Programmable Flow Injection for online solid phase extraction and more

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Programmable Flow Injection (pFI) was developed from the older FIA and SIA concepts [1]. It is based on a precise computer-controlled combination of flow reversals, flow accelerations, and stop flow periods of the µL zones of liquids mainly for volume metering, the sample collection, injection, confluence or sequence zone mixing for sample dilution, reaction or interaction with a solid phase, followed by detection of a result of the underwent processes in a flow cell or further transport to the next step of the analysis. pFI extends the capabilities of both a nonequilibrium flow-based and an equilibrium batch-flow based tasks that cannot be accomplished in a legacy continuous flow format, while maintaining the highly flexible software control of a clearly arranged manifold, high precision online detection, easy data evaluation, and automated feedback based on results.

The presentation provides examples and innovations in wet chemistry-based analysis of nutrients in seawater, automated sample preparation based on solid phase extraction, and hyphenation with separation techniques such as HPLC or sequential injection chromatography. News about the pFI concept is continuously published in the online tutorial “Programmable Flow Injection Analysis” [2] and relevant literature [3].

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Keywords: programmable Flow Injection, tutorial, automation, online solid phase extraction, sequential injection analysis

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Nano-liter Sample Pretreatment of Glycans for Capillary Electrophoresis Analysis

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Capillary electrophoresis (CE) is a powerful analytical technique that utilizes micro/nano-meter-scale inner diameter separation channels, facilitating efficient heat dissipation and minimizing analyte diffusion, thus significantly enhancing separation performance. However, the narrow channels also limit the sampling volume, posing a challenge to detection sensitivity. Typically, samples are handled in micro-liter scales, whereas CE sample injection volumes are only in the nano-liter range, resulting in substantial sample loss, often exceeding three orders of magnitude. To address this issue, large-volume sample stacking methods have been developed, which can increase the injected volume to a few micro-liters but still leave a significant portion of the sample unused [1].

This study introduces a novel approach by challenging sample pretreatment workflow using a nano-liter scale container. An open capillary tube, with dimensions of 5 mm length, 100 μm inner diameter, and a volume of 39 nL, was employed as a reaction container. The workflow for 8-aminopyrene-1,3,6-trisulfonic acid (APTS) derivatization of maltoligosaccharide standards was successfully realized within the capillary tube [2]. The molecular derivatization solution consisted of 100 mM glycan standards, 100 mM APTS, 7.5% acetic acid, 500 mM $NaBNCH_3$, and 50% tetrahydrofuran, and the derivatization process was conducted at 37°C overnight. The nano-liter reaction products were analyzed using CE coupled with laser-induced fluorescent detection (Fig. 1). The intensities of each oligosaccharide were found to be comparable with those from bulk-scale reaction. Future studies will focus on applying this nano-liter reactor to natural-source glycan sample pretreatment, aiming for whole sample injection. The proposed approach is expected to find applications in trace sample analysis, such as single-cell, tissue micro-section, minimally invasive clinical sample, and so on.

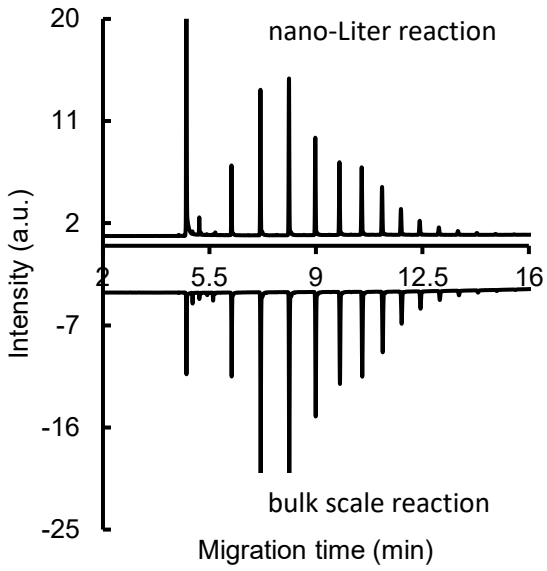

Figure 1. Electropherograms of malto-ligosaccharide standards in (a) nano-liter reactor and (b) bulk scale tube.

Keywords: capillary electrophoresis, glycans, sample preparation, nano-liter sampling.

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ELECTRODRIVEN ION FOCUSING AND STACKING ON A POLYMER INCLUSION MEMBRANE

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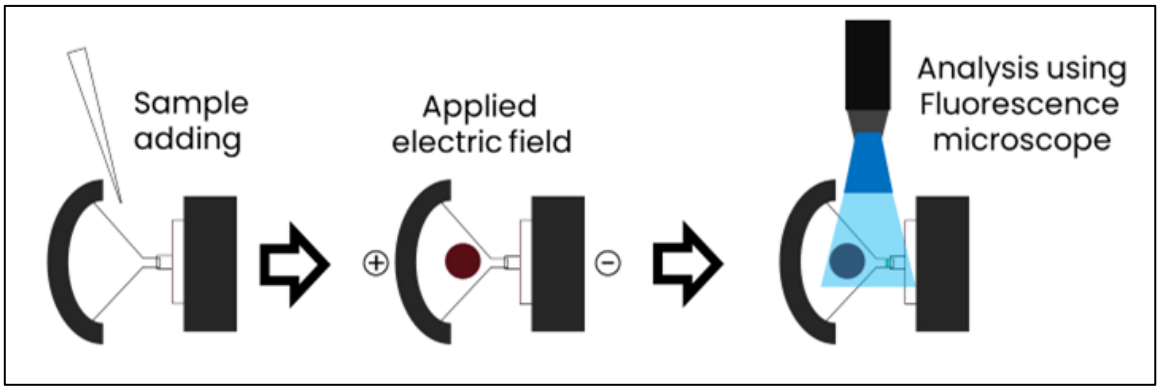
The detection of analytes from dried blood spots (DBS) is often hindered by low analyte concentrations and limited sample volumes, leading to weak signal responses. To address this challenge, a novel electrokinetic method has been developed to enable the focusing of cationic analytes at the interface of cationic and anionic polymer inclusion membranes (PIMs), as well as their separation across membranes with differing conductivities under an applied electric field. Two distinct PIMs were fabricated using cellulose triacetate (CTA) as the polymer matrix, 2-nitrophenyl octyl ether (2-NPOE) as plasticizer, and Di-(2-ethylhexyl) phosphoric acid (D2EHPA) and Aliquat 336 serving as functional carriers. The effects of CTA, carrier type, and plasticizer composition on ion mobility and stacking efficiency were systematically examined. Under optimized conditions (250 V/cm for 30 minutes), efficient focusing of Sanguinarine was achieved using PIM A, composed of CTA (100 mg), 2-NPOE (250 mg), and D2EHPA (40 mg), and PIM B, composed of CTA (100 mg) and Aliquat 336 (150 mg). A 20 µL aliquot of 1.0 ppm Sanguinarine was successfully stacked at the PIM interface in a fan-shaped configuration and visualized using fluorescence microscopy. Further enhancement of ion focusing and analyte separation was achieved by integrating Field-Amplified Sample Stacking (FASS) into the PIM platform. This enabled the electrophoretic separation of both cationic dyes and bioactive Sanguinarine. The results highlight the potential of this electrokinetic PIM-based method for miniaturized sample preparation and sensitive analysis, particularly for applications involving low-volume biological matrices such as DBS.

Keywords: Ion Focusing, PIM, Electrophoresis, FASS

Graphic:



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Aptamer selection based on microscale electrophoretic filtration

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Abstract

DNA/RNA aptamers are versatile recognition molecules with high specificity for their targets, making them attractive for applications in biosensing, diagnostics, and pharmaceuticals. However, conventional selection methods suffer from limitations such as complicated procedures, low separation efficiency, and the need for expensive equipment, which hinder their practical use. To address these issues, we developed a new selection method using a microscale electrophoretic filtration device [1].

In this method, a capillary device partially filled with hydrogel enables a sequence of aptamer selection steps: electrophoretic filtration of target proteins via molecular sieving, electrokinetic injection of a random DNA library to interact with the trapped proteins, washing to remove weakly or non-binding DNAs, and elution of strongly binding aptamer candidates.

To demonstrate the concept, immunoglobulin E (IgE) was chosen as a model target. Following the electrokinetic injection of fluorescently labeled IgE, strong fluorescence was observed near the upstream hydrogel interface, confirming successful filtration. When unlabeled IgE was filtered, subsequent introduction of a fluorescently labeled random DNA library produced fluorescence from DNAs bound to IgE. During the washing step, the fluorescence gradually decreased but remained detectable, and it disappeared after the elution step, indicating successful recovery of strongly binding DNAs. The recovered DNAs were then amplified by PCR to generate the next-generation library.

After three selection cycles, two aptamer candidates for IgE were obtained. Affinity capillary electrophoresis revealed strong binding, with dissociation constants (K_D) in the nanomolar range. These aptamers showed no binding to non-target molecules such as bovine serum albumin or immunoglobulin G. This method enables aptamer selection through simple operations, high-efficiency separation via electrophoresis and filtration, and low-cost instrumentation using disposable devices, offering strong potential for practical applications.

Keywords: Aptamer, aptamer selection, microscale electrophoretic filtering

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Polyacrylamide-modified Monolithic Silica Capillary Columns for the Separation of Polar Analytes

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Monolithic silica capillary column has been used for fine separation of challenging targets, such as peptides [1], isomers, and isotopologues in revered-phase mode [2]. It can be modified by polar functional group to be hydrophilic interaction chromatography (HILIC) column, too [3]. For better retentivity and selectivity, functionalization of the silica monolith by on-column polymerization by acrylamide [4], acrylic acid [5] and so on. To impart higher retentivity and better selectivity to the monolithic silica capillary column, higher concentration of monomers in the feed composition, however, such a polymerization conditions often resulted in column clogging. Capillary columns of 100 to 200 micron I.D. contain too small amount of silica to take samples of the polymer on the silica for molecular weight determination. For further improvement of the functionalization of monolithic silica capillary column, the nature of polyacrylamide bound onto silica surface was examined using polymer samples taken from silica particles modified by the same polymer, and the same polymerization conditions. Both of a monolithic silica capillary column and a particle-packed column modified by polyacrylamide showed similar separation characteristics, i.e., selectivity for OH group and CH_2 group, selectivity between vidarabine and adenosine, and theobromine and theophylline [6], were comparable that showed similar polymerization and similar binding of them to silica have undergone. The retention of the capillary column was one third of that by the particle-packed column, due to the lower silica content per one column. Molecular weight determination by size exclusion chromatographic analysis of the polymer on silica, and in solution resulted in 70,000 to 140,000 (M_W on silica), and 570,000 to 740,000 (M_W in solution), and the high molecular weight polymer in the solution part can be a reason of column clogging. High molecular weight, above 120,000 (M_W) was shown to be essential to impart good retentivity and selectivity for structural differences.

Keywords: HPLC, monolithic silica columns, polymerization, polyacrylamide

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Gold nanostructures as a tool for biothiols preconcentration from non-invasive samples

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Endogenous low-molecular-mass biothiols, such as homocysteine, cysteine and glutathione, are a promising group of potential biomarkers for oxidative stress and various pathological states [1]. Their quantification in non-invasive biological samples presents a significant analytical challenge, especially due to their extremely low concentrations, demanding highly sensitive analytical methods for their detection. In this work, we present a procedure that utilizes gold nanostructures in various arrangements for biothiol preconcentration.

We developed and compared three distinct approaches based on gold nanostructures for the selective capture and enrichment of biothiols. These include: (i) colloidal gold nanoparticles (AuNPs), (ii) composite gold magnetic nanoparticles (Au-MNPs) enabling simple magnetic separation, and (iii) AuNPs grafted on stationary phase particles for use in a micro-column solid-phase extraction (SPE) format.

The developed procedures were applied to preconcentrate endogenous biothiols directly from EBC samples. Following the selective capture, the adsorbed biothiols were efficiently desorbed by dithiothreitol (DTT), achieving significant enrichment (preconcentration factors ranging from 8 to 34). For subsequent analysis, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was employed. Prior to analysis, the thiol moiety was capped with N-ethylmaleimide (NEM), followed by a fluorescent derivatization via the amine group with naphthalene-2,3-dicarboxaldehyde (NDA) and potassium cyanide.

Gold nanoparticle-aided preconcentration procedure enhanced the overall sensitivity of the method, enabling the detection of biothiols at the nanomolar range (0.24-0.71 nM) [2]. This approach is a powerful tool for analysis of biothiols in non-invasive samples, especially with automation of the procedure by either magnetic manipulation or a flow-based sample treatment using grafted columns.

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Keywords: gold nanostructures, biothiols, capillary electrophoresis, exhaled breath condensate

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Bone Marrow Derived Mesenchymal Stem Cell Purification Using Thermoresponsive-Cationic Copolymer Brush Modified Beads Packed Column

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Cell therapy using mesenchymal stem cells (MSCs) has received attention as an effective regenerative therapy for treating intractable diseases [1]. To increase the therapeutic efficacy of cell therapy, an effective cell separation method is required that does not modify cells and maintains cell activity. In the present study, we developed a temperature-modulated mesenchymal stem cell separation column using thermoresponsive and cationic block copolymer brush-modified silica beads as packing materials (Fig.1). Temperature-responsive cationic block copolymer, poly(*N,N*-dimethylaminopropyl acrylamide)-*b*-poly(*N*-isopropylacrylamide) (PDMAPAAm-*b*-PNIPAAm) brush were grafted on silica beads through two-steps of atom transfer radical polymerization (ATRP). The prepared copolymer brush-modified silica beads were characterized by CHN elemental analysis and FTIR. Using the prepared copolymer brush-modified silica beads-packed column, the elution behavior of MSCs and BM-CD34$^+$ cells was observed at 37°C and 4°C. CHN elemental analysis and FTIR indicated that thermoresponsive cationic copolymer was successfully modified on silica beads through the ATRPs. The prepared silica beads were packed into a column, and the elution behavior of the cells from the column was observed. At 37° C, MSCs were adsorbed on the column through both hydrophobic and electrostatic interactions with the PNIPAAm and PDMAPAAm segments of the copolymer brush, respectively. By reducing the temperature to 4°C, adsorbed MSCs were eluted from the column by reducing hydrophobic and electrostatic interactions attributed to the hydration and extension of the PNIPAAm segment of the block copolymer brush. The suitable DMAPAAm composition in the block copolymer brush was determined from the temperature-modulated adsorption and elution behavior of MSCs. Using the column, a mixture of MSC and BM-CD34$^+$ cells was separated by simply changing the column temperature while maintaining the cellular activity of MSCs. The separated cells exhibited viability and differentiation potency. The results indicated that the prepared bead-packed column can separate MSCs from contaminant cells by simply changing the temperature and without modifying the cells.

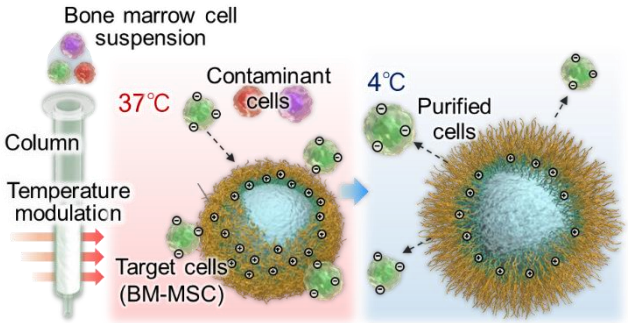

Fig.1 Temperature-responsive stem cell separation column for human bone marrow separation

Keywords: Stem cell, Cell separation, Cell therapy, Column separation, Bioseparation

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Salivary Lysozyme Determination by Simple CZE-UV: Possibilities and Challenges

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Saliva presents an attractive, noninvasive biological fluid for disease monitoring due to its ease of collection and its content of diagnostically relevant biomarkers. Lysozyme, an antimicrobial enzyme naturally present in saliva, has shown significant potential as a biomarker for various pathological and inflammatory conditions. In this study, we present the development and validation of a novel, rapid, and simple capillary electrophoresis (CE) method with UV detection for the quantitative analysis of human salivary lysozyme.

The method employs capillary zone electrophoresis with transient isotachophoresis as an in-capillary preconcentration technique to enhance sensitivity. Key method optimizations included the use of 1 M formic acid with 10% (v/v) isopropyl alcohol as the background electrolyte, and suppression of non-specific adsorption via surface deactivation using carbonic anhydrase. Sample pretreatment was minimized to simple dilution, supporting the method's applicability in clinical settings.

The method demonstrated very good analytical performance, with precision (RSD < 5%), accuracy (−2.03% to +5.36%), and a limit of detection of 0.5 µg/mL. Lysozyme stability in saliva stored at −80 °C for up to 7 days with protease inhibitors was confirmed, and short-term biological variability was evaluated over multiple time points and days.

The validated method was applied to saliva samples from 25 healthy volunteers, with results showing a mean lysozyme concentration of 14.3 ± 10.5 µg/mL. To our knowledge, this is the first CE-UV method developed for salivary lysozyme determination in its intact form. The method is characterized by its simplicity, speed (total time under 45 minutes), robustness, and suitability for real-world sample analysis, making it a promising tool for future clinical applications in disease screening and monitoring.

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Keywords: capillary zone electrophoresis, lysozyme, proteins, saliva, transient isotachophoresis

Pros and *Cons* of the Schlieren Effect in Flow-based Analysis and Its Application for Quality Control in Food and Pharmaceutical Formulations

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In flow analysis, schlieren effect is the phenomenon of light refraction at the boundary of two liquids with different refractive indices. The signal profile arising from this phenomenon often perturbs the measurement of light absorption in spectrometry. Albeit the '*Cons*' of the schlieren effect in flow-based analysis, there is a '*Pros*' side to this effect, since the schlieren signal correlates with the concentration of a solute in the solution. In this work, utilization of the schlieren effect is demonstrated for the quantitative analysis of alcohols, acetic acid and dissolved sodium chloride in binary-mixture solutions of rubbing alcohol, vinegar and normal saline, respectively. The analytical flow system is simple with injection of sample (200 or 400 µL) into a stream of water (flow rate: 1.8 mL min^{-1}). To avoid absorption of the sample in UV-Vis region, the light source was selected in the near-IR range (890 nm). Linear calibrations (r^2 > 0.99) were satisfactorily obtained with extremely high sample throughput of 60 to 200 injections h^{-1}. Applications of the method to selected food and pharmaceutical formulations gave results that corresponded well with the comparative methods. This proposed method is reagent-free, environmentally friendly and suitable for in-line quality control in food and pharmaceutical formulations.

Keywords: Schlieren effect, flow injection analysis, binary mixture, food and pharmaceutical products, quality control

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Amine-Functionalized Fe_3O_4@SiO_2 as Magnetic Dispersive Adsorbents for the Pre-concentration of Selective Serotonin Reuptake Inhibitor Antidepressants from Aqueous Solutions: Analytical Performance, Sorption Modeling, and Greenness Assessment

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Abstract

This study successfully synthesized, characterized, and applied a magnetic amine-functionalized adsorbent material, modified with an alkaline activator, for the pre-concentration of selective serotonin reuptake inhibitor (SSRI) antidepressant drugs namely escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline in water. A range of techniques, including X-ray diffraction (XRD), scanning electron microscopy (SEM), vibrating sample magnetometry (VSM), specific surface area analysis, and Fourier transform infrared spectroscopy (FTIR), were employed to investigate the physical and chemical properties of the synthesized material. Response surface methodology, specifically the 2^7-run Plackett–Burman and the 2^4-run Central Composite design, was utilized to explore synergistic factors influencing the magnetic dispersive micro-solid phase extraction method. The optimal conditions for pre-concentrating the target analytes as suggested by model as follows: a sample volume of 15 mL, water temperature of 22 °C, sorbent weight of 0.5 g, effervescent precursor amount of 1 g, solution pH of 11.0, contact time of 7 minutes, and desorption solvent volume of 250 µL. Under these conditions, a high recovery of 90% was achieved, with a desirability value of 0.90. Quantification of the analytes was performed using high-performance liquid chromatography (HPLC) equipped with a diode array detector. The method demonstrated low limits of detection (LOD) and quantification (LOQ), ranging from 0.01 to 0.08 µg/mL and 0.03 to 0.28 ng/mL, respectively. Extraction recoveries at three spiked concentration levels ranged from 79% to 95%, while intra- and inter-day precision showed low variability, with relative standard deviations (RSDs) below 10%. Regeneration studies revealed recovery losses of less than 10% after four cycles of use. The Freundlich isotherm model (R^2 >0.990) and the pseudo-second-order kinetic model (R^2 >0.980) provided the best fit to the experimental data. Through molecular dynamics simulations, adsorption configurations and energies were systematically calculated to investigate the interaction between SSRI molecules and the adsorbent. Theoretical calculations indicated that adsorption was mainly driven by electrostatic interactions and π–π stacking. Furthermore, the magnetic dispersive micro-solid phase extraction method proved to be environmentally sustainable, achieving an overall AGREEnness score of 0.70, a Blue Applicability Grade Index of 67.5, and a Sample Preparation Metric Sustainability score of 7.37 highlighting its green credentials.

Keywords: pharmaceutically active drug, magnetic adsorbent, greenness metric assessment

Integrated Multidimensional Analytical Strategies for Quality Assessment of Valuable Agarwood (*Aquilaria* spp.)

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Agarwood is an aromatic and valuable wood containing resin, mainly produced by plants of the *Aquilaria* species (Thymelaeaceae family). The formation of resin is induced by the defense response of *Aquilaria* plants to external injuries. The extensive use of agarwood and the depletion of resources make the quality control of agarwood particularly important. Unsystematically identified components in the ethanol extract of *Aquilaria agallocha* were explored by mass spectrometry-guided separation and identification techniques. The multi-modal mass spectrometry integration approach was used to analyze agarwood from multiple perspectives, thereby unearthing the intrinsic components related to quality evaluation. An ambient ionization pre-processing technique combined with miniature mass spectrometry was employed for on-site detection and rapid authentication of agarwood. Multidimensional analytical separation techniques were used to separate the ethanol extract reflecting the resin content to magnify the differences. Meanwhile, based on the technique of combining optical microscopy and mass spectrometry imaging at the spatial level, the compositional distribution of the resin aggregation sites in agarwood slices was visualized. Furthermore, a comprehensive strategy combining targeted and non-targeted methods was adopted to efficiently enrich the aroma of different zones as well as wild Kynam agarwood, which comprehensively revealed the aroma components related to the production area and quality. The gas-liquid microextraction (GLME) technique was further used to capture the volatile target aroma components at different temperatures in real-time and explore the changes in the related odor components. In conclusion, the above research provided data support for the quality control and scientific quality evaluation of agarwood.

Keywords: Agarwood, multi-modal mass spectrometry, aroma, quality evaluation

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Multi-platform mass spectrometry for in-depth chemical profiling and anti-platelet compound identification in *Panax Notoginseng*

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Mass spectrometry (MS), with its high sensitivity, superior structural elucidation capabilities, and broad applicability, has become an essential tool in natural product research. Our group developed an integrated workflow combining multiple MS techniques to comprehensively characterize the chemical constituents and identify antiplatelet components in the medicinal herb Panax notoginseng (PN). Through integrated analysis of diagnostic ions and neutral losses, 180 ginsenosides were identified. Coupled with mass spectrometry imaging (MSI), the spatial distribution of these ginsenosides was successfully visualized. To resolve common isomeric ambiguities of ginsenosides, complementary dissociation methods—collision-induced dissociation (CID) and electron-activated dissociation (EAD)—were employed, enabling precise determination of sugar substitution sites and linkage sequences on ginsenoside molecules for detailed structural characterization. For non-saponin constituents, a “MS molecular networking–molecular fingerprinting–metabolomics” (3M) strategy identified over 200 compounds, including amino acids, sugars, and nucleosides, with MSI further revealing their spatial distributions. Chemical profile–bioactivity relationship analysis facilitated the discovery and validation of key antiplatelet ginsenosides, such as Rk1 and Rg5. Finally, a quality evaluation method for PN was established using active components as indicators by mass spectrometry analysis. This study highlights the comprehensive utility of mass spectrometry in natural product analysis including constituent identification, bioactivity screening, and quality control, advancing research and development in this field.

Keywords: Mass Spectrometry, Mass spectrometry imaging, *Panax Notoginseng*, Ginsenosides, Antiplatelet activity

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Development of a Simple Analytical Method for *Legionella pneumophila* Using Novel DNA Aptamer-Gold Nanoparticle Conjugates

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Monitoring pathogenic microorganisms in public and environmental waters is important for protecting ecosystems and human health. However, current detection methods such as culture method, qPCR method, and immunoassay require long times, complicated operations, and expensive reagents. In this study, we developed a simple analytical method for *Legionella pneumophila* (*Lp*) as the model of pathogens using DNA aptamer-gold nanoparticle (AuNP) conjugates to determine the *Lp*.

We propose a single-round polymer-enhanced capillary transient isotachophoresis (SR-PectI) technique to obtain *Lp*-binding DNA aptamers [1,2]. The single peak of *Lp*-DNA complexes was separated from randomized DNA library, and was fractionated the single peak area (the dotted area in Fig. 1) (Fig. 1). After fractionation, the *Lp*-binding DNA sequences were determined by next-generation sequencing (NGS) technique. As a result, four DNA aptamers showed higher affinity (K_d = 1.0-3.6 nM) than previous *Lp*-binding DNA aptamers (K_d = 7.6, determined in our experiments) [4].

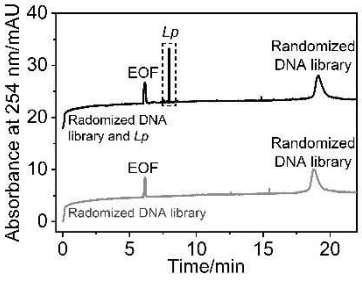
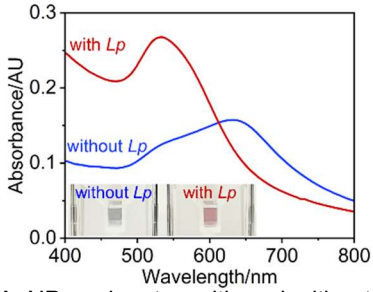


Fig. 1 Typical electropherograms of randomized DNA library, and the complex of *Lp* and randomized DNA library using PectI. The dotted area is the fractionation area (60 seconds).

The conjugates between our obtained aptamer, which has the highest affinity among the determined aptamers, and AuNPs were prepared (named AuNP conjugates). In the absence of *Lp*, the AuNP conjugates aggregated and turned blue. In the presence of *Lp*, the AuNP conjugates bound to *Lp*, remaining dispersed to give red colour (Fig. 2). By measuring the ratio of the absorbance at 650 nm and 520 nm (A_{650}/A_{520}), the number of *Lp* could be analysed. After investigating the aggregation and dispersion conditions of the AuNP conjugates, the calibration curve was obtained and the detection limit was determined to be 89 cfu. The AuNP conjugates could demonstrate the measurements of *Lp* in environmental samples.



AuNP conjugates with and without *Lp*. Inset shows the colour change of AuNPs with and without *Lp*.

In conclusion, novel *Lp*-binding DNA aptamers which have higher than the reported DNA aptamers and a simple analytical method for *Lp* using the obtained *Lp*-binding DNA aptamer were developed. The advantages of our method are rapid (> 1 hour) and easy-to-use (not relying on culture, extraction of DNA and RNA, and PCR) with high sensitivity and selectivity.

Keywords: capillary electrophoresis, DNA aptamer, gold nanoparticles, *Legionella pneumophila*

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Enhancing LiP-MS Structural Proteomics with Multi-Protease Complete Digestion Strategies

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Protein higher-order structure is closely linked to molecular function, making proteome-scale structural analysis essential for understanding how environmental stimuli induce protein conformational changes. Limited Proteolysis-coupled Mass Spectrometry (LiP-MS) combines partial digestion with proteinase K (PK) followed by complete digestion with trypsin and mass spectrometry to facilitate structural proteome analysis. However, the cleavage specificity of trypsin limits peptide diversity and hinders comprehensive identification of PK cleavage sites, restricting the method's ability to detect protein conformational changes across the proteome.

In this study, we developed multiLiP-MS, which employs multiple proteases for complete digestion, generating diverse peptide fragments and improving the detection of PK cleavage sites. HEK293T cell lysates were treated with proteinase K (1:100 enzyme-to-substrate ratio) for 5 minutes at 25 °C, followed by protein denaturation using 5 % sodium deoxycholate. Samples were then digested with one of five proteases (trypsin, LysC, Arg-C, LysargiNase, or V8 protease) and analyzed by nanoLC/MS/MS. The peptides were identified using Spectronaut 18 (Sagan).

We first evaluated the coverage of PK cleavage positions. MultiLiP-MS identified 37,300 PK cleavage positions using five proteases—approximately four times more than conventional trypsin-only LiP-MS. This dramatic increase demonstrates that the diversity in protease specificity significantly enhances PK cleavage site identification. The additional cleavage sites tended to be located near lysine and arginine, the target residues of trypsin digestion, suggesting that PK cleavage sites in these regions could not be identified using trypsin alone. Furthermore, applying multiLiP-MS with trypsin and LysargiNase to rapamycin-treated samples successfully identified residue-level structural changes in the rapamycin target protein FKBP1A, validating the utility of this method for advancing LiP-MS-based structural proteomics.

Keywords: LC/MS/MS, proteomics, protein structure, protease

Evaluating process development strategies through a multi-attribute mass spectrometry approach to minimize disulfide bond-related modifications in monoclonal antibodies (mAbs)

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Introduction

mAbs rely on the structural integrity provided by interchain disulfide bonds linking the light and heavy chains, as well as intrachain disulfide bonds within the constant or variable domains. These bonds are susceptible to various modifications, including reductive cleavage, trisulfide formation, cysteinylation, and disulfide scrambling, due to their relatively low dissociation energy. Such alterations can affect antigen binding, Fc effector functions, and renal clearance, and may result in aggregation, raising safety concerns. While Protein A purification is commonly used, it faces challenges such as incomplete host cell protein removal and aggregation. We have developed new strategies to enhance mAb properties and minimize disulfide bond modifications. To assess these strategies compared to the conventional method, we extensively applied multi-attribute mass spectrometry.

Methods

This study explores a modified purification strategy aimed at reducing disulfide bond-related modifications in mAbs. The effectiveness of this strategy is assessed by evaluating disulfide linkages and maintaining the structural integrity and quality of therapeutic mAbs. Peptide mapping using liquid chromatography-mass spectrometry (LC-MS) with Multi-attribute monitoring (MAM) approach was employed, specifically targeting cysteine-stressed and non-stressed IgG1 antibodies to identify potential disulfide bond alterations. To support the LC-MS findings, additional analytical techniques were applied, including size exclusion chromatography (SEC) for protein size distribution, differential scanning calorimetry (DSC) for thermal stability assessment, and polyacrylamide gel electrophoresis (SDS-PAGE) for examining protein purity and aggregation. These complementary methods provided a comprehensive evaluation of the purification strategy's impact on mAb quality and disulfide bond modifications.

Results

Reduced and non-reduced peptide mapping fragment data, when analyzed using the MAM module, enables the simultaneous assessment of various critical quality attributes (CQAs). The results for in-house synthesized mAbs produced using both methods revealed that both samples had similar intact mass and exhibited similar glycoform profiles, indicating consistent production and similar post-translational modifications.

Non-reduced peptide mapping demonstrated comparable trends in disulfide bond shuffling between the C-mAb (conventional method) and the M-mAb (modified method), suggesting that both samples underwent similar levels of disulfide bond rearrangements. However, when subjected to reduced peptide mapping, a notable difference emerged. The C-mAb showed significantly higher levels of deamidation, suggesting that the modified method was effective in reducing such modifications, which are often associated with reduced stability and altered functionality.

Further analysis using SDS-PAGE indicated that the C-mAb was more prone to disulfide bond-related modifications, particularly under cysteine stress conditions. The M-mAb, on the other hand, showed fewer signs of degradation and aggregation, confirming that the new purification strategy effectively minimized disulfide bond-related alterations. SEC-HPLC provided additional confirmation, where the M-mAb exhibited a more stable profile compared to the C-mAb, with fewer aggregates observed.

Thermal stability assessments through DSC revealed that the M-mAb exhibited a higher melting temperature (Tm) and molar heat capacity, both with and without cysteine stress. This indicates that the M-mAb possesses superior stability, likely due to fewer disulfide bond modifications. In contrast, the C-mAb showed a lower Tm and reduced stability, further highlighting the benefits of the modified purification strategy in enhancing mAb quality. Overall, these preliminary results suggest that the modified purification method significantly improves the stability and integrity of mAbs, reducing disulfide bond-related modifications.

Novel Aspect

The novel process modification enhances monoclonal antibody stability, utilizing MAM techniques to precisely analyze and reduce disulfide bond modifications.

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Enhanced-Sensitivity Profiling of Natural Products from TLC Plates Using a Facile Graphite-Based LA-DART-MS Platform

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The isolation and analysis of natural products are crucial to ensure their safety and efficacy, particularly in natural medicine. Thin-layer chromatography (TLC), with advantages in operational simplicity and visual results, is widely employed for natural product detection. Integrating established quality control methods like TLC with ambient ionization mass spectrometry allows targeted characterization of unknown components on TLC plates, markedly enhancing the accuracy and validity of natural products quality assessment. We present a novel graphite-assisted platform to improve the performance of laser ablation direct analysis in real-time mass spectrometry (LA-DART-MS) for TLC plates. This facile technique significantly enhances laser ablation efficiency, boosting MS signal intensity for key compound classes such as flavonoids, alkaloids, volatile oils, and organic acids. To further verify the applicability of this method, we applied this method to the analysis of active components in Chenpi and Qingpi citrus herbs, identifying 14 compounds directly from the TLC plate. When combined with multivariate statistical analysis, the data enabled clear differentiation of Citrus samples based on geographical origin, highlighting its utility for quality control. This work establishes graphite-assisted LA-TLC-DART-MS as a highly sensitive and broadly applicable method for the rapid screening and identification of compounds in complex natural product mixtures.

Keywords: Natural products; Thin layer chromatography; Direct analysis in real time mass spectrometry; Ambient ionization mass spectrometry; Quality control

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