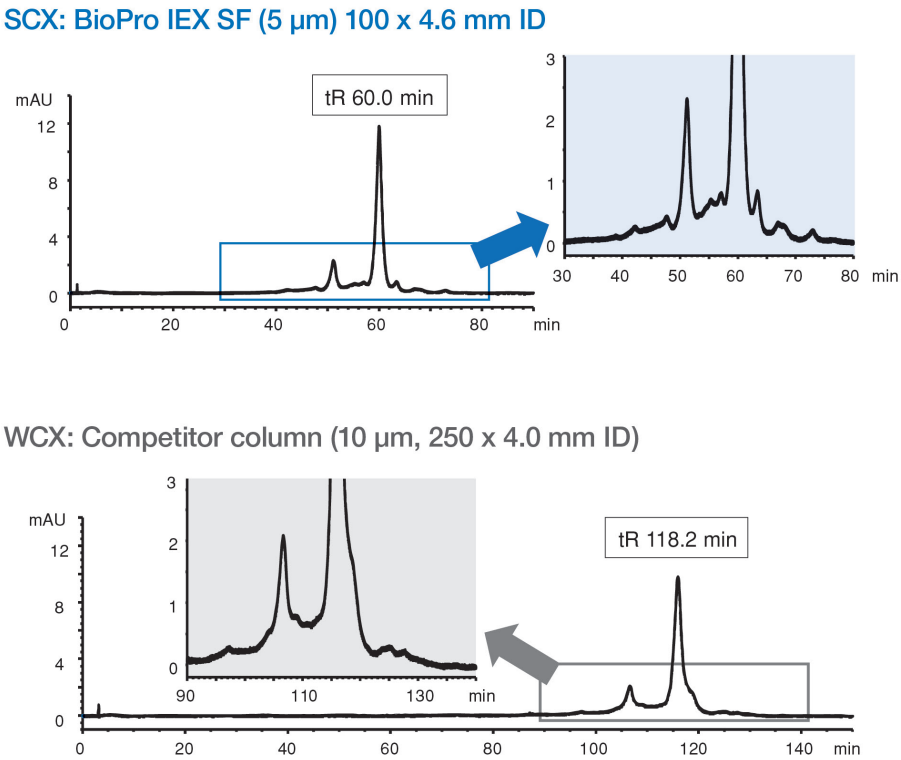

Figure 5: Separation of MAbs using non-porous BioPro IEX SF column and a weak exchanger column often used for such analysis. The strong exchange column can achieve higher resolution in shorter analysis time. Eluent: A) 20 mM MES-NaOH (pH 5.6) B) 20 mM MES-NaOH (pH 5.6) containing 0.2 M NaCl; gradient: 35 %B (70 mM NaCl); Gradient slope: 0.25 %B/min (0.5 mM NaCl); Flow rate: 180 cm/hr; temperature: 30 °C; detection: UV at 280 nm; injection: 10 µL (1 mg/mL IgG1).

5 µm non-porous BioPro IEX QF/SF columns offer higher performance when compared to other IEX columns (Figure 5). Due to the low backpressures, higher flow rates can often be applied while maintaining the desired resolution. This fact together with the particle robustness and reproducibility makes the 5 µm non-porous exchangers an ideal choice for QC.

Conclusion

The use of smaller, modern particles usually allows shorter analysis times and therefore higher sample throughput for all separation modes typically used in BioLC. With the increasing number of therapeutic biologicals, this is welcomed by R&D as well as QC labs. Modern solutions based on different particle technologies and materials produce the flexibility required for most BioLC separations and depending on the separation mode employed the small particle sizes available (between 1.9 and 3 µm) allow high resolutions and speedy run times for each mode.

1. M. Kesik-Brodacka, Progress in biopharmaceutical development, Biotechnol. Appl. Biochem., 65(3) (2018) 306-322.

2. Evaluate Pharma, World Preview 2019, Outlook to 2024, 12 (2019).

3. I. Novák, B. Buszewski, J. Garaj, D. Berek, Influence of pore structure of silica packing on HPLC column characteristics, Chem Papers, 44 (1) (1190), 31-43.

4. A. Goyon, S. Fekete, A. Beck, J.L. Veuthey, D. Guillarme, Unravelling the mysteries of modern size exclusion chromatography – the way to achieve confident characterization of therapeutic proteins, J. Chrom. B, 1092 (2018) 368–378.

5. Proteins Under Pressure, Chromatography Today, 12(4) (2020) 44-46.

6. V. D'Atri, R. Pell, A. Clarke, D. Guillarme, S. Fekete, Is hydrophobic interaction chromatography the most suitable technique to characterize site-specific antibody-drug conjugates?, J. Chrom. A, 1586 (2019) 149–153.

7. A. Murisier, M. Lauber, S. J. Shiner, D. Guillarme, S. Fekete, Practical considerations on the particle size and permeability of ion-exchange columns applied to biopharmaceutical separations, J. Chrom. A, 1604 (2019) 460-487.