



# Supporting a New Dawn in Biologic Therapies

Explore our expertise across all stages of therapeutic antibody development, beginning with early research and traversing the production line through to method development, upscaling and production.

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New Antibodies on  
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Conjugates

Glycan Analysis

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## The New Antibody Formats on the Block

More complex alternatives to monoclonal antibodies are demanding innovation from manufacturing and purification techniques

By Patrick Endres

Monoclonal antibodies and other biopharmaceutical products, as well as their manufacturing processes, are inherently at risk of viral contamination, making viral safety testing critical. Viral safety testing is mandated by regulators worldwide, and although technologies for biomanufacturing have rapidly advanced, viral testing methods remain largely the same today as they were thirty years ago. Traditional virus detection approaches – cell-based assays – have served the biopharma industry very well over the years, but they have limitations; for example, some assays have turn around

times as long as 28 days. In addition, although cell-based assays can detect contaminants, they generally cannot directly identify them and it can be slow to obtain results.

Albert Einstein once said, "Once

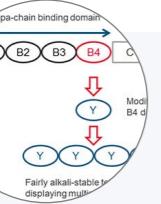
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"we accept our limits, we go beyond them." In an age where speed is the key to success, we believe it is time to accept the limitations of traditional testing and to focus on newer technologies that focus on speed, sensitivity and reliability. Faster assay results will lead to more rapid batch disposition, reduced interruption of processing, and also meet the needs of more intensified processing – a key capability given the increasing interest that manufacturers are paying to continuous manufacturing strategies.

### Working together

One of the biggest challenges for everyone in biopharma is that the industry is very slow to change. Developing a new biopharmaceutical takes years, and once completed, companies prefer not to replace it unless there is a very good reason to do so! And this is something we take to heart at Tosoh: we have a very strong commitment to continue producing and supplying established products for a very long time. Our customers don't want to wake up one morning and realize that Tosoh has suddenly stopped producing a material that is essential to their process and product! For example, the on-going expansion of production capacity by 50 percent is part of this commitment.

In the same way, close cooperation with the biopharma

industry is crucial for us when we want to develop new prototypes or products, and we are always talking with companies to find out what innovations they would like to see, both in the near future and looking much further ahead.

Current buzzwords in downstream processing are "single-use technology" and "continuous production".

Continuous technologies offer the potential for using smaller volumes of highly efficient resins and getting more productivity out of the resin – a win-win situation.

It's clear there is an exciting future for the industry; biopharmaceuticals are becoming more advanced, manufacturing methods are improving, and there is also the advent of cell and gene therapies that could completely change how patients are treated. But we need to work on development timelines. It can take in excess of 10 years for a product to go from idea to market. Surely this can be reduced if regulators, manufacturers, resin vendors and other suppliers work together to optimize bioprocessing.

Patrick Endres is Senior Laboratory Specialist at Tosoh Bioscience, Germany.



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## Process Analytics and Intermediate Purification of Bispecific Antibodies with a Non-Affinity Platform

TOYPEARL resins afford high binding capacities for the purification of bispecific antibodies

Modern chromatography resins were evaluated for the purification of a bispecific antibody - entities widely acknowledged to overcome some of the limitations associated with monoclonal antibodies. Hydrophobic cation exchange chromatography and hydrophobic interaction chromatography can replace two subsequent affinity chromatography steps for the purification of a  $\kappa\lambda$ -body (a fully human antibody devoid of linkers or mutations) from the monospecific monocloonal antibody by-products. The three-step process shows comparable yields to a 3-step affinity platform process currently used to purify a  $\kappa\lambda$ -body. The excellent selectivity of TOYPEARL MX-Trp-650M and TOYPEARL Butyl-600M paves the way for future implementation at research, clinical and commercial manufacturing scales. This approach combining reduced cost of goods and higher binding capacities offers an attractive new version of the purification process for the future manufacture of  $\kappa\lambda$ -bodies.

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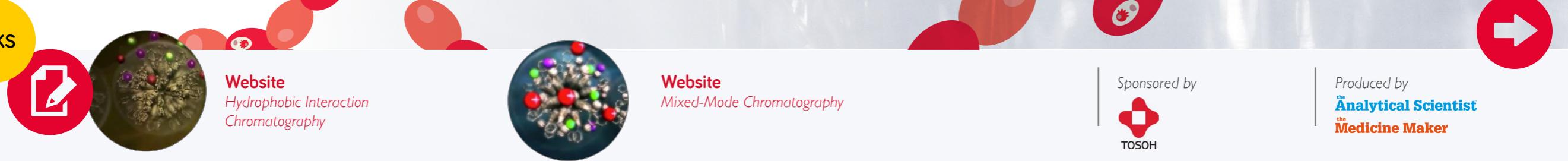


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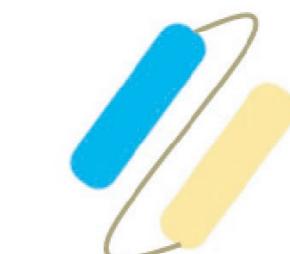
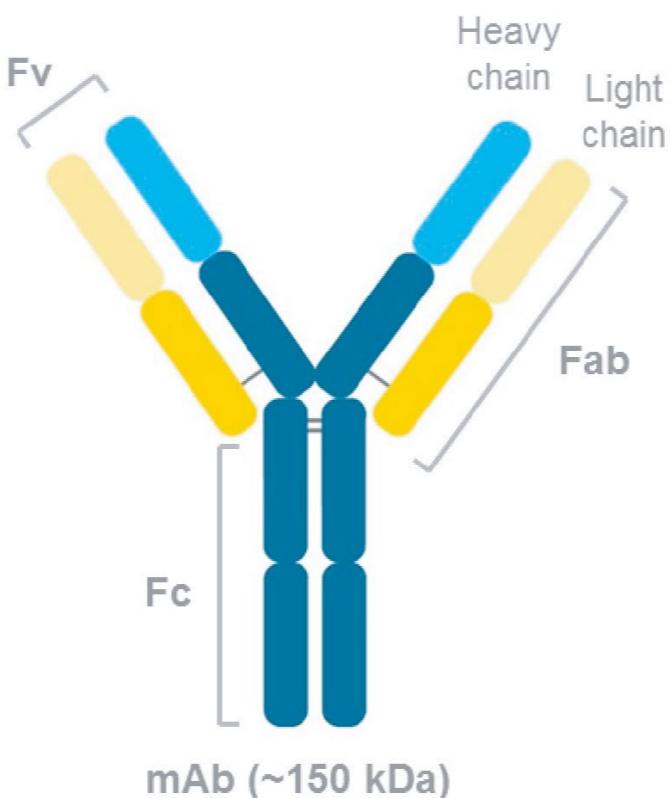
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## Capturing of a Single-Chain Variable Fragment from E. Coli F

An applied example of general method setup and optimization using TOYOPEARL AF-rProtein L-650F

The optimization of single-chain variable fragment (scFv) capture on TOYOPEARL® Protein L resin using a robotic parallel chromatography system leads to significant purity improvement. More specifically, post-load washing with chaotropic agents can significantly reduce host cell protein (HCP) content. It may further support on-column refolding of misfolded and aggregated product.

ScFvs are antibody-derived molecules comprising the variable part of the light chain and the heavy chain, connected by a peptide linker. The molecular weight of the whole construct is approximately 30 kDa. ScFvs are well suited to production in E.coli, since they lack the glycosylation site of a full-length immunoglobulin G (IgG). Other advantages over a full-length IgG include rapid target access and good tissue penetration. However, renal clearance limits serum half-life to hours instead of days, when compared with the typical serum half-lives of monoclonal antibodies. In some therapeutic strategies, this may be an advantage. Hence, straight-forward and efficient capturing solutions similar to Protein A for monoclonal antibodies may pave the way for the future success of this class of molecules. This application note describes general conditions for capturing an exemplary scFv from E. coli with TOYOPEARL AF-rProtein L-650F, with optimized HCP removal through a parallel chromatographic approach.

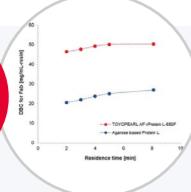


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## Toward Better Biotherapeutics

When digging into the inherent complexity of antibody-drug conjugates, choosing the right chromatography column is key - we spoke with the University of Geneva's Alexandre Goyon about the evolution of UHPLC, and how to streamline separations for complex biotherapeutics

### How did you get into analytical chemistry?

My first experience with analytical chemistry instrumentation was during my Master's degree in Philip Jessop's lab at Queen's University, Canada. I knew straight away that it was an area I'd like to explore more. I joined the Nestlé Research Centre and spent two years developing and validating LC-MS/MS methods for determination of chemical contaminants in food materials. Next, I joined the pharmaceutical analysis lab in Geneva for my PhD, working with three well-known scientists – Jean-Luc Veuthey, Davy Guillarme and Szabolcs Fekete. While there, I used different LC and capillary electrophoresis approaches for biopharmaceutical analysis.

### What keeps you moving forwards?

It's easy to be motivated about improving therapies for cancer patients! Of course, my research is a long way from the patient, which is one reason why I wanted to spend time in biopharmaceutical companies. There, I can see first-hand the impact of analytical chemistry on drug discovery, development and manufacture.

### Your work has focused on antibody-drug conjugates (ADCs). Why is analysis of these biotherapeutics particularly challenging?

ADCs combine a lipophilic drug with a monoclonal antibody, meaning that the hydrophobicity of an ADC is much more pronounced than that of an unconjugated antibody. In theory, size-exclusion chromatography (SEC) separates different species based on their size, but in practice the picture is complicated by a range of non-specific interactions, including hydrophobic interactions.

To reduce hydrophobic interactions, biopharma scientists typically include isopropanol in the mobile phase, but this introduces two drawbacks: i) you



may no longer be working under native conditions, and ii) isopropanol has been found to have deleterious effects on some antibodies.

### How have recent innovations in SEC columns helped tackle the complexities of ADC analysis?

The ability of the previous generation of ultrahigh-pressure SEC columns to limit non-specific hydrophobic interactions with little or no isopropanol has helped to establish the validity of these analyses. In addition, most ultrahigh pressure columns are packed with sub-micron particles, which has allowed for smaller columns and faster separations. You can now do in a 4.6 mm ID, 150 mm length column what used to require 7.8 mm ID and 300 mm length, and a separation that used to take 45 mins can be completed in 10 mins. Such improvements start to look attractive in other areas of biopharma development, such as process control.

I would go as far to say that, when it comes to ADC analysis, the

biggest advances over the last five years have been seen in SEC columns.

### You published an article in the Journal of Chromatography A this year on "Extending the limits of SEC" (1) – what was your aim?

A critical quality attribute of ADC products is the amount of free payload in solution. Typically, the payload molecule is cytotoxic, so you don't want it being released before the ADC reaches its target. Establishing the free payload content of a candidate drug in solution by SEC alone can be challenging. During SEC of an ADC, the high-molecular-weight species (whole ADC, monoclonal antibody, etc) will emerge first, followed by smaller molecules (payload, linker, etc). The smallest molecules all elute as a single band, making it impossible to accurately quantify the payload. Previously, researchers had tried a 2D-LC method – coupling SEC to reverse-phase chromatography to separate the smaller molecules – but we were able to find a faster, more streamlined method.

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### How did you develop the new method?

I was conducting experiments using an ultrahigh pressure SEC column (Tosoh) with colleagues at the University of Geneva and a major pharmaceutical company based in Basel. We found that the payload species were highly hydrophobic, and were therefore absorbed onto the stationary phase. It gave us an idea: why not try a two-part separation using the same SEC column? We allowed the largest proteins to elute, then applied an acetonitrile gradient to elute the smaller molecules. We were pleasantly surprised to find that, thanks to secondary hydrophobic interactions, the acetonitrile gradient allowed separation of the smaller molecules. For some ADCs, this meant we were able to quantify both the high-molecular-weight species and the free payload in a single run – in under 10 mins. By taking advantage of (usually undesirable) hydrophobic interactions, the streamlined method could allow R&D scientists to quickly rule out ADC candidates that release payload in solution. It's a great example of how analytical chemistry can help in the development of new biotherapeutics.

### What technology is likely to have the biggest impact in biopharma – now and in the future?

Right now, the most important trend is towards multidimensional LC, as demonstrated by the large number of presentations on the topic at HPLC 2018 in Washington. Most vendors now sell at least some 2D-LC-specific solutions, although software development for these systems has lagged behind somewhat. I believe that over the next five years, most companies will be using multi-dimensional LC approaches, coupled to high-resolution MS instruments.

Looking ahead 10 years, I think we will see separations going beyond two dimensions and into three, four or five dimensions. In parallel, I hope that suppliers will be able to improve column chemistry even further, in particular to reduce non-specific interactions in SEC and allow it to be coupled directly to MS.

Pharma is (rightly) a cautious industry and, before any new technology is adopted, companies and regulators must be sure it won't cause unexpected issues. Nevertheless, despite the technical and regulatory challenges, all the company scientists I have worked with have been very open to new technology.

#### Reference

1. A Goyon et al., *J Chromatogr A*, 1539, 19-29 (2018).

## Keeping Apace with Biopharma Trends

*Regina Roemling, Senior Marketing Manager, Separations at Tosoh Bioscience GmbH, Germany fills us in on the company's latest innovations for the biopharma market*

What challenges do vendors face in developing products for biopharmaceutical applications?

The biopharmaceutical industry is highly regulated and established methods are not easily replaced. Consequently, products such as chromatographic resins used in manufacturing or columns applied in QC of an approved biologic have to maintain a consistent quality over a very long period. On the other hand, the development time for new analytical tools needs to be reduced to cope with the increasingly rapid development of new, complex biopharmaceuticals. Looking just at therapeutic antibodies, there is a huge range, from antibody-drug conjugates (ADCs) to small, single-chain variable fragments (scFv). Analytics need to keep pace with this variety.

What do biopharma customers want?

R&D labs dream of multidimensional analytical platforms allowing the thorough characterization of tiny amounts of candidate molecules overnight. In production, throughput is of higher importance, but robustness and reproducibility are essential too.

What trends in biopharma analysis have you seen in recent years? As Alexandre describes, hyphenation and multidimensional chromatography have been amongst the hottest topics at recent HPLC meetings. Another trend is the increasing use of affinity-based separations, not only in purification but also in HPLC analysis. A good example is Protein A affinity, which can be applied for fast analysis of cell titers or as a kind of sample pretreatment of a crude feedstock in process analytics when it's coupled with techniques like size exclusion chromatography (SEC) for monitoring aggregate contents. In fact, we will soon launch a new affinity column designed to analyze the antibody-dependent



cell mediated cytotoxicity (ADCC) activity of antibodies with high selectivity and reproducibility.

Why develop a column for measuring ADCC activity?

ADCC plays an important part in the mechanism of action of therapeutic antibodies, particularly cancer-targeting monoclonals. When developing an antibody for cancer therapies it is extremely important to select a clone delivering the required ADCC activity, but current methods (*in vitro* cell-based assays or surface plasmon resonance techniques) are less reproducible than chromatography. In addition, there are many more potential applications for a fast and highly reproducible HPLC method; for example, monitoring lot-to-lot differences for antibody products or comparing the ADCC activity of an antibody biosimilar to the innovator. We are keen to hear further ideas from academia and industry for potential applications and are looking forward to releasing the new affinity column by the end of 2018, which we are confident will be a real asset to biopharma scientists (!).

#### Reference

1. M Kiyoshi et al., *Nature Scientific Reports*, 8, 3955 (2018).





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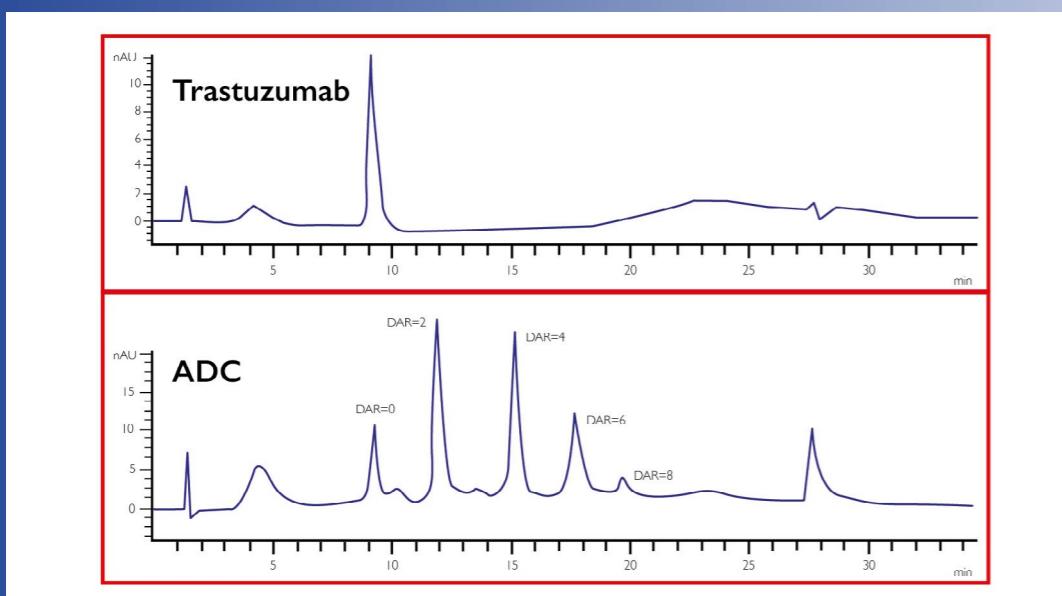
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## DAR Analysis of Antibody-Drug Conjugates

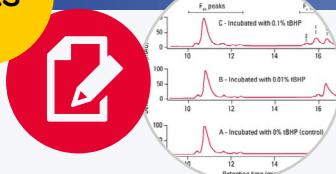
An applied example of general method setup and optimization

The antibody drug market has continued to expand in recent years, and antibody drugs held six of the top ten blockbuster drug spots for 2015. The most promising antibody drug candidates for next-generation biopharmaceuticals are antibody-drug conjugates (ADCs). ADCs have a structure in which a cytotoxic drug is chemically bonded to an immunoglobulin G antibody.

Since low-molecular weight drugs are more hydrophobic than monoclonal antibodies, differences in hydrophobicity arise when the numbers of bonded drug molecules differ. This property can be utilized to determine the drug-to-antibody ratio (DAR) by hydrophobic interaction chromatography (HIC). In this application note, an ADC was analyzed using a TSKgel Butyl-NPR column, which can be used with both high-pressure LC and ultrahigh-pressure LC instruments.



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TSKgel Butyl-NPR



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## TOYOPEARL PPG-600M: the HIC Resin for ADC Purification

Antibody-drug conjugates (ADCs) are promising anticancer biopharmaceuticals, with four ADCs receiving market approval to this point. They combine the high selectivity and affinity of an antibody with the toxicity of chemotherapeutics.

ADCs consist of a monoclonal antibody, covalently bound via a linker to a highly potent cytotoxic drug. Due to the highly toxic payload, very high safety standards should be implemented during method and process development. ADC-mimics contain a non-toxic payload with similar structure and physicochemical properties as the toxic payload of an ADC. Therefore, they can be used as a model to develop a suitable purification process or analytical method. The ADC-mimic in this work consists of adalimumab bound to fluorescein 5-isocyanate (FITC).

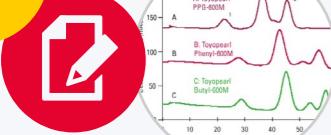
The purification process of ADCs is complex due to the heterogeneity of the conjugates. The main challenges are in the isolation of the unconjugated antibody and free drug, and the separation in different drug-antibody-ratios (DARs), which correlate with the potency of the ADC. High DARs are associated with high cytotoxic levels and can cause aggregation, affecting the stability of the ADC. On the other hand, low DARs can have an impact on the efficacy of the therapeutics.



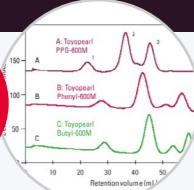
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## Sugar Rush

The addition of polysaccharide chains to therapeutic monoclonal antibodies can have a dramatic impact on their safety and efficacy – and that's why glycosylation is a critical quality attribute. Here, we speak with Tomasz Walski, Project Group Leader in the R&D department at Mabion S.A., to find out how the biosimilar developer is keeping glycan analysis short and sweet.



### What exactly does your team do – and what analytical technology do you rely on?

In short, my team develops chromatographic methods to analyze the quality attributes of the therapeutic antibodies we develop. Our workhorse analytical tools are (ultra)high-pressure LC ([U]HPLC) and LC-MS/MS systems.

### What keeps you motivated?

As a company, our priority is creating safe, effective and accessible drugs for the patients who need them. The safety of a biological drug relies on the quality attributes of the antibody, so having sensitive and accurate methods to analyze them is of outmost importance during the drug development phase. It is incredibly rewarding to feel that our work is contributing directly to patient safety.

My favorite part of the job is when we are able to improve a method to generate less waste, be more analyst-friendly and less time-consuming – all while still generating high-quality results. Ultimately, that enables us to develop projects faster and deliver safe drugs to patients at an affordable price. We are constantly looking for technological innovations that help us to achieve that.

### Why is N-glycosylation so important in biopharma analysis?

N-linked glycosylation describes the attachment of oligosaccharides (sugars) to a protein, via an asparagine residue. We pay very close attention to N-glycosylation of biologics – the composition of the sugar chains attached to the drug molecule is one the key quality attributes for therapeutic antibodies.

N-glycosylation strongly affects the way the drug interacts with its target (for example, cancer cells). It determines how efficient it is in its therapeutic action, how long it remains active and how long it circulates in the bloodstream. In addition, making sure that the N-glycosylation stays the same during development, through clinical trials and onto the market helps to minimize the possibility of any adverse reaction. It is crucial to carefully monitor the N-glycosylation



throughout project development and then in the production process to ensure that patients always receive safe, high-quality medicine.

### What challenges does glycan variant analysis typically present?

There are a number of powerful, gold-standard methods for the accurate analysis of antibody N-glycosylation. These methods typically require sample purification followed by multi-step preparation of some sort; for instance, proteolytic digestion or glycan separation and labeling, several clean-up steps, and finally UHPLC or LC-MS analysis. All of these steps can be automated, of course, but it still takes significant time. The ideal approach would





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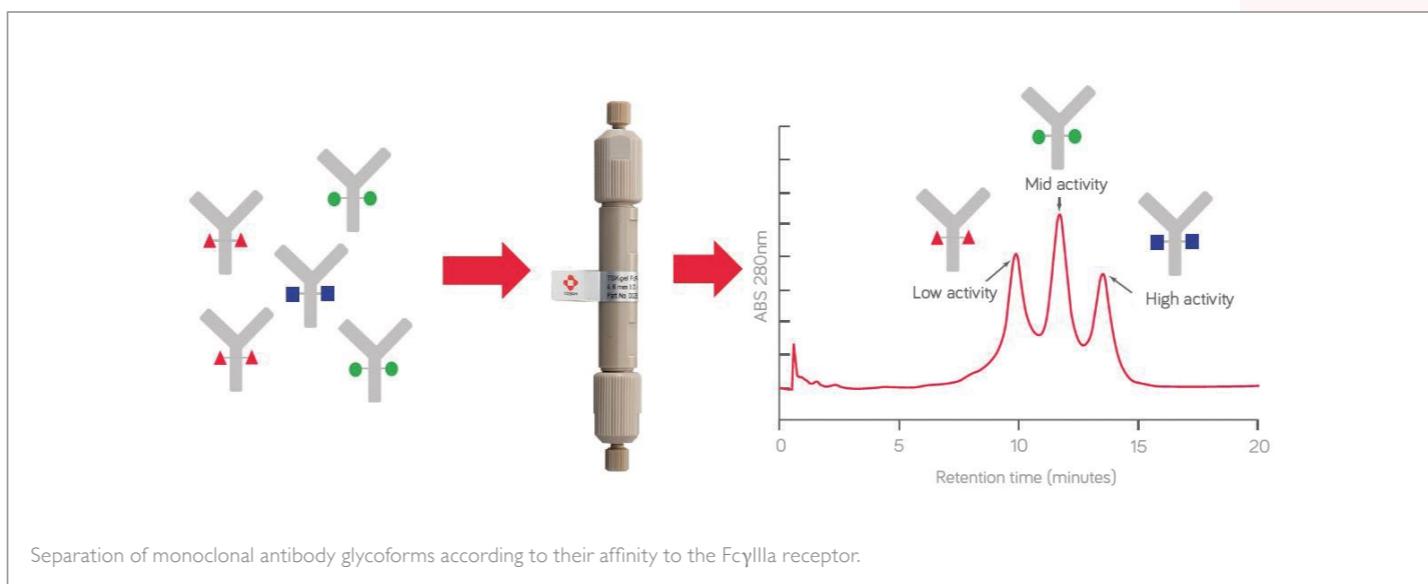
keep the reliability and high quality of results while simplifying and accelerating the workflow.

**You've used a number of different LC columns over the years.  
How is the FcR column different?**

By using an FcR HPLC column, we can make the whole process much simpler. For a significant number of therapeutic antibodies, interaction with the Fc $\gamma$  receptor is crucial for their activity. This interaction is strongly dependent on the type of N-glycosylation. By making good use of the unique characteristics of the TSKgel FcR-IIIA-NPR column, which uses the Fc $\gamma$  receptor as the ligand, we are able to get information on the N-glycosylation of the antibody and its biological activity. The resulting pattern of chromatographic peaks strongly correlates with the N-glycosylation composition.

The accuracy of the data really surprised us. We could predict the content of galactosylated glycans with less than five percent error in most cases, compared with the orthogonal method – hydrophilic interaction chromatography-UHPLC using released and labeled N-glycans.

The method was sensitive enough to use low microgram amounts of sample and, on top of that, we got virtually the same results regardless of whether we used highly purified sample or the antibody still in the cell medium. And that means we can perform high-throughput and highly informative screening of clones, cell culture conditions and lead molecule selection with minimal sample handling. Not to mention that the lack of sample preparation makes this approach cost-efficient and environmentally friendly.



Separation of monoclonal antibody glycoforms according to their affinity to the Fc $\gamma$ llla receptor.

Given these characteristics, I am certain that the FcR column will be extensively used in our current and prospective biosimilar development projects.

**How else could the column be used?**

As well as targeted screening for activity and glycosylation, I believe that the column has potential in a number of areas. Estimation of

binding affinity on non-purified samples is one thing that comes to mind. The fact that the Fc region of the antibody interacts with FcR can be used to detect antibody fragmentation or changes in the quaternary structure. FcR could make a great ligand for antibody purification, so a preparative-scale FcR-IIIA column is something I'd be interested in exploring. I imagine that our colleagues in the Quality Control department might find some new uses for the column too; for example, in one of the batch-release methods.

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Learn more about TSKgel FcR-IIIA-NPR HPLC column for fast ADCC activity assessment

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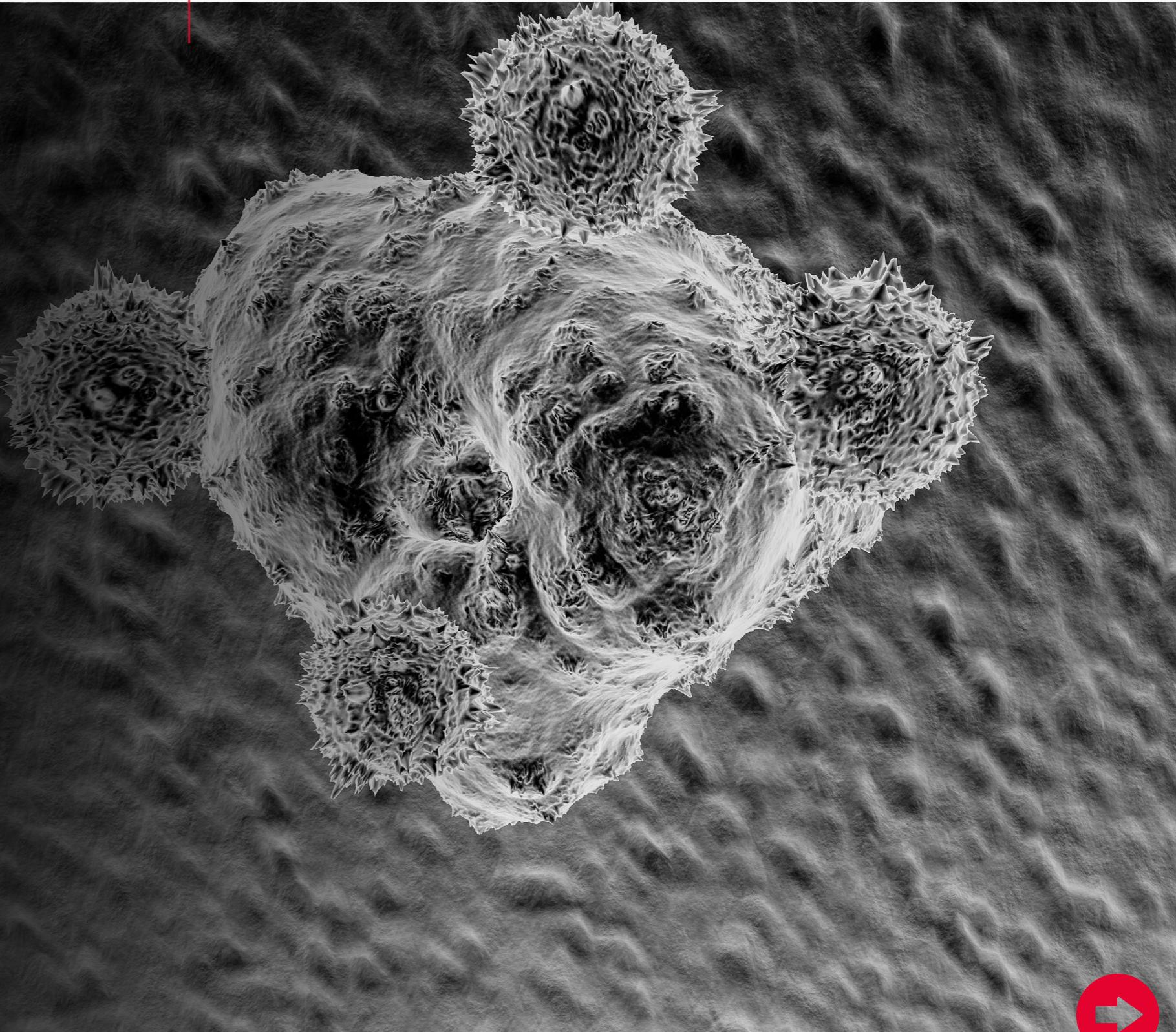
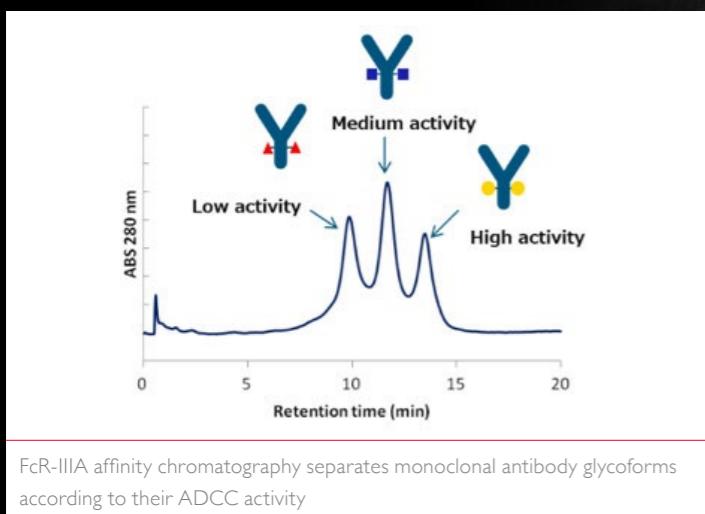
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## Evaluation of Antibody-Dependent Cell-Mediated Cytotoxicity of a Monoclonal Antibody by Fc Receptor Affinity Chromatography

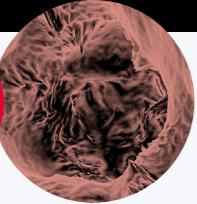
Data on the analysis of therapeutic mAbs with the new TSKgel FcR-IIIA-NPR HPLC column

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism of action of anti-tumor antibodies in which the Fcγ receptor (FcR) plays a key role. Hence, affinity chromatography using recombinant FcR can deliver valuable information about ADCC activity.

The N-glycans of antibody Fc domains are known to play an important role in Fc-mediated effector functions. Accordingly, separation patterns of therapeutic antibodies on TSKgel FcR-IIIA-NPR can be correlated to monoclonal antibody glycoforms. Early eluting peaks (low affinity to Fc receptor) represent monoclonal antibody glycoforms with low ADCC activity while late eluting peaks represent glycoforms with high ADCC activity. More information and chromatographic conditions can be found in the full application note.



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Evaluation of mAb ADCC Activity through  
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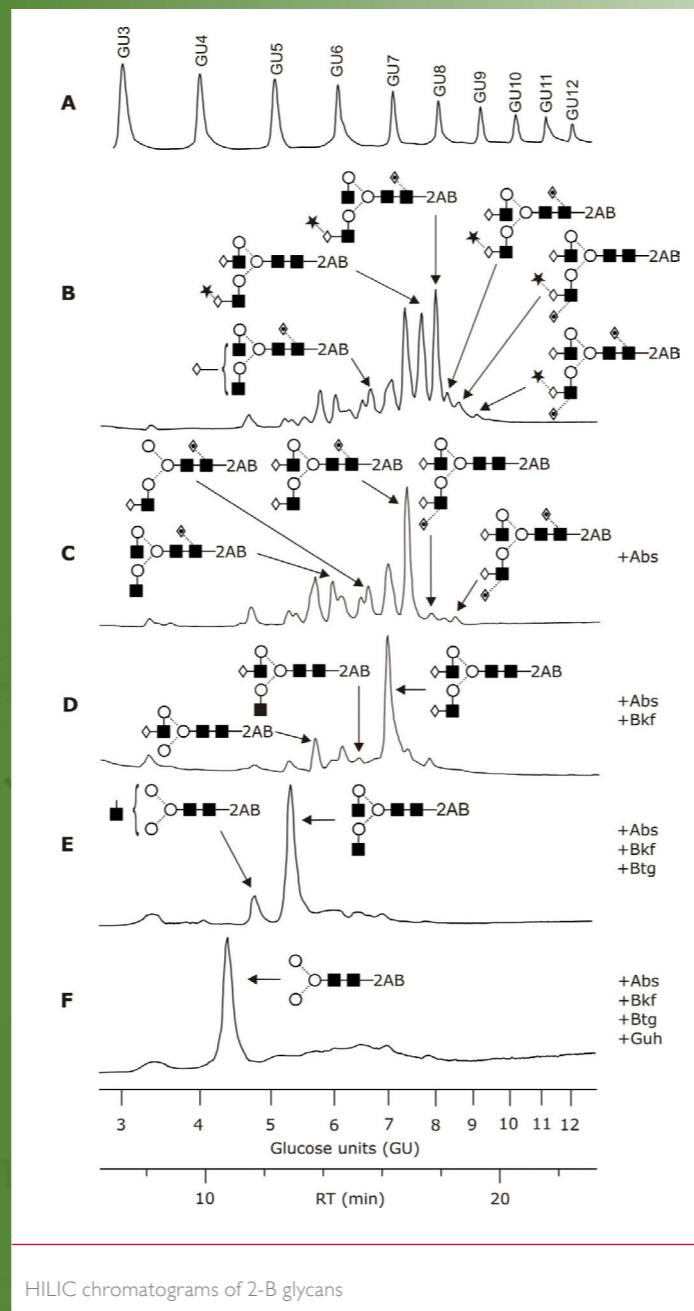
## UHPLC Glycosylation Analysis with TSKgel Amide-80 2 µm HILIC Columns

Glycosylation is one of the most common forms of post-translational modification of proteins. The polysaccharide side chains (glycans) play critical roles in physiological and pathological reactions ranging from immunity to cell signaling. Besides the interest in characterizing glycosylation pattern of proteins for structure/function analysis, the thorough characterization of glycosylation is also a major quality parameter in the production of biotherapeutics. Hydrophilic interaction liquid chromatography (HILIC) is a well-recognized technique that effectively separates and quantifies isolated glycans.

Glycoprotein analysis involves characterizing complex N- and O-linked structures composed of sugar moieties. Besides mass spectrometric techniques, HILIC using amide-based stationary phases is a well-established, robust technique used by many laboratories to obtain high-resolution separation of N-linked glycans released from glycoproteins. Tagging the glycans with a fluorescent label such as 2-aminobenzamide (2AB) or aminopyridin (PA) allows the sugars to be detected at femtomole levels.

TSKgel Amide-80 column chemistry is ideally suited for the separation of charged and neutral fractions of glycan pools in one run. The retention of labelled polysaccharides by TSKgel Amide-80 enables the identification of glycan structures by comparison to a labeled dextran ladder that is used to normalize retention times in order to calculate the number of glucose units (GU values) of the separated glycans. The GU values obtained after separation of sequential exoglycosidase digests can be used to predict the glycan structure by database query (Glycobase, autoGU).

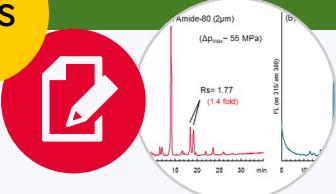
Packed with 2 micrometer spherical silica particles that are covalently bonded with non-ionic carbamoyl groups, TSKgel Amide-80 provides the same unique selectivity as TSKgel Amide-80 3 µm or 5 µm that are applied for glycan analysis in many QC labs for years. The new 2 µm material improves peak capacity and sensitivity for both ultrahigh-pressure LC (UHPLC) and LC-MS analyses, and allows a smooth transfer of established methods from HPLC to UHPLC. The columns are especially suited for use in UHPLC systems, as their reduced system volume and optimized detector specifications help to maintain the high resolution that can be achieved with 2 micron stationary phase.



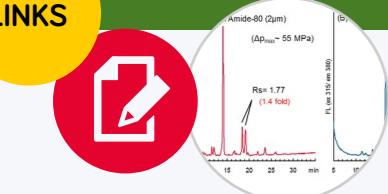
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## Fast Monoclonal Antibody Titer Determination with TSKgel® Protein A-5PW

The antibody therapeutics market is enjoying high growth rates, the major areas of therapeutic application being cancer and immune/inflammation-related disorders. Six of the top ten best-selling global drug brands are monoclonal antibody-based. This market is predicted to show continued growth for many years to come, with more monoclonal antibodies designed and produced for treatments of specific diseases.

Early in monoclonal antibody development, many harvested Chinese hamster ovary cell supernatant samples must be screened for their antibody titers. Antibody titer determination by Protein A affinity high-pressure LC (HPLC) is much more robust, reliable and reproducible than enzyme-linked immunosorbent assays (ELISAs). During upstream processing, the optimal time for harvesting monoclonal antibody from cell culture supernatant can also be detected by using Protein A HPLC. In addition, partial purification of mAb can be accomplished using an Protein A affinity column initially to establish the right cell lines and to partially characterize a newly produced product. With many samples to be screened for different purposes, a reliable and high throughput column is needed for this workflow.

In this application note, the quick capture and accurate titer analysis over a wide concentration range of monoclonal antibody is demonstrated using a TSKgel Protein A-5PW analytical column. Packed with 20  $\mu\text{m}$  hydroxylated methacrylic polymer beads coupled with a recombinant Protein A ligand (a code-modified hexamer of the C domain), this 4.6 mm ID  $\times$  3.5 cm PEEK column can be used with high flow rates for high throughput analysis and still maintains chromatographic efficiency, peak width and resolution. In addition, the TSKgel Protein A-5PW column can perform for more than 2,000 injections with no sign of deterioration and without cleaning.

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FAST CAPTURE OF IgG FROM CHO CELL SUPERNATANT

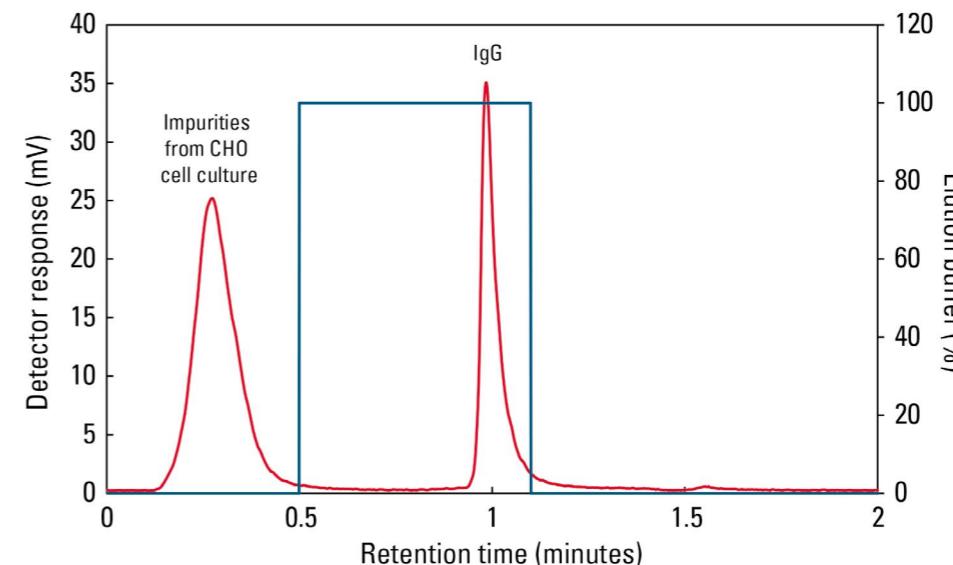


Figure 1

Column: TSKgel Protein A-5PW, 20  $\mu\text{m}$ , 4.6 mm ID  $\times$  3.5 cm  
Binding buffer: 20 mmol/L sodium phosphate buffer, pH 7.4  
Elution buffer: 20 mmol/L sodium phosphate buffer, pH 2.5  
Stepwise gradient: 0 – 0.5 min: binding buffer; 0.5 – 1.1 min: elution buffer;  
1.1 – 2.0 min: binding buffer  
Flow rate: 2 mL/min; Detection: UV @ 280 nm  
Sample: 20  $\mu\text{L}$  of CHO cell culture supernatant spiked with polyclonal IgG (0.5 mg/mL)

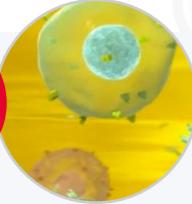
Fast Capture of immunoglobulin G from Chinese hamster ovary cell supernatant

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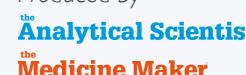


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TSKgel FcR-IIIA-NPR Affinity Column for fast Evaluation of ADCC Activity



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## Easing Ion-Exchange Chromatography

Why salt-tolerant resins make for “happier” biomolecules with better binding

By Romain Dabre

Process chromatography is one of the main components of downstream processing. One of the most commonly-used chromatography techniques for the purification of recombinant biomolecules is ion-exchange chromatography. It is, for example, the preferred step after protein A capture for the intermediate purification of antibodies and antibody constructs. Ion-exchange chromatography uses the ionic charges on the surface of the proteins to bind and elute, and requires specific buffer conditions. Often, a desalting or dilution step is required to ensure proper binding of the target to the ion exchanger.

The introduction of salt-tolerant ion-exchange chromatography resins has been appreciated by the industry. Salt tolerant means that the binding of biomolecules will work at higher salt concentrations than on traditional ion exchange. Normally in ion-exchange chromatography, you bind the biomolecule on the column and elute your target by increasing the salt concentration. Typically, that means the feedstock needs to be salt-free before going onto the chromatography steps, adding time and money to the process. With salt-tolerant ion-exchange chromatography resins, you can work with a higher salt concentration for binding, allowing for more straight-forward processes with fewer steps and/or less dilution while maximizing productivity through high dynamic binding capacity (DBC). For example, the salt-tolerant cation exchanger Toyopearl Sulfate-650F exhibits DBCs up to 120 g/L at salt concentrations as high as 0.3 mol/L. Another example of the unique performances of these resins is the capacity of the salt-tolerant anion exchanger Toyopearl NH2-750F to remove monoclonal antibody-aggregates along with viruses, endotoxins, DNA and host cell protein, paving the way for reducing the number

of steps in antibody purification.

It is also important to point out that biomolecules are natural structures – and when existing in humans or plants there is always the presence of salt. Being without salt, as required in conventional ion-exchange chromatography, goes against the laws of nature, and some proteins do not like these conditions. In this way, salt-tolerant ion-exchange chromatography has a huge advantage. Although they have only been on the market a few years, our salt-tolerant resins are already being used in phase III pre-commercial manufacturing steps. In some ways, it is remarkable how these resins have made it to this stage so quickly – but there's a simple reason for the rapid uptake: they solve previously intractable problems. And I would say that they are perfect for newer antibody formats coming through pipelines.

### Collaboration works

Collaboration is very important to us at Tosoh, and we have contact with many companies and research institutes. Yes, we sell our products, but I like to say that our expertise is something that we offer for free! It's very rewarding that customers see us as experts who they can ask for help when they have a problem with developing methods, or choosing the best material conditions for a specific program. One of our collaborative projects involved the Max-Planck Institute for biochemistry in Martinsried (Germany), one of the largest institutes within the Max Planck Society, and we're proud to share their feedback:

Leopold Urich, a scientist in the group of Sabine Suppmann (Head Recombinant Protein Production), works in the Institute's core facility

– one of many service facilities devoted to supporting the scientists during their work. Among the many areas of expertise, the Institute is working in the field of “recombinant protein production” – essentially, developing strategies for protein expression and purification. As a result, all of Urich's projects require some form of chromatography.

Urich described Tosoh's Toyopearl Sulfate-650F and Toyopearl NH2-750F as breakthroughs for his work because ion-exchange was something the group had been struggling with. “The biggest issue has always been the binding conditions with other ion-exchange resins, meaning very low salt concentrations,” says Urich. “Most of the material we are working with simply can't withstand such low amounts of salt. This problem has since been eliminated and, on top of that, we're seeing some great separation results in our testing, even with simple linear gradients especially with the Toyopearl anion exchange columns.”

Tosoh has also worked with Sanofi, and generated more positive feedback. Benoit Mothes, Head of Sanofi's Global DSP Breakthrough Technologies Skill Center, says, “The Toyopearl NH2-750F anion exchange resin is the greatest innovation from the past 10 years in the downstream processing field.”

We are always open for collaboration with the industry. If you are interested in working with us, please email: [romain.dabre@tosoh.com](mailto:romain.dabre@tosoh.com).

Dr. Romain Dabre is Product Manager, Process Business at Tosoh Bioscience, Germany.

### Reference

- I. Tosoh, Application Note, “Antibody-Drug Conjugate Mimic Purification with Toyopearl® PPG-600m HIC Resin for DAR-Separation”. Available at <https://bit.ly/2PVztWY>.



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## Increasing Monoclonal Antibody Purity with Salt-Tolerant Cation Exchange

Ion-exchange chromatography is often used as an intermediate purification step in monoclonal antibody (mAb) purification for the removal of protein aggregates, host cell proteins (HCP), and leached protein A ligand. Industry trends are focusing on the development of continuous downstream processing. Typically, scientists in biopharmaceutical settings use cation-exchange (CEX) and anion-exchange (AEX) chromatography steps in series to further polish a purified monoclonal antibody after the protein A capturing step. In this study, we focus on the development of a post protein A CEX step for the removal of aggregates, host cell proteins and leached protein A to improve the purity of the antibody eluate in a single polishing step.

A strong cation exchange resin, TOYOPEARL Sulfate-650F, is used in this study. It is a novel resin with the following benefits: strong capture of monoclonal antibody aggregates, high salt-tolerance, wide working pH range, and high dynamic binding capacity. The protocol used with this resin has a minimal pH adjustment of the after the protein A step.

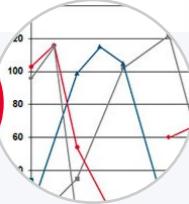
Leopold Ulrich, a scientist at the Max-Planck-Institute of Biochemistry, summarises the benefits of the TOYOPEARL salt-tolerant resins as follows:

Ion-exchange has been something we've struggled with for a while now. The biggest issue has always been the binding conditions with other ion-exchange resins, meaning very low salt concentrations. Most of the material we are working with simply can't withstand such low amounts of salt. This problem has since been eliminated [since we started using TOYOPEARL salt-tolerant ion exchangers] and on top of that, we're seeing some great separation results in our testing, even with simple linear gradients.

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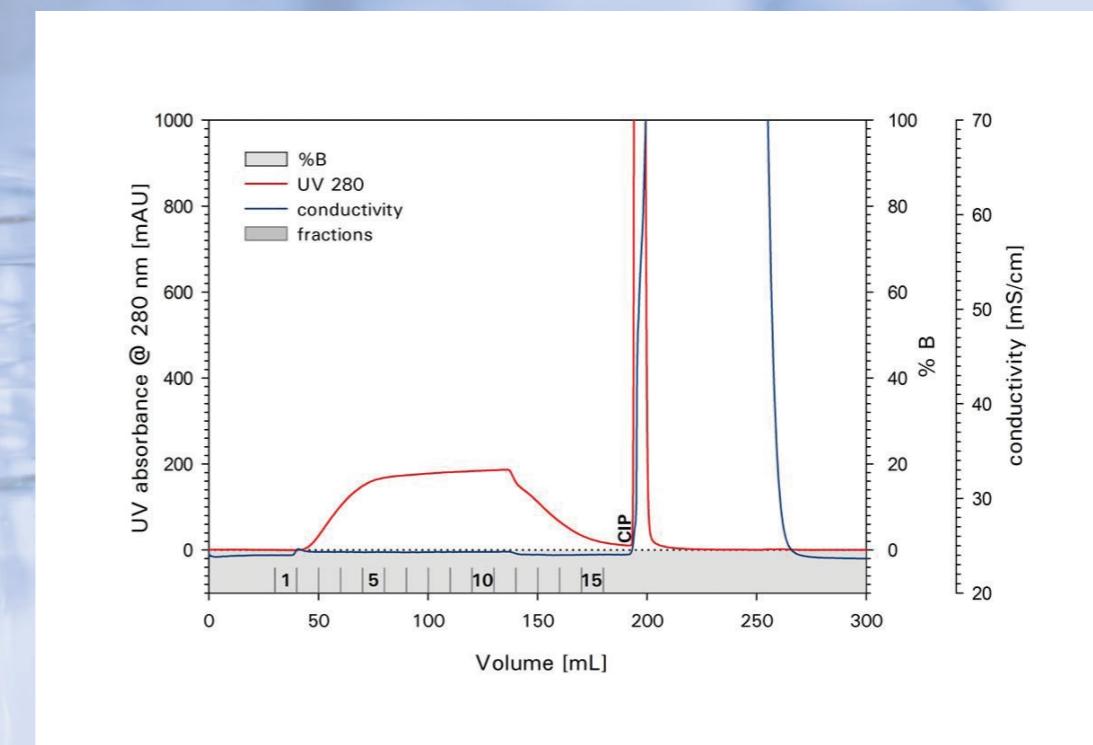
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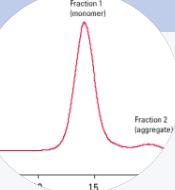
## Monoclonal Antibody Aggregate Removal Through Ion-Exchange Chromatography in Bind/Elute and Flow-Through Mode

Purification schemes for monoclonal antibodies typically consist of three chromatographic steps accompanied by filtration steps. The common Protein A capturing is typically followed by ion-exchange chromatography, hydrophobic-interaction chromatography (HIC) or mixed-mode polishing steps. Residual DNA, viruses, and host cell proteins are usually removed by flow-through anion exchange chromatography, while aggregates can be reduced through a cation exchange, mixed-mode, or HIC step.

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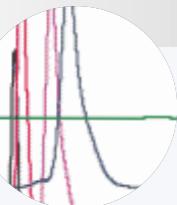
## Effective Removal of Endotoxins with AEX RESIN TOYOPEARL® NH2-750F

Endotoxins are remnants of bacterial cell walls that may contaminate drug products and cause an immunogenic response. They are often referred to as "pyrogens" due to their fever-inducing effects. Endotoxins may be found in drug products either due to contamination from host cells used to produce a drug product in a bacterial expression system or due to adventitious bacterial contamination in non-microbial products. Thus, endotoxin clearance is a requirement of downstream processing of biologics, especially those derived from microbial expression systems that contain endogenous host cell endotoxin. In this application note, we evaluate the ability of TOYOPEARL NH2-750F for the removal of endotoxins by anion-exchange chromatography.

TOYOPEARL NH2-750F	
Particle size ( $\mu\text{m}$ )	30-60
Pore size (nm)	> 100
Ion Exchange capacity (eq/L resin)	0.07 – 0.13
SBC (g/L resin)	$\geq 70$

» Table I

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TOYOPEARL NH2-750F

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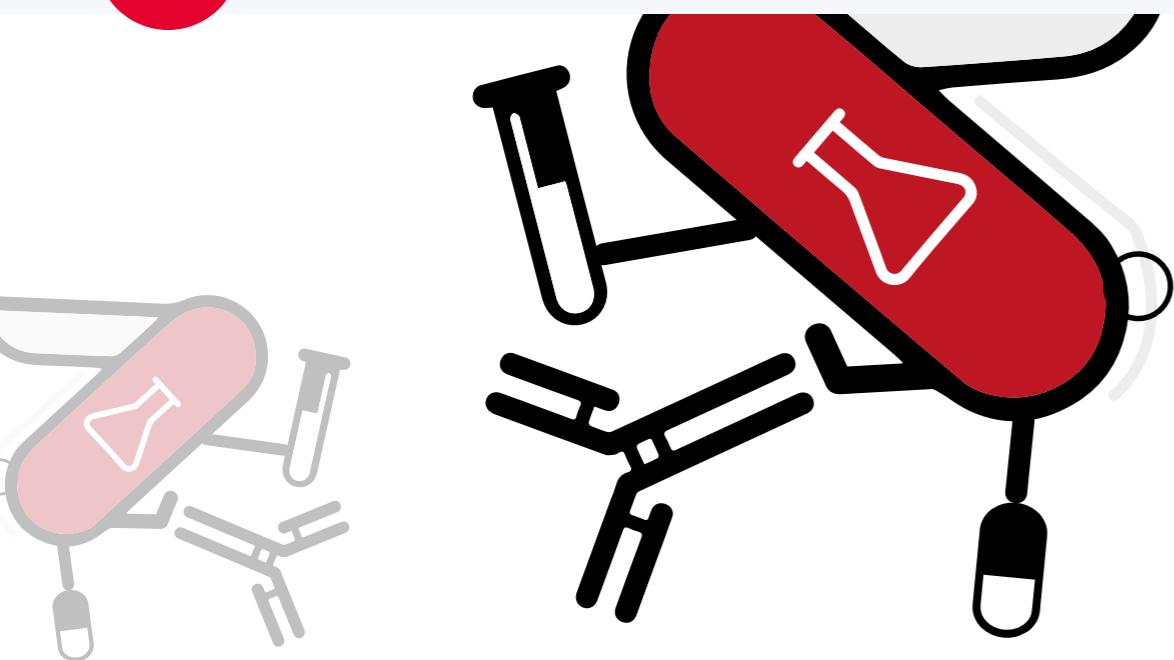
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## The Biopharma Multi-tool

Size-exclusion chromatography – long a mainstay in routine biopharmaceutical analysis – receives an upgrade

Quick and straightforward, size-exclusion chromatography (SEC) has become a Swiss Army knife for the analysis of protein aggregates. It is a “mild” technique that usually preserves biological activity and structural integrity – and could almost be considered a platform technology. Coupling of SEC with various advanced detectors, such as a MS, light scattering or surface plasmon resonance, makes it a versatile tool for numerous applications beyond aggregates. With new biopharmaceutical formats in the pipeline – bispecific monoclonal antibodies, antibody-drug conjugates and virus-like particles, for example – rapid and thorough characterization will be even more important.

Timelines in analytical laboratories are increasingly tough – on the other hand, there is a strong drive to explore and understand biologics in more detail. In separation science, this means higher resolution and plate counts in shorter analysis times.

View the full application note online

### Aggregate Win

Senior scientist Xianwen (Susan) Chen and her colleagues in the process analytical group at Boehringer Ingelheim Fremont, Inc. are making the switch from HPLC to UHPLC for some routine SEC analyses. We caught up with Chen to find out more.

What is the focus of your work?

It's always been my goal to work in pharmaceuticals – and to help deliver drugs to patients. My PhD was on drug screening and afterwards I joined the pharmaceutical industry as a development scientist. I've been at Boehringer Ingelheim for two years, working in the process analytical group. We support process transfer, optimization and development, starting from the cell culture through purification, right up to formulation. We develop assays to test product quality; for example, if our purification group need to optimize their column conditions, they will try different buffers and processes, and send samples to us to test the product quality – aggregate level, charge variance, fragmentation, and so on – and we will provide feedback on the process that gives the best results.

What challenges do you face?

The samples we deal with are varied and often arrive in large numbers – if we're working with a team doing a Design of Experiments project, hundreds of samples might be submitted at once. So it's crucial that we use reliable instrumentation that allows us to carry out very robust, high-throughput assays.

How do you use SEC?

SEC is one of the essential assays we run every day, particularly for monoclonal antibodies. It tells you aggregate and degradation levels of the product, which is absolutely key information that is required by the FDA. There is a safety concern, in that aggregates could trigger an immune response that could render the drug ineffective or even cause adverse events.

How has SEC technology advanced in recent years?

The main advance in the past decade has been the arrival of ultrahigh-pressure LC (UHPLC) with sub-2 µm-particle columns. The first UHPLC column for SEC was released in 2010; now, a number of new columns are coming onto the market, such as the Tosoh TSKgel UP-SW3000.

With existing UHPLC columns, we've noticed lot-to-lot variation and a tendency to lose resolution when analyzing low-molecular-weight species. To combat those problems, we had to spend a lot of time troubleshooting, trying different cleaning solutions to get the resolution back. So when the new sub-2 µm column was released from Tosoh, we were happy to try it out. In a poster presented at the HPLC 2016 conference, we showed that the column is robust, with minimal lot-to-lot variation.

Is UHPLC the future for SEC?

UHPLC brings many benefits – assays run faster and with higher resolution. Over time, I think it will replace some of the older HPLC assays, which have been around for 10–20 years. Many pharmaceutical companies are currently in the evaluation stage, deciding whether to switch. Changing assays can be hard work, especially for commercial drugs, and you want to make sure the new technology lives up to its promise. I think the two technologies will be used in parallel for a long time to come.

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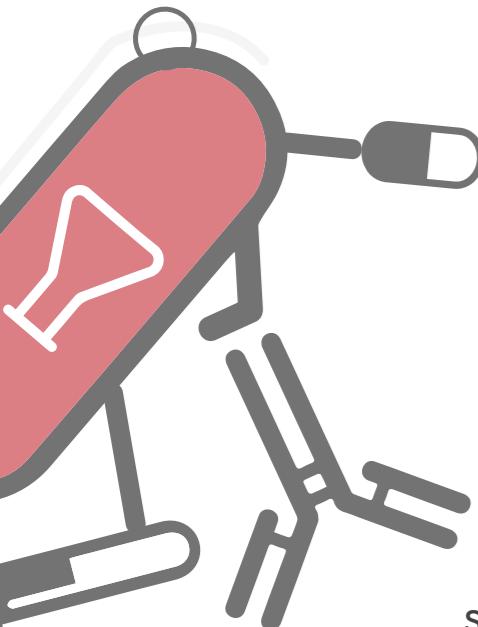
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*“With new biopharmaceutical formats in the pipeline, rapid and thorough characterization will be even more important.”*

#### Small is beautiful

Over the past 30 years, miniaturization has been the key trend both in stationary phases and instrumentation. Smaller particles provide better separation performance, so we can either shorten the required separation path lengths, or provide better performance at the same path length. The use of smaller particles also pushed the optimum column dimensions towards smaller values, with the welcome side-effect of saving solvent.

When it comes to biopharmaceuticals, HPLC and UHPLC cannot be as strictly segregated as in other disciplines. In this setting, UHPLC is defined as “ultra-high performance LC” rather than the more common definition – “ultra-high pressure LC”. Especially in SEC, the small (2 µm) particles of UHPLC do increase the pressure – but rarely exceed the pressure limits of modern HPLC. Any higher, and the resulting frictional heating and shear forces would damage the macromolecules and lead to artefacts. Instead, bio-UHPLC is focused on optimization of valves, capillaries and flow cells to decrease dead volumes and

subsequent Eddy diffusion – which also holds true for conventional HPLC columns.

#### Upgrading biopharma’s Swiss Army knife

UHPLC is already a standard technology in the analysis of small-molecule drugs so it is no surprise that biopharmaceutical manufacturers are now looking to follow suit. Our TSKgel 3000SWXL column is a standard tool for QC aggregate analysis by SE-HPLC, and we noticed an increasing demand for a 2 µm-particle column that enables easy method transfer to UHPLC.

There were many challenges in developing such a column. The minimum particle size for HPLC is believed to be 1 µm (1), so at 2 µm we are skirting the edge of what is possible – and that’s never easy going. But chromatographers really appreciate the benefits in terms of speed and resolution.

Of course, UHPLC is not a magic bullet. Coupling a SE-UHPLC column to a non-optimized system with dead volumes is an unhappy marriage. The smaller internal diameters result in lower volumetric flow rates, which leave more room for undesirable Eddy diffusion. In some cases, this may result in worse separation performance than with a conventional SE-HPLC column, so it’s important to consider the system as a whole.

Thorough characterization of every new drug is crucial for patient safety – and complex next-generation biopharmaceuticals present a challenge. I expect to see diversification of columns – columns capable of delivering high performance for fundamental research and columns dedicated to rapid in-process control and drug release. With regard to instrumentation, the use of LC-MS and other hyphenated techniques will continue to grow.

#### Reference

- I. Halász et al., “Ultimate limits in high-pressure liquid chromatography” *J Chromatogr A*, 112, 37–60 (1975). Available at: <http://bit.ly/2p4pVfh>. Accessed May 9, 2017.





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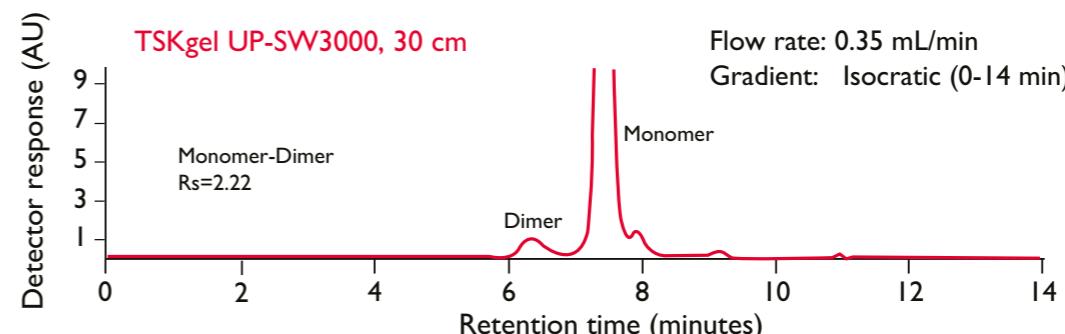
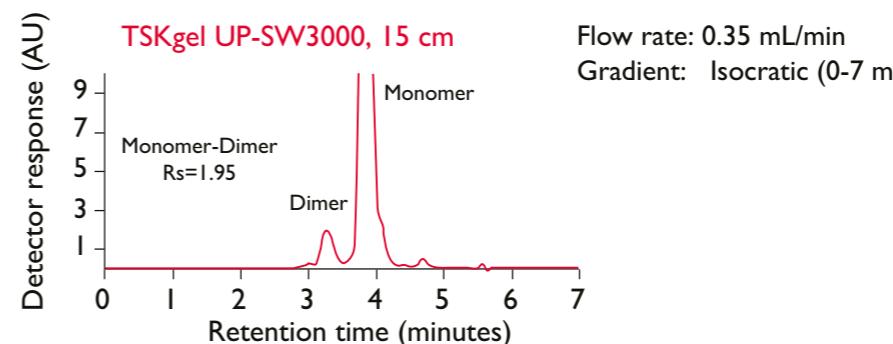
AGGREGATE ANALYSIS

## Rapid and Accurate Therapeutic Monoclonal Antibody Aggregate Analysis Using TSKgel® UP-SW3000, 2 µm, SEC Column

High-pressure LC (HPLC) and analytical size-exclusion chromatography (SEC) columns are widely used to determine the ratio of aggregates, dimers, monomers, and fragments in monoclonal antibodies. Columns are expected to deliver high resolution, excellent reproducibility in a short analysis time. In order to achieve these parameters, SEC columns must have the appropriate particle size, pore size, good bonding chemistry, and suitable column dimensions. In addition, the columns must be packed well. Traditionally, SEC columns with 30 cm length are used for high resolution analysis because the length allows different molecular sizes to be separated with a longer run time. However, because of the long length, a typical analysis can take up to 30-40 minutes for each analysis. With the demands for high sample throughput, there is a need for shorter analysis time. There are many available SEC columns with 15 cm length currently available for this usage. However, these columns typically suffer from low resolution.

This application describes the use of a 4.6 mm ID × 15 cm TSKgel UP-SW3000 SEC column for fast and accurate monoclonal antibody aggregate analysis without compromising the quality of the aggregate determination or reproducibility. Unlike many other available 15 cm length SEC columns, these columns are packed such that they can be operated with both HPLC and ultraHPLC systems. The 4.6 mm ID × 15 cm TSKgel UP-SW3000 SEC column has a particle size of 2 µm and a 25 nm pore size. The particles are coated with a hydrophilic diol-type bonded phase in order to minimize the interaction between the silica surface and proteins. The column is designed to be operated with a simple and well-established method (sodium phosphate mobile phase, pH 6.8). A comparison study was done between a TSKgel UP-SW3000, 15 cm column and a 30 cm length column, both 4.6 mm ID. Results show that the run time of the 15 cm column was completed in 4 minutes without compromising the resolution of the chromatogram.

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