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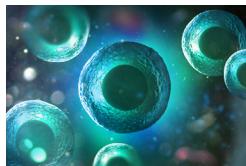
Advances in Separation Science

JULY 2018



Advancing Health and Disease Research with Efficient Analytical Methods

An interview with Guowang Xu



Separations-Based Chemical Cytometry

An interview with Nancy Allbritton



New UHPLC–MS/MS Methods Advance DNA Research

An interview with Hailin Wang



Studying the Kinetic Performance of Columns in Fast LC

An interview with Attila Felinger



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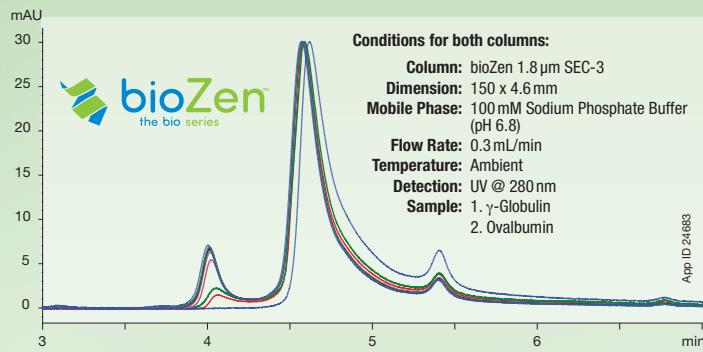


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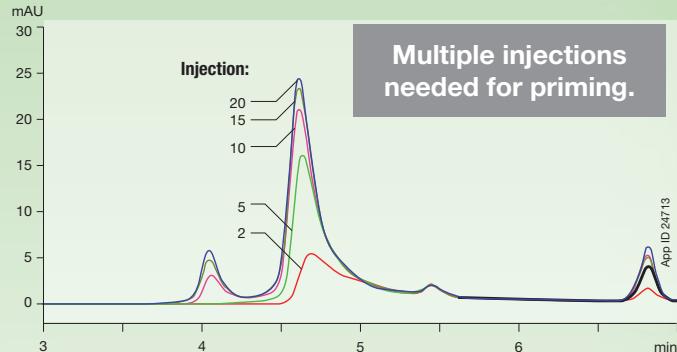
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INTRODUCTION

The International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC) is one of the most high-profile events for chromatographers. The organizers of this year's event, HPLC 2018, have collaborated with the *LCGC* editorial team to share some highlights from this year's event through this ebook, which brings you interviews with several keynote speakers.

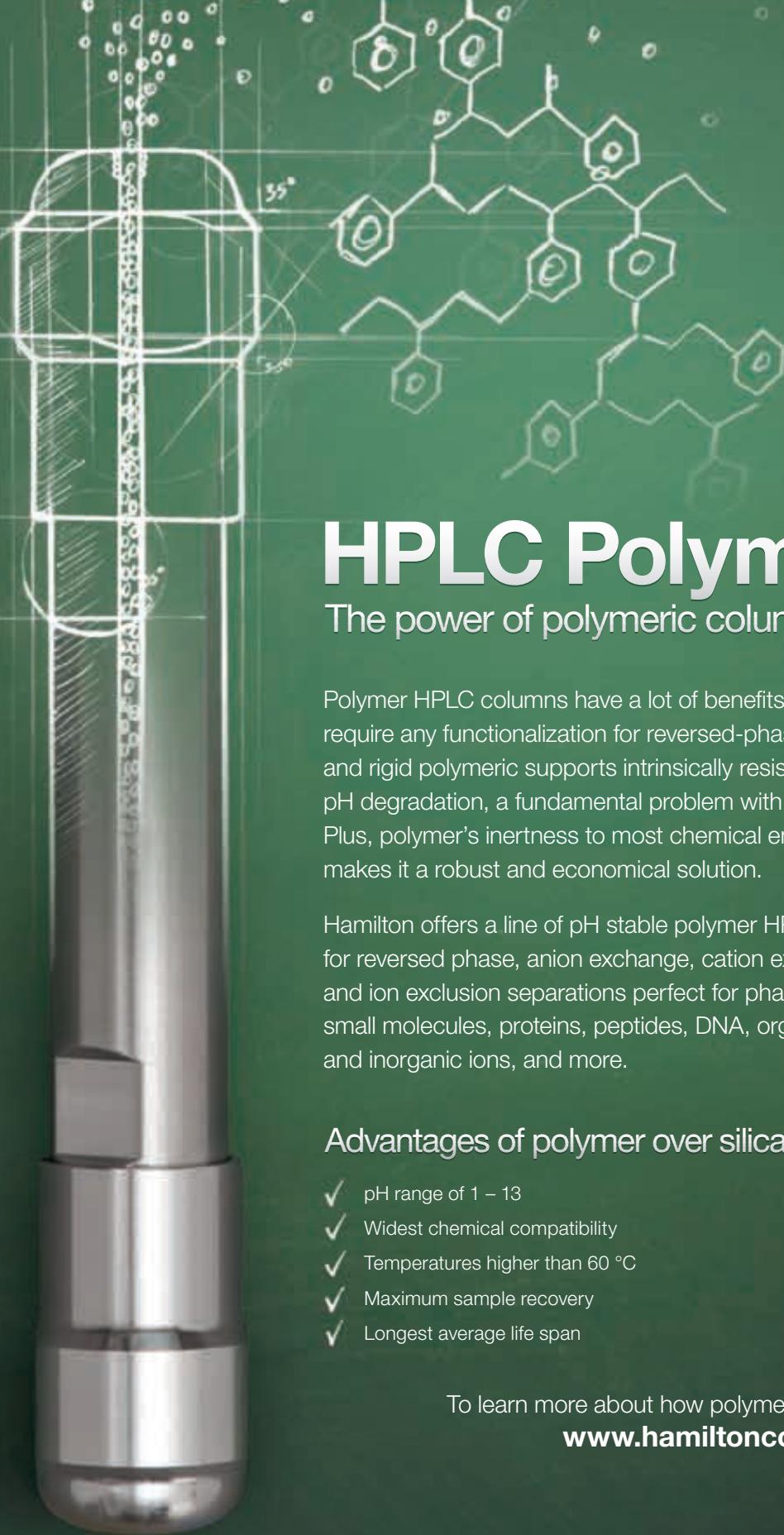
First, Guowong Xu of the CAS Key Laboratory of Separation Science for Analytical Chemistry at the Dalian Institute of Chemical Physics, of the Chinese Academy of Sciences, discusses his work to develop methods that can advance health and disease research by more providing better separation of closely related compounds or by separating related classes of compounds in a single run to save time.

Nancy Allbritton of the University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, spoke with *LCGC* about her approaches to single-cell analysis with capillary electrophoresis, including how she addresses the complexities of developing the specific probes needed for different compounds under study.

Next, Hailin Wang of the State Key Laboratory of Environmental Chemistry and Ecotoxicology, at the Research Center for Eco-Environmental Sciences, which is part of the Chinese Academy of Sciences in Beijing, explains the methods he is developing, using LC with high-resolution mass spectrometry (LC–HRMS), for his studies of DNA modifications.

Lastly, Attila Felinger of the University of Pécs, in Hungary, discusses his recent work studying the kinetic performance of columns in fast LC, with a particular focus on local performance at the column ends.

These interviews highlight a few of the ongoing advances in separation science techniques that are enabling scientists in many fields to achieve greater resolution and gain new insights into important areas of research.



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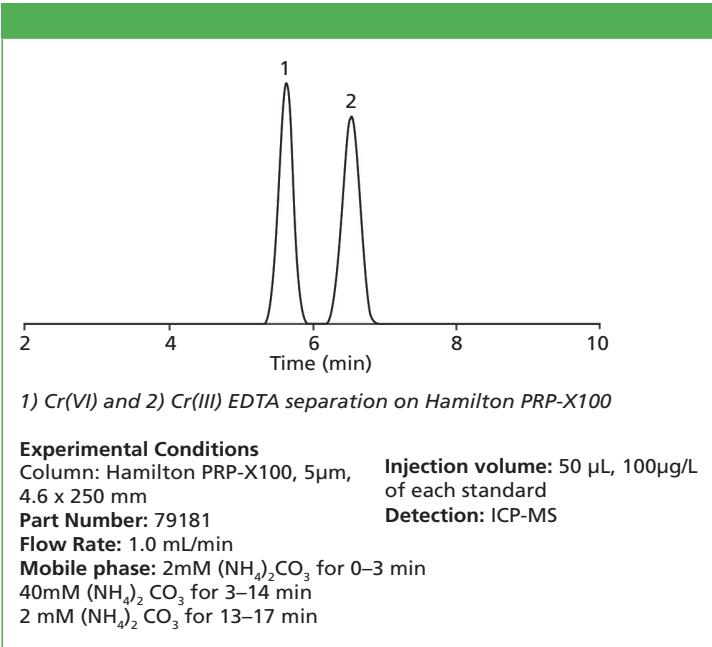
Hexavalent Chromium Determination

Hamilton PRP-X100 Anion Exchange HPLC Column

Derek Jensen and Mark Carrier, Hamilton Company

Chromium (Cr) is a metal with an interesting relationship to the environment. Whereas trivalent chromium (Cr(III)) is an essential nutrient, hexavalent chromium (Cr(VI)) is a poison to humans and aquatic life and poses serious environmental and ecological threats. Recent studies of various ground and drinking water sources have detected toxic levels of Cr(VI). This dangerous trend has gained the attention of national and worldwide health organizations, such as the United States Environmental Protection Agency (EPA) and the US Food and Drug Administration (FDA), who seek to understand how widespread the problem is. The California Department of Public Health included Cr(VI) as an unregulated chemical requiring monitoring in 2001. Based on recent data, 3107 of 6565 public wells in Los Angeles, San Bernardino, and Fresno counties had Cr(VI) concentrations above 1 µg/L. A Public Health Goal of 0.02 µg/L was published in July 2011.

Analysis of chromium species is made challenging due to the nature of the element and diverse sample matrices. Because chromium exists in two oxidation states, it is important to differentiate between the nutrient, Cr(III), and the poison, Cr(VI) in samples. An HPLC-ICP-MS method using the Hamilton PRP-X100 has been developed in order to determine relative abundance of Cr(III) and Cr(VI) in diverse sample matrices.



Trivalent chromium (Cr(III)) is stabilized as a chelation complex by incubating the sample at 70 °C in 0.2 mM EDTA. The Cr(III)-EDTA complex is suitable for binding to an anion exchange resin. Resolution of the two species then becomes a straightforward isocratic separation. Although UV and conductivity are suitable for detecting chromium species, inductively coupled plasma-mass spectrometry (ICP-MS) is the method of choice for trace analysis.

PRP-X100 HPLC Column Ordering Information

Column Type	Hardware Size (mm)	Particle Size		
		5 µm	10 µm	12 – 20 µm
PRP-X100	2.1 x 150 PEEK	79852		
PRP-X100	4.6 x 150 PEEK	79174	79354	
PRP-X100	4.6 x 250 PEEK	79181	79455	
PRP-X100	Bulk Resin (1 Gram)	79584	79585	79586



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PRP-X100 HPLC Guard Column Ordering Information

Part Number	Description
79383	Analytical Guard Column Starter Kit (1 holder, 2 cartridges), PEEK
79385	Analytical Guard Column Replacement Cartridges (5/pk), PEEK



Biological and health research often involves the analysis of highly complex classes of similar compounds that are difficult to distinguish. Analytical methods that can distinguish between similar forms give researchers more power, while methods that can separate related classes in a single run save time. Guowang Xu, the Director of the CAS Key Laboratory of Separation Science for Analytical Chemistry at the Dalian Institute of Chemical Physics, of the Chinese Academy of Sciences, has been developing methods that can do both, and using those approaches to advance research on a variety of compounds, such as acyl-coenzyme A, acylcarnitines, and a wide range of metabolites and lipids. He recently spoke to us about some of this work.

You have developed a two-dimensional (2D) liquid chromatography (LC) method coupled with high-resolution mass spectrometry (UHPLC-

Advancing Health and Disease Research with Efficient Analytical Methods

An interview with Guowang Xu

HRMS) for the simultaneous analysis of short-, medium-, and long-chain acyl-coenzyme A (CoAs) (1). Why are these compounds important to study?

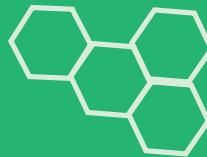
Acyl-CoAs are essential substrates. These compounds are involved in many metabolic pathways, such as the Krebs cycle, lipid synthesis and remodeling metabolisms, fatty acid oxidation, and so on. The dysregulation of acyl-CoAs plays a very important role during the development of many diseases, including cancer and diabetes.

Why was a better method needed for the analysis of these compounds?

Traditional methods covered only a limited range of acyl-CoA species in one analysis, usually either short-, medium- or long-chain acyl-CoAs. Thus, a comprehensive method was needed to simultaneously analyze short-, medium-, and long-chain acyl-CoAs. This method could help researchers to understand physiological and pathological processes better.



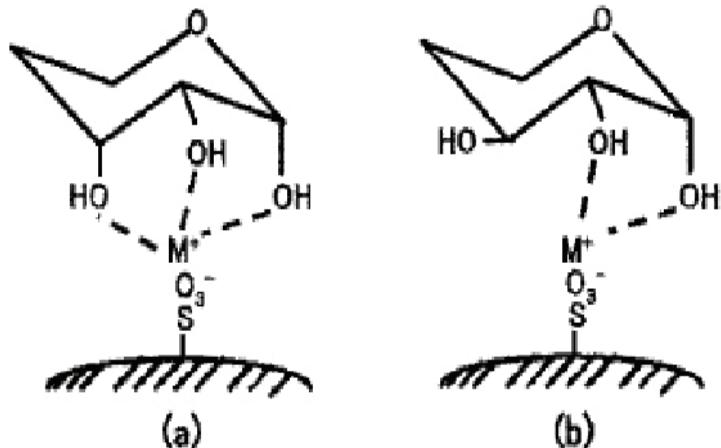
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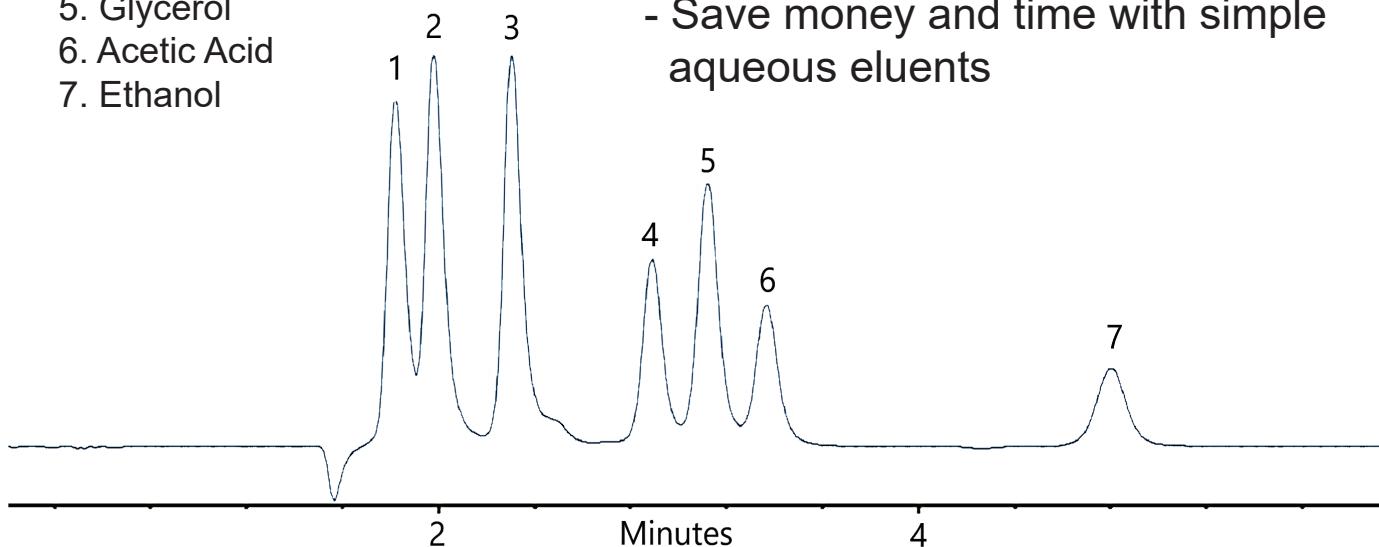
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2. Maltose
3. Glucose
4. Lactic Acid
5. Glycerol
6. Acetic Acid
7. Ethanol



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- Save money and time with simple aqueous eluents





A key challenge in developing this method was the incompatibility of the mobile phases needed for separation of each type of CoA. How did you address this challenge?

We introduced a precolumn and a makeup flow into the construction of a two-dimensional (2D) LC–MS system. The acyl-CoAs could be trapped on the precolumn, and meanwhile, the residual second-dimension mobile phases in the precolumn were replaced by the makeup flow, thus making the precolumn and the remaining buffer suitable for the next analysis. Thus, the incompatibility of mobile phases was solved.

You then used the 2D LC–MS method to profile all acyl-CoAs in mouse liver tissues to investigate metabolic differences of acyl-CoAs in glioma cells. What did these initial applications of your method show?

These applications further demonstrated the power and practicability of the 2D LC–MS method. In our study, more than 90 acyl-CoAs were identified in mouse liver tissue, much more than those from previously reported methods. With this method, we designed a metabolomics study of malignant glioma cells with an IDH1 mutation. As expected, we found some helpful clues of cell status and significant changes of enzyme activity, which influences cancer malignancy and progression.

You have also developed a 2D LC–MS technique to conduct simultaneous

metabolomics and lipidomics analysis (2). Why is it valuable to be able to do both types of analysis at once?

It is very important for an untargeted metabolomics method to increase metabolite coverage. First, researchers can better understand related metabolic mechanisms and identify biomarkers because more information about the metabolites is obtained. Second, the analysis throughput is significantly improved by comparison with dual conventional metabolomic and lipidomic analyses. This is particularly important for large-scale metabolomics studies with small sample amounts.

Prefractionation and fractionation of the samples seem to be important aspects of this technique. Can you explain how the prefractionation and fractionation were carried out, and their role in the technique?

Prefractionation was carried out using a precolumn. Complex metabolites in a biological sample were divided into two fractions including metabolome and lipidome based on their different polarity. Fractionation was implemented respectively with a C18 column and acetonitrile–water mobile phases for the metabolomics analysis as well as a T3 column and isopropyl alcohol–acetonitrile–water mobile phases for the lipidomics analysis.

You developed an LC–MS method for comprehensive identification of acylcarnitines using high-resolution targeted MS (3). How were you able

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to use a targeted approach for such a large group of analytes?

We performed layer-layer progressive MS/MS spectra acquisitions. Initial full-scan MS–data-dependent MS/MS mode produced MS/MS spectra for the top 10 peaks in every cycle. Some low-concentration acylcarnitines were detected in MS but without MS/MS spectra, they could not be identified. To make up this defect, each precursor ion that might be identified as an acylcarnitine was extracted in MS mode, and the time window was scheduled for targeted high-resolution MS/MS. Based on the targeted MS/MS spectra, a large number of peaks were able to be labeled as acylcarnitines. These analytes might have been detected before, but nobody knew what they were or focused on them. We have proposed a strategy to annotate a series of unknown compounds.

You also used parallel reaction monitoring (PRM) mode, rather than selected reaction monitoring (SRM) mode for MS data acquisition. Why did you take this approach? What challenges did you face in using it?

Compared with SRM, which provides precursor–product ion pair unit resolution, PRM mode can provide high-resolution MS/MS spectra. In PRM mode, the related product ions as well as precursor ions were detected in the high-resolution orbital trap mass analyzer, which was helpful for identification. Since PRM mode was limited by scanning speed, it was difficult to fulfill the need for the simultaneous

MS/MS spectra acquisition for all potential acylcarnitines in a single run. We devoted efforts to solving this problem using approaches such as time window scheduling and adjusting other parameters.

What process did you use to identify the acylcarnitines in each class?

First, we extracted the chromatograms of precursor ions as well as characteristic product ions in PRM mode. If their chromatographic behavior were similar, we checked the high-resolution MS/MS to infer acylcarnitine structure. The compounds identified as acylcarnitines had at least four characteristic product ions. Moreover, the retention times of homologue acylcarnitines should conform to the structure–retention relationship. For example, the acylcarnitine with 16 carbons should be eluted later than the homologue acylcarnitine with 14 carbons in a reversed-phase separation.

How will this work be useful in future work, by you or other researchers, who are investigating these compounds?

In this work, we established the most comprehensive database of acylcarnitines reported to date, which includes more than 700 acylcarnitines, along with their exact mass, retention time, and high-resolution MS/MS information. Applying this database, acylcarnitines were rapidly and reliably annotated in biological samples. Some acylcarnitines were omitted before, but now they can be



annotated and studied for related disease research on health problems such as inborn errors of metabolism, diabetes, and so on. Moreover, this large-scale identification strategy has the value to the future identification of other metabolite groups, for example, fatty acids, acyl-CoAs, and so on.

What are your next steps in this work?

The sufficient information including exact mass, retention time, and MS/MS spectra, will be added to our in-house database, which can identify metabolites automatically. At the same time, we might reuse the existing raw data and focus on additional acylcarnitines to discover their biomarkers. Moreover, we hope to find acylcarnitines with new groups, such as nitro groups.

Guowang Xu, PhD, is the administrative vice-director of the Biotechnology Division, the Director of the Metabonomics Research Center, and the Director of Chinese Academy of Sciences (CAS) Key Laboratory of Separation Science for Analytical Chemistry, all at the Dalian Institute of Chemical Physics, CAS, in Dalian, China. His main research fields are in chromatography-related research and mass spectrometry-based metabolomics applications in disease biomarker discovery, traditional Chinese medicines, and food safety. Prof. Xu has co-written five books, published more than 380 peer-reviewed papers, and holds more than 50 Chinese patents. He is a member of permanent scientific committee of the HPLC conference and a member of editorial boards of more than ten journals including *Anal. Chim. Acta*, *Metabolomics*, and *Anal. Bioanal. Chem.*



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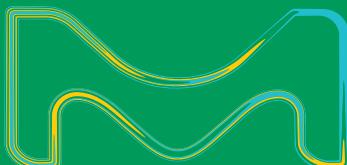
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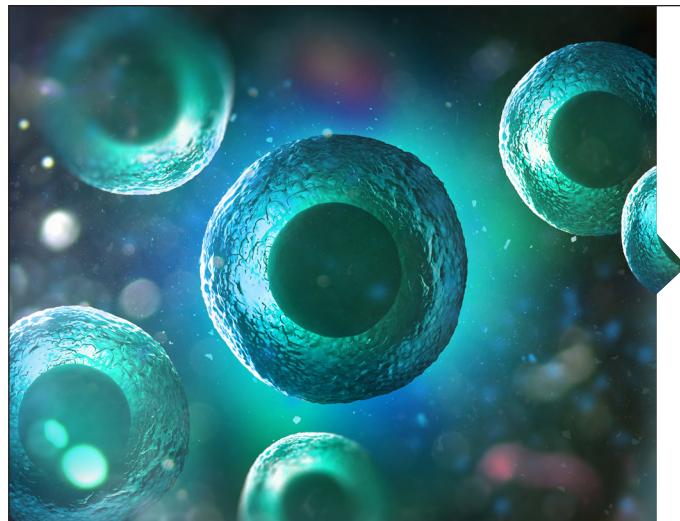


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Studying activity within cells can provide powerful information about biology and disease, but such studies present significant analytical challenges. Separations techniques like capillary electrophoresis can be highly useful tools for these studies. Nancy Allbritton of the University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, talked to us about her work in this area.

First, what is the definition of chemical cytometry? And what instrumental setups are used to perform this technique?

I would define it as the use of analytical tools to measure the components within a single cell. That's a pretty broad definition, so it covers a lot of different technologies and strategies and the list is ever growing. But when I think of chemical cytometry and the instrumental setup, I most often think of separation technologies or technologies that incorporate a separation

Separations-Based Chemical Cytometry

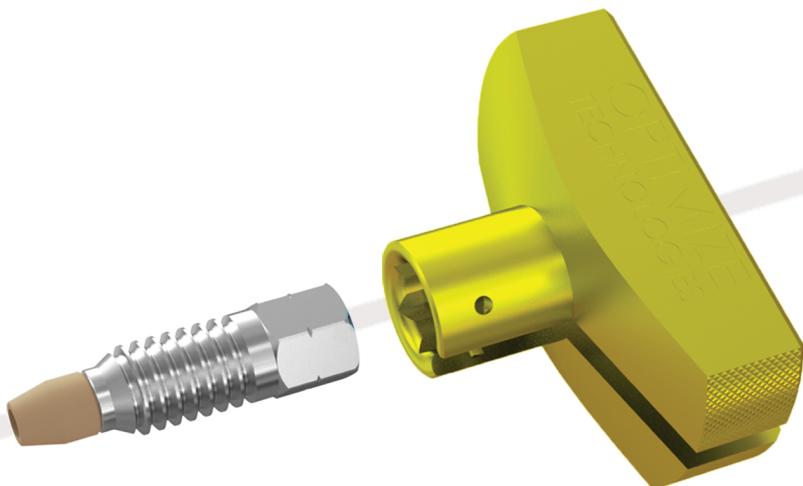
An interview with Nancy Allbritton

step to quantify the different components of a cell. I think of techniques like capillary electrophoresis (CE) and micro or ultra-nano liquid chromatography (LC). But it also includes techniques like fluorescence microscopy and mass spectrometry (MS). It's getting broader and broader as more and more people get excited about looking at the diversity of single cells and how they behave and why they're different from each other.

What are the advantages of separations-based chemical cytometry over other types of single-cell assays?

There are many advantages. First of all, many separation techniques, like CE, are really geared to handle picoliter-volume samples, which is the volume of your typical mammalian cell. The other thing is that we know that there is a fantastically large number of compounds within a cell. So if you use a microscope, for example, it's hard to see the tens of thousands of compounds in a cell, but with CE you can

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Separations-Based Chemical Cytometry

really get nice separations and can identify much, much larger numbers of chemicals at the same time. The potential for multiplexing and looking at many analytes at once with separations-based techniques is pretty powerful.

Also, a lot of the species in cells are isomers so they can be really, really hard to assay except by a separation method. And if you think about CE, people have used it to separate ions, proteins, peptides, carbohydrates, metabolites, sugars—you name it and it's probably been separated by CE. So when you consider the number of different chemical constituents that are present in a cell, CE clearly has one of the most powerful ranges in terms of the types of chemicals for which it is suitable.

And of course, you don't just have to use CE by itself and depend on intrinsic absorbance or fluorescence. You can combine CE with reporter molecules that act as sensors, adding to the power of this separation technology.

What advantages and disadvantages do CE and LC have compared to each other for this type of analysis?

CE is more geared, as I mentioned before, to the picoliter volumes of cells. When I think about LC, I don't really think of picoliter volumes, I think of more nanoliter or microliter volumes typically. That's not 100% true but typically the two techniques have different volume input requirements.

Also in CE you don't have band broadening; you have the flat electro-osmotic flow instead of the parabolic flow that you see

in pressure-driven chromatography techniques like LC. You can get much higher sensitivity and much better resolution. So when you face the challenge of the picoliter volume of a cell, with its vanishingly small amounts of each type of chemical, you really need to push the limits of sensitivity. In that situation, I think the benefits of CE clearly outweigh those of LC.

Are there any advantages that LC has for this type of analysis?

I think it's a little easier because it's an older, more mature technology. For example, for a lot of peptide separations, you know to use a C18 column in LC. For CE, you often need to survey different modes. If you want to separate peptides there's no obvious buffer to use so you may need to do a large buffer screen, or you might have to try capillary wall coatings. It's a technique where there are fewer established approaches; you need to make educated guesses that point that way forward. In LC it's a little more straightforward to see the pathway to follow.

Both CE and LC techniques can be coupled to three major detection strategies, electrochemistry, MS, and fluorescence detection. For chemical cytometry, which detection strategy tends to be best?

It really depends on what you want to measure. For example, if you have an electrically active molecule, you can't beat electrochemistry. The technology for making the probes is really quite extraordinary.

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Application Note

UHPLC Analysis of Biosimilars Using Size Exclusion Chromatography

Introduction

In order to provide new treatment possibilities, increasing accessibility to life-saving medicines and possibly decrease health care costs through generic substitution, pharmaceutical companies have developed path for biological products that are proven to be biologically similar to an FDA-approved reference product. The manufacturer of the proposed biosimilar must generate an array of analytical data to demonstrate that the proposed biosimilar is similar to the product; a detailed analytical characterization and comparison of the proposed product with the reference product.

A series of analytical chromatographic techniques are used to characterize the similarity such as size exclusion chromatography (SEC), reversed phase chromatography (RPC), high performance liquid chromatography (HPLC), and ion exchange chromatography (EX). SEC is a chromatographic technique that separates molecules based on their size and shape. It is used to analyze the characterization of a biosimilar molecule versus the reference product drug by comparing the molecular weight distribution of each molecule. In the application note, a 15kg/m³ UP-SW2000 SEC column was used to separate the Yervoy® biosimilar from the reference product Yervoy®. Yervoy® and the corresponding biosimilar/reference products were kindly supplied by Takeda.

Experimental HPLC Conditions

Bioanalytical Reference Product

Column: 15kg/m³ UP-SW2000, 2 μm, 10 mm ID × 30 cm length, 15 kg/m³ SW2000 Guard Column, 2 μm, 10 mm ID × 30 cm length.
Mobile phase: 0.05 M Tris-HCl, pH 7.4, 0.02% Bovine Serum Albumin (BSA), 0.02% DMSO, 0.005% Na₂HPO₄, 0.005% Na₃VO₄.
Gradient: Flow rate: 0.35 mL/min
Detector: UV 280 nm
Temperature: 25 °C
Injection volume: 100 μL
Samples: Yervoy® Investor Reference Product (5 mg/mL) Yervoy® Investor Reference Product (5 mg/mL) Yervoy® Investor (5 mg/mL)

Results and Discussion

Yervoy® as a biosimilar was subsequently injected onto a 15kg/m³ UP-SW2000 column. Figure 1 shows how closely it assesses the similarities between the two products. Figure 1 shows the chromatogram of the relative retention times at t₀₀₂ minutes and t₀₂₀ minutes respectively.

Figure 1: UHPLC Analysis of Yervoy and Biosimilar with TSDkg/m³ UP-SW2000. Retention Time Comparison

In Figure 2 a zoomed-in plot provides a closer look at the baseline separation between the two products. The relative percent area values of the major peaks and minor impurities in each sample, both monomers are ~99%.

Figure 2: UHPLC Analysis of Yervoy and Biosimilar with TSDkg/m³ UP-SW2000. Zoomed-in Plot

Table 1: Comparison of Retention-Time and Percent Peak Area for Yervoy and Biosimilar

	Retention time (min)		
	Major	Minor	Percent Peak Area
Yervoy Investor	8.70	8.00	9.97
Yervoy Biosimilar	8.1, 8.2, 8.4	8.97	8.87
Percent area (%)			
Yervoy Investor	99.9	0.0	99.9%
Yervoy Biosimilar	9.8	9.8	99.8%

UHPLC Analysis of Biosimilars Using Size Exclusion Chromatography

LC/MS Analysis of Monoclonal Antibody Glycans using a Novel FcR Receptor Affinity Stationary Phase Paired with High Resolution Mass Spectrometry

Figure 1: Novel Dissociation Kinetics of a Typical IgG1 mAb

Figure 2: Chromatogram of a Mixture of IgG1 mAb Glycan Formations

Figure 3: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 10% Glycan Concentration

Figure 4: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 50% Glycan Concentration

Figure 5: Kinetics of the Dissociation Process from the IgG1 mAb Glycan Formations

Figure 6: Chromatogram of a Mixture of IgG1 mAb Glycan Formations

Figure 7: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 10% Glycan Concentration

Figure 8: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 50% Glycan Concentration

Figure 9: Chromatogram of a Mixture of IgG1 mAb Glycan Formations

Figure 10: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 10% Glycan Concentration

Figure 11: ESI-MS Spectrum of IgG1 mAb Glycan Formations at 50% Glycan Concentration

Figure 12: Chromatogram of a Mixture of IgG1 mAb Glycan Formations

Table 1: Sample Flow Conditions for Chromatography

Sample	Flow Rate (mL/min)	Column	Detector
IgG1 mAb	0.5	FCR-Affinity	ESI-MS
IgG1 mAb + 10% Glycan	0.5	FCR-Affinity	ESI-MS
IgG1 mAb + 50% Glycan	0.5	FCR-Affinity	ESI-MS
IgG1 mAb + 10% Glycan + 10% FcR	0.5	FCR-Affinity	ESI-MS
IgG1 mAb + 50% Glycan + 10% FcR	0.5	FCR-Affinity	ESI-MS

Characterization of Biosimilars using Different Modes of Chromatography

LC/MS Analysis of Monoclonal Antibody Glycoforms

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The sensitivities are impressive. The drawback is you have to have an electroactive molecule, which most molecules in cells aren't.

But CE has been paired with a lot of different detection strategies so it's fairly versatile.

Fluorescence detection is clearly one of the favorites for chemical cytometry because you can get down to yoctomole detection limits, which means down to a limit of just a few molecules of an analyte. But the molecule has to be fluorescent to get to that kind of a detection level. So that's the disadvantage.

You can measure anything with MS if you have enough of it. The challenge with MS is that often you're seeing the more highly abundant analytes in cells as opposed to some of the molecules that exist in 10 copy numbers per cell. So sensitivity remains an ongoing challenge. It's getting pretty exciting in the MS world, but the method still has a ways to go before it can look at many of the critical signaling molecules within single cells that are really driving what the cell is doing.

All of these detection technologies have their pros and cons, depending on what the exact analyte is and where the analyte is located within the cell and the type of sensitivity you need.

Some types of single-cell analysis require the use of a reporter molecule

(1). What characteristics does a reporter molecule need to have?

One of the challenges when you're using a reporter molecule is getting the reporter

into the cell. It turns out not to be so easy to get molecules across the plasma membrane—the lipid bilayer—of a cell, because it's charged on the outside, greasy on the inside, and then charged on the inside. So just getting the reporter in the cell can be a big challenge.

It's also a challenge convincing the cell to behave appropriately when these molecules do get through the barrier of the cell membrane. For example, cells can sometimes sequester the reporters in little bitty compartments and small subregions of the cell such as lysosomes or other small vesicles, which is not going to yield an accurate measurement.

You also need to make sure the reporter molecule is selective. The reporter molecule has to measure what you think it's measuring or if it's measuring many different analytes at once, you need to at least have an idea of what the different analytes are. That challenge can be overcome but it's something to be aware of.

Reporter stability is definitely a challenge also. Cells are constantly metabolizing and recycling proteins and peptides and other small molecules. So developing something that a cell isn't going to chew up and recycle can be a challenge. That might mean using non-native amino acids or peptides or very stably folded structures like green fluorescent protein or some of the other protease-resistant structures.

Then if you can have control over your reporter molecule—if you can turn on and off its ability to act as a reporter—that's icing on the cake. We'd like to have the reporter go into a cell and sit in the



cell being inert, and then be able to give it a signal to turn it on and have it start measuring, and then be able to turn it off. That's the hardest thing of all to design—a start-stop point. But if you're able to do that, it can give you really good temporal resolution and much greater insight into the connections between what a cell's doing and exactly when it's doing it. That is a great asset if you're able to engineer it.

There are all sorts of design challenges, but it can be a lot of fun.

How challenging is it to develop a reporter with the best set of features for a given application of chemical cytometry? And what are some of the strategies you use to do that?

It can be really easy or it can be really hard, depending on the analyte you're trying to measure.

For example, we've worked on reporters for lipids and it's not so hard to make reporters for those. The enzymes in the cells that metabolize lipids can be relatively tolerant of small additions like a fluorescein or a click moiety. And the fluorescent moiety doesn't mess up all the intracellular reactions for that probe.

At the other extreme is the challenge of developing reporters for measuring protein kinases and phosphatases. Cells have an amazing recycling system for breaking down the peptide substrates for these enzymes, turning the reporters into amino acids and then using those amino acids to make more proteins. Trying to beat that system is really tough. To do that can require screening libraries to find reporters

that are substrates of whatever enzyme or process you want to measure and yet are robust in the face of all the assaulting enzymes or irrelevant enzymes in the cytosol. The larger the library used, the more likely one will see success (but also the more time and effort required).

One type of analyte that has been studied using chemical cytometry is reactive oxygen species. Why is it valuable to study these compounds? Can you provide an example of the kind of information or insights chemical cytometry has been able to provide in the study of reactive oxygen species?

This area is pretty exciting. Reactive oxygen species are associated with inflammation, as immune cells become activated and the reactive oxygen species are used by the immune cells to kill bacteria and other pathogens invading our bodies. An additional problem though is that this inflammation can sometimes damage our own cells more than it damages the pathogen or invader. We can also have immune reactions directed at our own cells even when there's no pathogen, which is what happens in autoimmune diseases. So we want to gain insight into how immune cells attack their targets and what can go wrong when the immune system gets really active and how we might mitigate that.

These reactive oxygen molecules are all very short lived, so unless you have a probe to sample very close to the site of action, you can't learn anything about their kinetics



or spatial locations. Chemical cytometry using probes can help us do this.

We now understand that reactive oxygen species play a large role in a lot of different processes, from aging to cancer. Chemical cytometry that analyzes reactive oxygen species can help us begin to understand what's useful about these species as well as the undesired effects. It can begin to give us some nice insights into the biology of these very diverse processes that ultimately result in tissue damage like aging or cancer or uncontrolled immune processes.

The study of signaling lipids presents various challenges for most analytical techniques. Why is CE with fluorescence detection a good technique for this type of analysis?

Lipids are an interesting group of molecules. First, they are greasy and tend to stick to everything, so they're really difficult to handle. In CE you can use organic solvents and get the lipids really well solubilized and minimize their sticking to surfaces.

The other reason lipids are interesting is that lipids come in a wide range of different classes and with different chemical side groups within these groupings. And many of lipids can be interconverted, creating fairly complex signaling pathways, so there are a lot of different types of lipid molecules with a single cell. It is likely that many lipids have yet to be identified. CE has the power to begin separating out all these different classes.

And often, the key signaling lipids are only active as a certain isomer. A good example is phosphatidylinositol-trisphosphate. The 3,4,5-trisphosphate form is a potent signaling molecule within cells whereas some of the other isomers are not active; they're just intermediate metabolites. So if you just measure the total amount of this molecule in the cell and you miss the isomers, you will be completely misled about what's going on. CE can separate all these different isometric forms and show you the full range of what's going on in the cell.

Also, lipids can have very large dynamic ranges in terms of their concentrations in the cell. The housekeeping lipids are at super high, probably millimolar, concentrations. Signaling lipids are at subnanomolar concentrations and lower.

Another point is that there are very few antibodies or affinity reagents for lipids. For proteins, we have a vast array of antibodies that we can use to bind the proteins and light them up using immunochemical staining or capture them for further study. But many lipids are conserved across species, so you can't really make good antibodies to use as probes or for capturing lipids for further study. There are very few tools like that for lipids right now.

So how do you address that problem when you're working with lipids?

The good thing about many lipids is they'll tolerate small modifications to the lipid. A good example is sphingosine. You can put a click moiety down at the end of the acyl chain or even a fluorescein. And kinases



and other enzymes will still act on it with only slightly altered K_m and V_{max} values. So that's one good strategy—to exploit the tolerance of the enzymes that metabolize a given lipid.

You developed an approach to analyze protein kinases (2). One of the challenges involved was developing a suitable reporter. How did you address this problem?

There are several big challenges in developing reporters to analyze protein kinases, and one is specificity. Most kinases bind to another protein to phosphorylate it, and they recognize the three-dimensional (3D) structure. But for CE, we're using peptides because it's so much easier to separate the peptides. But in doing so, we lose the 3D structure.

So you try to make up for this by getting the sequence of the amino acids just right and really optimizing it to be a substrate for the kinase. We put a lot of effort and thought into doing screens of small-scale libraries to find the right substrate. Additionally, as mentioned earlier, the peptides are sometimes metabolized quickly within a cell. So we judiciously put in non-native amino acids that are tolerated by the kinases but are not tolerated by the proteases and peptidases.

But there are other enzymes that perform the reverse function of kinases—they remove a phosphoryl group instead of adding one. The challenge in this case was that the tyrosine phosphatases were orders of magnitude faster at dephosphorylating the reporter than the kinases

were at phosphorylating the reporter. So we developed a modified reporter that the kinases were still able to phosphorylate, but that is too constrained for the phosphatases to dephosphorylate readily. That was a strategy to beat the phosphatases and get the kinases on par with the phosphatases so that we can actually measure the kinase activity in a cell.

Another challenge in this work was addressing the low throughput of typical methods for delivering a reporter to cells. How did you address this challenge?

We've done that in a number of ways. First, if you can deliver your agent to all the cells at once by making it membrane-permeant, that's the way to go. One strategy to achieve that is to cage the charged groups on a peptide. We add a light-activated moiety that is a little on the hydrophobic side and it will basically help the reporter move through the cell membrane. Once it's through the membrane, we can use a laser beam to activate or remove that moiety since it's light activated. You get membrane permeability and also have a way to start the reaction or turn the reporter on. And in this case, the approach also helped to protect the reporter from peptidases and proteases. So that's one strategy, to get all the cells loaded at once in a very efficient manner.

Another strategy has been to add in automation. The more you can get people out of the process, the faster and more reliable the measurements are. We've done a lot of automation of the CE pro-



cess and of the sampling of single cells, so you can sample one cell after the next very fast and then lyse them individually, and separate their contents. We try to hit at every little point in the process, so that when you put it all together, you can get throughputs that are close to 100-fold faster than we used to get. There is still room for improvement but it's getting much better and much more reliable with all of these different tweaks and improvements that we've added into the process.

When you use the strategy that involves activating the reporter with a laser, is it difficult to do that without damaging parts of the cell?

No. You can use a very low power laser at a wavelength at which most of the cell's constituents don't absorb well. We have shown that we can essentially light-activate 100% of the reporter within a cell without damaging the cell. Usually if cells are not happy with what you're doing to them they'll activate what's called *stress pathways* that help them survive situations that might be damaging to them. We looked at the stress pathways to see if they were activated in the cell after this light pulse or light activation of the reporter, and those stress pathways weren't turned on at all. The cell didn't seem to be bothered by this brief pulse of light.

If you consider what people are doing when they're doing standard fluorescence microscopy, which people do all the time in biomedical research, tracking living cells for many hours, we're putting in tremendously less energy into the cell with the

light activation of the reporter. It's orders of magnitude less than in fluorescence microscopy. So that's what was great news. It makes the technique completely doable.

What results did you see in this work on protein kinases?

Basically, we looked at primary human tumor cells. There's an enzyme called Akt or protein kinase B, and it's often hijacked by tumor cells. We wanted to look at the tumor cells to understand how tumor cells were using this enzyme to fuel their inappropriate proliferation as well as to resist inhibitors of this enzyme. For example, in one tumor cell, this enzyme might be highly activated, while in another there might be very little activity of this enzyme, and in yet another there might be intermediate level of activity, suggesting that the tumor growth might be fueled by multiple different mechanisms. There might even be groups of tumor cells that you could place in different buckets or categories based on their Akt signaling behavior. The reason that's important to know is that when you treat a cancer patient you would like to kill all of their tumor cells—not just a subset of the tumor cells.

We were able to use a light-activated reporter to look at cancer cells and show that the heterogeneity and signaling was actually quite amazing even amongst otherwise seemingly identical cells. The different cells each had a distinct level of signaling activity, so if you used a protein kinase B or Akt inhibitor you were going to kill or inhibit some of the cancer cells but



other cancer cells seemed to be relying on other pathways and they would likely not be the least bit impacted by an Akt inhibitor. This result points to the need for using combined inhibitors or multiple different strategies to treat cancer patients successfully. That's probably not news to anybody but it's really nice to begin to look at this at the molecular level, to see what's going on in these cells and just how extreme the diversity and signaling behavior is in these cells.

And then, some cells will develop resistance to these drugs. And you can use the analytical strategy to see, for example, that the enzyme is turned off in certain cells but that in other cells, the enzyme is staying active for some reason. You can look at the diversity of resistance mechanisms to drugs.

Is there anything else you would like to add?

To look at single cells and the different constituents and the activities of those constituents in the cells, we need a whole suite of technologies with different pros and cons. Fluorescence microscopy has one set of pros and cons. And chemical cytometry or separations-based strategies to analyze cells have a very complementary set of pros and cons, so I see them as an incredibly valuable addition to the single-cell biology tool chest.

In the future, they could fit quite well into clinical workflows. It's an exciting time. We've got much work to do still but as single-cell biology expands and the

technologies become more readily available, we'll see these chemical cytometry techniques come into their own.

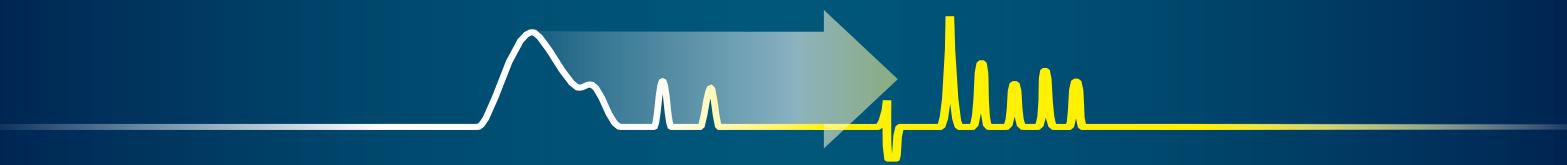
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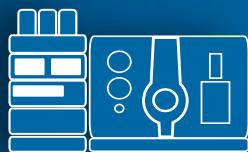


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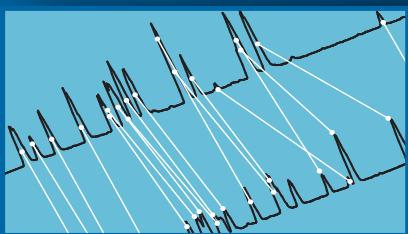


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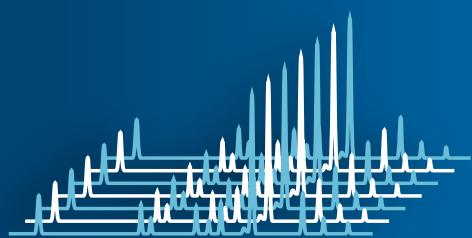
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The study of epigenetic DNA modifications is important for understanding fundamental questions in biology and disease. Studies of DNA methylation, for example, are important for understanding processes like gene suppression and activation, embryonic development, and nuclear reprogramming. Hailin Wang, of the State Key Laboratory of Environmental Chemistry and Ecotoxicology, at the Research Center for Eco-Environmental Sciences, which is part of the Chinese Academy of Sciences in Beijing, focuses on studying DNA modifications, and has developed many advanced separation techniques to further his work. He recently spoke to us about how the techniques he has been developing are enabling the progress of his research.

You and your group have studied the effects of the vital nutrient ascorbic acid (vitamin C) on the oxidation of 5-methylcytosine (5mC) and DNA demethylation in mammals (1).

New UHPLC–MS/MS Methods Advance DNA Research

An interview with Hailin Wang

How was ultrahigh-pressure liquid chromatography (UHPLC) with mass spectrometry (MS) detection used in this study?

In this landmark epigenetic study, we explored a newly developed UHPLC–MS assay to detect the levels of 5mC and 5mC oxidation products. Essentially, UHPLC provides highly efficient and rapid separation of nucleosides generated from digestion of genomic DNA, and the coupled triple-quadrupole mass spectrometry provides structure-specific quantitation of target nucleosides. The assays were performed with a 100 mm x 2.1 mm, 1.8- μ m C18 column (for separation) and electrospray MS/MS. Therefore, the adopted coupling analytical technology guarantees the accuracy and sensitivity for detection of 5mC and 5mC oxidation products. Using the developed UHPLC–MS assay, we showed, for the first time, the enhancing effect of vitamin C (ascorbic acid) on Tet proteins catalyzing the oxidation of genomic 5mC in vitro, in transfected cells, and in mouse embryonic



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cells and model animal mice. Moreover, we demonstrated that the enhancing effect of vitamin C could be observed through whole oxidative reaction chain, 5mC→5hmC→5-formylcytosine (5fC) and 5-carboxycytosine (5caC).

We also validated the identity of 5hmC using UHPLC–high-resolution mass spectrometry analysis. The corresponding fractions collected from UHPLC separation of digested genomic DNA of the untreated and vitamin C-treated cells had accurate mass-to-charge ratios of 258.1080 and 258.1092 ($M + H$), which match the theoretic monoisotopic mass of 5hmC (258.1084) with a deviation of 1.6–4.0 ppm. We further examined 5hmC using T4-glucosyltransferase-catalyzed glucosylation, which converts 5hmC in genomic DNA to glucosyl-5-hydroxymethylcytosine (5ghmC), which is detectable by UHPLC–MS/MS analysis with multiple-reaction monitoring (MRM) of a transition pair of m/z 420 → 304. Indeed, the 5hmC peak completely disappeared, and a new peak corresponding to the glucosylated product (5ghmC) appeared for the genomic DNA from both vitamin C-treated and untreated embryonic stem cells, but the level of glucosylated 5hmC in vitamin C-treated ES cells was fourfold higher than that in untreated embryonic stem cells. These results consistently support the vitamin C-induced increase of 5hmC in mouse ES cells.

In conclusion, UHPLC–MS made extremely important contributions to discovery of the role of vitamin C in the oxidation of 5mC and regulation of 5mC.

Without the developed UHPLC–MS approach, we could not have carried out this successful epigenetic study.

You continued the study of DNA methylation by examining the effect of redox-active quinones, which are found in natural products and cancer therapeutics, on Tet dioxygenases in human cells (2). What levels of the various compounds were you able to analyze using UHPLC–MS/MS? What challenges were faced in the analysis?

By coupling UHPLC–MS/MS- with MRM, we were able to detect trace amounts of 5hmC in somatic cells, which are 10–100 times lower than that in embryonic stem cells.

In an earlier study (3), you used a stable isotope dilution approach with UHPLC–MS/MS to detect 5-methylcytosine and DNA demethylation intermediates in human urine. Can you please briefly describe the solid-phase extraction (SPE) sample preparation process used in this research? How did you refine or optimize the SPE approach to prepare samples for high performance liquid chromatography (HPLC)–MS/MS analysis?

There are more than 3000 chemicals found in human urine, and the four most abundant ingredients are Na^+ , Cl^- , K^+ , and urea, the levels of which range from 4.6 to 22.5 mM/mM creatinine. To avoid possible ionization source contamination and ion



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suppression caused by coexisting ingredients in urine, we tested two types of SPE cartridges (C18 and HLB) for the cleanup and enrichment of the target deoxynucleosides. Using off-line coupling of SPE with stable isotope dilution HPLC–MS/MS, we demonstrated, for the first time, the presence and the abundance of free 2'-deoxy-nucleoside 5hmC in human urine.

Experimentally, the C18 and HLB cartridges with equal amounts of packing were tested. In both cases, the cartridges were first preconditioned with 10.0 mL of methanol followed by 6.0 mL of water. Then a 1.0-mL urine sample (or standard solution) was loaded into the preconditioned cartridges. The cartridges were washed with 5.0 mL of water and successively eluted with 5.0 mL of 1:9 (v/v) methanol–water and 5.0 mL of 3:7 (v/v) methanol–water. After methanol was removed by nitrogen gas at room temperature, the eluted fractions from each step were lyophilized. The residues were redissolved in 50 µL of water and centrifuged at the RCF of 13,000 for 10 min. The supernatants were collected. To evaluate the SPE protocol, the collected SPE fractions were further analyzed using HPLC–UV analysis. We found that the C18 cartridges were unable to effectively adsorb 5hmC. During cartridge washing by pure water, approximately 95% of 5hmC and 5hmU were washed away. In comparison, the HLB cartridges, filled with a water-wettable stationary phase mixed with the immobilized hydrophilic ligands and lipophilic ligands, showed much better performance in the separa-

tion and enrichment of all the targets from urine. Less than 20% 5hmC was lost during washing with water, and most of 5hmC, 5hmU, and 5fC were retained in the HLB cartridges until they were eluted with 10–30% methanol.

Of note, we could observe a matrix effect even when we used HLB cartridges to enrich 5hmC from urinary samples. The MS signals of 5hmC and 5mC enriched from urinary samples were repressed over 17 times and 2.7 times, respectively. However, the accurate quantification of urinary 5hmC and 5mC is obtained by adding stable isotopic standards of [D_3]5mC and [D_3]5hmC in known amounts to the urinary samples.

Your group also examined N⁶-methyladenine DNA modification in *Drosophila* (4). Various chromatographic approaches were used in the study, including UHPLC–MS/MS, HPLC–UV, and UHPLC–quadrupole time-of-flight (QTOF) MS/MS. How were these techniques used in the study? What additional information were you able to obtain using the QTOF system?

It remained elusive for decades whether DNA methylation was present and functioning in *Drosophila*. Scientists further wondered whether DNA methylation was essential to eukaryotic organisms. Addressing these fundamental questions, we speculated that there was probably another DNA modification as an alternative to the classic DNA methylation mark 5-methylcytosine. Therefore, we took advantage of various LC–MS technolo-

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gies to screen and identify possible DNA modification in *Drosophila*. Essentially, we used UHPLC–MS/MS for accurate quantitation of DNA modifications; HPLC–UV was used to collect pure fractions of modified 2'-deoxynucleoside generated from digestion of genomic DNA, and we further used UHPLC–QTOF MS/MS for high-resolution mass measurement of presumed DNA modifications. As a result, we discovered DNA N⁶-methyladenine modification in *Drosophila*, at the stage of embryonic development, and in adult tissues such as the brain and ovaries. Also of note, QTOF provided high mass resolution analysis of the fragmentation pattern of DNA N⁶-methyladenine, showing the complete agreement of presumed DNA N⁶-methyladenine with that of the standard.

Prior to our work, there were no data showing the presence of DNA N⁶-methyladenine modification in *Drosophila*. Therefore, these LC–MS technologies contributed greatly to the discovery and validation of DNA N⁶-methyladenine modification in *Drosophila*.

In a study published in May 2017 (5), you analyzed the inhibitive effects of nickel(II) on Tet dioxygenases that regulate the dynamics of DNA methylation. You used a size-exclusion chromatography (SEC)–inductively coupled plasma–mass spectrometry (ICP–MS) method to measure protein–metal binding events. What were the benefits of using SEC–ICP–MS in this study? Were any special sample

preparation processes needed for this analysis?

The principle of SEC is that it separates molecules according to size. Essentially, the molecules of larger sizes can access few pores widely distributed along with the stationary particles, showing shorter retention times; on the other hand, molecules of smaller sizes will access more pores of the stationary particles, showing longer retention times. By this mechanism, the protein-bound metal ions can be well separated from unbound (free) metal ions. Moreover, ICP–MS can be used for selective detection of metals. Because of its ability to detect unbound metal ions and protein-bound metal ions, SEC–ICP–MS can be used to measure these protein–metal binding events. By this measurement, we found that the estimated affinity of natural Ni(II) to demethylase Tet protein is 7.5-fold higher than that of the cofactor Fe(II). This is the first evidence to support that Ni(II) displays a much higher affinity than the native cofactor of Tet dioxygenases Fe(II) *in vitro*.

Of note, in these measurements, excess free metal ions were removed by ultrafiltration to eliminate possible interference prior to SEC–ICP–MS analysis, and ferrous-stabilized hemoglobin, which had the same retention time as Tet1CD, was used to indicate the position of Tet1CD-bound metal ions.

A second study of yours from 2017 implemented a stable isotope–



labeled deoxynucleoside as an initiation tracer along with UHPLC–MS/MS analysis to identify target DNA N⁶-methyladenine in human cells (6). What were the advantages of this approach? What challenges did you face in performing this analysis?

In reality, animals always live together with various bacteria. Noticeably, mycoplasmas, a genus of the smallest bacteria known, lack a cell wall around their cell membranes and are unaffected by many common antibiotics, and thus are often found in experimental cells and animals. This presents a great challenge to rationally interpret experimental results and to identify DNA N⁶-methyladenine (6mA)-related gene activities. To unlock the 6mdA mystery, an analytical technique was needed to identify and quantify DNA 6mdA of different origins and to further the study of diverse gene activities in mammalian cells.

To this purpose, we explored stable isotope-labeled deoxynucleoside [¹⁵N]₅-2'-deoxyadenosine ([¹⁵N]₅-dA) as a tracer, combined with UHPLC–MS/MS analysis, for accurate and rapid identification and detection of target DNA 6mdA. Unexpectedly, we found that the initiation tracer [¹⁵N]₅-dA could be converted into [¹⁵N]₅-dA in mammalian cells. In contrast, mycoplasma failed to perform this conversion. Based on this finding, we further utilized stable isotope-labeled deoxynucleoside [¹⁵N]₅-dA as an initiation tracer for monitoring the metabolism of endogenous 2'-deoxyadenine and its modification (6mA) in DNA. This unique tracing approach en-

abled us to specifically and quantitatively evaluate the origins and metabolism of DNA 6mA.

A paper from your group that was published earlier this year described an immunoprecipitation approach to select N⁶-methyladenine-specific fragments for genome-wide sequencing (7). How was UHPLC–MS/MS implemented in this study? What approach was used for sample preparation for the UHPLC analysis?

In this study, we exploited UHPLC–MS/MS, an advanced analytical technology, to evaluate 6mA-IP efficiency. Benefiting from its excellent sensitivity (attomole level) and structure-dependent measurement, we could detect 6mA accurately in the final pulled-down DNA (down to 1 ng).

Therefore, it is feasible to evaluate 6mA-IP using UHPLC–MS/MS throughout the whole immunoprecipitation procedure. Moreover, we demonstrated that a large portion of the pulled-down DNA does not contain any 6mA, particularly for dealing with the genomes with 6mA of very low abundance. To eliminate possible bias caused by the predominance of 6mA-free fragments, we designed, for the first time, an elegant, multiple-round 6mA-IP to allow for the possible predominance of 6mA-specific fragments in the final pulled-down fractions.

Another study from 2018 discussed an ultrafiltration method that enabled the removal of inorganic salts without DNA loss before UHPLC–MS/MS



analysis (8). Can you please briefly describe this sample preparation technique?

It is crucial to remove inorganic salts to improve the DNA digestion efficiency, which is required prior to sensitive HPLC–MS analysis. There are several ways to remove salts, for example, cartridge-based spinning-down, gel-filtration chromatography, and ultrafiltration. The major concern is that all these treatments may cause a significant DNA loss while removing the inorganic salts. Since UHPLC–MS/MS analysis uses only nanograms to a few micrograms of DNA, DNA digestion also uses only the same amount. Therefore, DNA loss caused by inorganic salt removal-related treatment becomes more serious. If we used our standard laboratory practice, a few micrograms of DNA would be largely lost during spinning down and gel-filtration chromatography. So we explored an ultrafiltration approach to remove the inorganic salts. To this end, we first tested routinely used ultrafiltration tubes. Surprisingly, as detected by agarose (2%) gel electrophoresis analysis, we failed to recover any DNA after ultrafiltration. This is probably related to the setting of the routine ultrafiltration (with horizontal membrane). With this horizontal setting, direct flow ultrafiltration (without a dead end) was exerted by centrifugation, and thus no liquid was retained above the membrane, forcing the partial entering of DNA threads into the pores of the membranes. Therefore, the DNA adhered to the filtration membrane and could not be recovered.

We further speculated that an ultrafiltration tube with vertically set membranes might keep DNA in the residual solution because of its bottom void volume (20 µL). We tested vertical ultrafiltration of three different molecular weight cutoffs (3, 10, and 30 kDa). Indeed, DNA in all tests was satisfactorily recovered. As desalting with this vertical ultrafiltration, DNA was efficiently digested by DNase again. This result proves that the desalting by vertical ultrafiltration can effectively facilitate DNA digestion.

What are the next steps in your research?

We expect more than 10,000 samples per year in our lab. To deal with the large number of samples, we need make the analysis time as short as possible, while minimizing matrix effects. Rapid and high-throughput UHPLC–MS/MS methods are highly desirable. Also, prior to UHPLC–MS/MS analysis, labor-intensive pretreatment is involved. Therefore, another step will be to develop high-throughput pre-treatment of genomic DNA.

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Studying the Kinetic Performance of Columns in Fast LC

In liquid chromatography (LC), particularly in ultrahigh-pressure LC (UHPLC), packing materials and column packing procedures are continuously being studied, improved, and optimized. Less attention is paid, however, to the column ends and frits. As part of his work studying fundamental issues in chromatography, Attila Felinger of the University of Pécs, in Hungary, has studied the kinetic performance of column in fast LC, with a particular focus on local performance at the column ends. Felinger recently spoke to us about this work.

You recently conducted a study to compare the kinetic performance of various columns, both silica and monolith, for fast LC (1). How is “fast” LC defined? Is that definition important for this study?

Fast liquid chromatography is associated nowadays with ultrahigh-performance liquid chromatography (UHPLC). With the advent of LC systems capable of working

at pressures of 1000 bar and greater, vendors have introduced many abbreviations. Fast liquid chromatography seems to be a good term to encompass all those 1000+ bar separation systems. It is funny to note that 50 years ago, Csaba Horváth used the same term, *fast chromatography*, for the HPLC technology that was just emerging at that time.

In our study we compared a number of 2.1×50 mm columns, but the method we introduced is applicable for the study of “old school” columns as well.

Why did you feel it was important to conduct this study?

We wanted to understand whether the column packing procedure influences the local efficiency at the respective column ends. For that purpose, we needed an experimental protocol with which the local efficiency at any axial position of the column can be determined.

One factor you evaluated was the de-



pendence of column efficiency on the retention factor. What did you find?

We found that for weakly retained compounds, the frits of the packed columns significantly lessen efficiency, and the efficiency of the fritless monolithic columns outperforms the packed columns when retention factor is small.

What prompted you to study the effect of frits on column efficiency?

Usually, very little attention is paid to the frits, whereas the packing materials and the column packing procedures are continuously being improved and optimized. It was surprising to see that for an unretained analyte, the band broadening caused by the two 1-mm-long frits can be the same as the band broadening caused by the 50-mm packed bed.

In your study, you used the flow-reversal method to measure band broadening. Can you briefly explain this procedure?

We install the column in the chosen flow direction, which is either the direction suggested by the vendor, or the opposite. Then we inject an unretained analyte and stop the flow when the zone has migrated a given distance. After that, we disconnect the column and reinstall it in the opposite flow direction. The flow is restarted, and the zone elutes at the same end as it entered the column.

You conducted a series of experiments to characterize band broadening that resulted from peak parking. How did you carry out those experiments? And what did you find?

Peak parking is an inherent part of our experimental protocol. When the flow is arrested, the pressure must relax before the column is disconnected and reinstalled, and that takes time. Furthermore, peak parking is an important tool to determine the effective diffusivity of the analyte in the column.

Peak parking also serves as a tool to estimate the axial heterogeneity of the bed if flow is arrested at various penetration distances of the sample band. An interesting outcome of our experiments was that the local plate height values obtained with flow reversal are always smaller than the ones measured for the same column without reversing the flow. For hydrodynamic reasons, reversing the flow sharpens the bands.

What results did you find overall about the difference in kinetic performance for the three types of columns? How significant were the differences?

There are rather small differences between modern LC columns as long as the separation efficiency for well-retained small molecules is considered. For less retained analytes, the fritless monolith technology has a lot to offer.



What direction should these results point to? Are these results useful for making column selections? Or is their primary purpose to expand our understanding of the factors contributing to column performance?

The primary focus of our research was indeed to better understand the limits of column efficiency. When the various contributions to the band broadening in a well-packed column are compared, we see that the observed overall, uncorrected plate number of an unretained analyte is only one-third of the efficiency of the well-packed bed itself, even if the best equipment is used. Furthermore, we were able to demonstrate that for packed columns, the local efficiency at the two respective column ends is different, which is the consequence of the packing procedure.

What are your next steps in this work?

We are continuing our research by studying the influence of column length or

packing procedure on the axial heterogeneity of LC columns. Furthermore, we are using macromolecules as test compounds, combined with the flow-reversal experiments.

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