3D Printed Immobilized pH Gradient (IPG) Carbon Microbead Arrays for Isoelectric Focusing Pre-Fractionation of Protein

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Abstract

Two-dimensional electrophoresis and multidimensional chromatography rely on an initial prefractionation step to reduce proteome complexity before detailed analysis. Isoelectric focusing (IEF) with immobilized pH gradient (IPG) gel strips is the most widely used approach for charge-based prefractionation but transferring focused proteins from gel strips into orthogonal or hyphenated analytical platforms can be labor-intensive and can lead to unacceptable sample loss when working with nanogram- to picogram-scale inputs. The suitability of existing bulk (microgram and above) prefractionation methods for single-cell or similarly low-input proteomics remains uncertain, motivating the development of new IEF formats that operate efficiently at small sample volumes.

Here we describe carbon microbead arrays as an alternative to conventional IPG gel strips for IEF prefractionation. Ampholyte-blended carbon microbeads with defined pH-gradient profiles (NanoPak-C-IPG) were synthesized using a microfluidic co-nozzle process and then assembled into two-dimensional (2D) and three-dimensional (3D) arrays by 3D printing. Each microbead functions as a localized immobilized pH-gradient site, enabling spatially organized, pI-directed protein prefractionation. We demonstrate proof-of-principle IEF prefractionation of a three-protein mixture spanning acidic, neutral, and basic isoelectric points, followed by reverse-phase HPLC separation of the eluted fractions on NanoPak-C all-carbon columns.

Under the conditions tested, NanoPak-C-IPG microbeads achieved extraction efficiencies greater than 90% for 100 µm beads and greater than 85% for 40 µm beads while maintaining pI-selective fractionation across different sample volumes. These results suggest that 3D printed IPG carbon microbead arrays provide a structurally and chemically tunable IEF platform that may better support low-input proteomics workflows and integration with downstream analytical techniques than conventional IPG gel strips.

1. Introduction

The complexity of the proteome spans several orders of magnitude in dynamic range and contains proteins that differ subtly in charge, size, and hydrophobicity [1, 2]. Direct analysis of unfractionated lysates often results in undersampling of low-abundance and difficult-to-ionize species. This challenge has motivated the use of prefractionation strategies (**Figure 1**) to reduce sample complexity before detailed mass spectrometric or electrophoretic analysis [3]. Two-dimensional electrophoresis (2DE) is a well-established approach in which proteins are first separated according to isoelectric point (pI) by isoelectric focusing (IEF) and then by molecular weight using SDS–PAGE [3].

Polyacrylamide gels containing a covalently bound pH gradient called immobilized pH gradient (IPG) gel strips are the most common platform for IEF in 2DE and related workflows [3]. These gel strips eliminate the gradient drift and instability associated with traditional carrier ampholyte systems. This elimination enables high-resolution prefractionation by overall protein charge.

During use, dried IPG strips are rehydrated in a solution containing urea, detergent, and the protein sample. Upon application of an electric field, each protein migrates through the gradient until it reaches its isoelectric point (pI), where the net charge of the protein is zero. The protein focuses at pI.

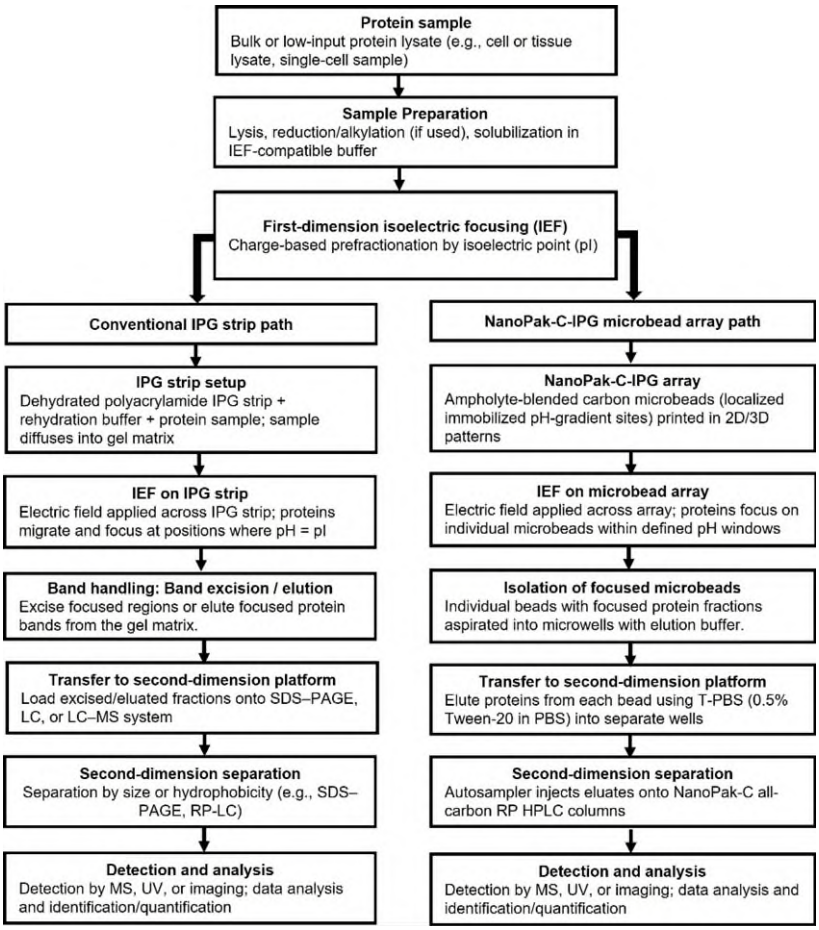

Figure 1. Schematic of a typical proteomics workflow highlighting the role of isoelectric focusing (IEF) pre-fractionation in reducing sample complexity before downstream separation and detection. The diagram illustrates sample preparation, IEF prefractionation, second-dimension separation (e.g., SDS–PAGE or LC), and mass spectrometric analysis.

While IPG strips are highly effective for bulk (microgram and above) samples, their suitability for nanogram- to picogram-scale inputs, such as single-cell proteomes, is not fully established. In particular, the elaborate sample transfer procedures required to move pre-fractionated protein bands from IPG strips into orthogonal or hyphenated analytical platforms introduce significant opportunities for sample loss. These handling losses are increasingly unacceptable as workflows shift towards low-input and single-cell applications, where every nanoliter of sample is valuable [4].

To address these challenges, IEF methods are being explored that operate efficiently at minimal sample amounts, retain compatibility with microfluidic and microwell formats, and simplify integration with downstream analytical techniques [5].

In this study, we describe the development of 3D printed IPG carbon microbead arrays as an alternative IEF prefractionation platform. Using a microbead synthesis setup, we first manufacture ampholyte-blended carbon microbeads with distinct pH-gradient profiles (NanoPak-C-IPG). We then employ these custom 3D-printable stationary-phase materials and additive manufacturing tools to build pH-tunable IPG carbon microbead arrays. Finally, we present proof-of-principle IEF fractionation and reverse-phase HPLC separation of proteins to illustrate the capabilities and potential advantages of this platform.

2. Experimental

2.1 Synthesis of NanoPak-C-IPG microbeads

A viscous suspension was prepared containing natural micrographite powder, a liquid crosslinker (binder), and small quantities of ampholytes (weakly acidic and basic buffering

groups) . This suspension was passed through a microfluidic co-nozzle device to generate microdroplets containing the starting materials. The droplets were collected and cured at 90 °C for 12 hours to achieve in situ binding and form solid microbeads. The resulting NanoPak-C-IPG microbeads were washed thoroughly to remove unreacted components and dried prior to use.

The synthesis of the NanoPak-C microbeads for reverse-phase chromatography is described elsewhere. These microbeads were subsequently packed into columns and served as the all-carbon stationary phase for RP-HPLC analysis.

2.2 Fabrication of 3D-printed microbead chains and arrays

To fabricate 3D-printed arrays, microbead ink was prepared by homogenizing NanoPak-C-IPG microbeads with tetradecane (non-polar solvent) and a small amount (0.01 wt%) of benzoyl peroxide (BP) as a radical initiator at 2,500 rpm for 5 minutes. BP enabled crosslinking between adjacent microbeads and between the beads and the substrate surface during curing.

An off-the-shelf micro-extrusion bioprinter was used to deposit the microbead ink onto substrates in programmed patterns. We used microbeads with diameters of 40 µm and 100 µm as starting materials to develop methods for 3D printing beads into 2D and 3D periodic structures. UV curing was then applied to join the microbeads into continuous chains and to anchor them to the substrate in both 2D and 3D configurations. The printing setup provided flexibility to create complex patterns, including arrays with different bead spacings, geometries (e.g., square and circular arrangements), and numbers of layers.

2.3 Isoelectric focusing (IEF)

The printed NanoPak-C-IPG substrate was placed in a custom IEF holder device fabricated from aluminum oxide ceramic. This holder accommodated a single fused silica substrate and incorporated channels for hydration buffer, a movable sample holder to interface with the printed microbead channels, and electrode clips to fit substrates of variable length. The channels in the holder were filled with hydration buffer, and protein samples were introduced into the IEF system (**Figure 2**).

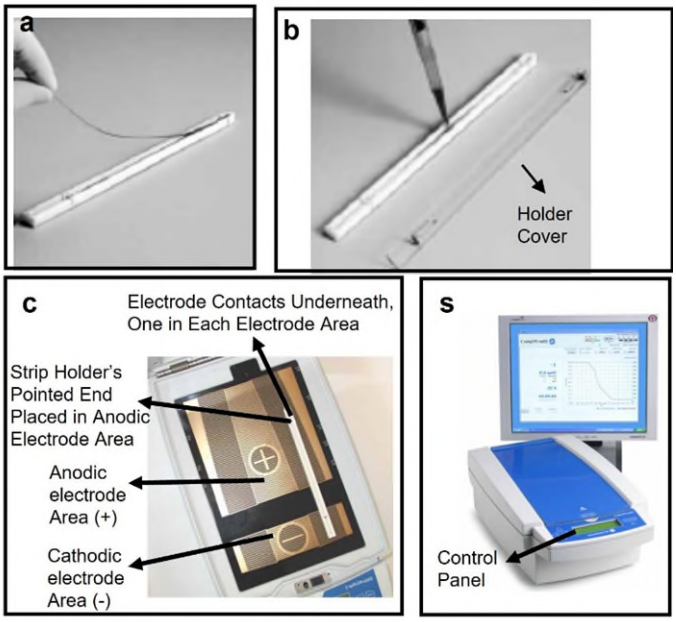

Figure 2. Digital image of IEF components. (a) NanoPak-C-IPG on silica substrate placed in the aluminum oxide holder, (b) filled with hydration buffer solution. (c) Internal components and (d) control panel of the IEF system.

IEF was performed according to the instrument manufacturer's protocol. After the focusing step, each microbead was aspirated individually using a 10 µL pipette tip and transferred into an individual microwell containing 5 µL of elution buffer. Proteins fractionated on the surface of each microbead were eluted with 0.5% (v/v) Tween-20 in phosphate-buffered saline (T-PBS).

2.4 RP-HPLC analysis

An autosampler injected 1 µL of each eluted protein fraction from the microwell plate into an

HPLC system for analysis. NanoPak-C all-carbon columns (150 × 4.6 mm, 6 µm) were used as the reverse-phase stationary phase. The mobile phases were: Phase A, 0.1% trifluoroacetic acid (TFA) in water; and Phase B, 0.1% TFA in acetonitrile.

The following linear gradient was applied: 0–6 min, 35–75% B; 6–7 min, 75–35% B; followed by re-equilibration to the initial conditions to give a total run time of 10 min. The flow rate was 1 mL/min. The injection volume was 20 µL. UV absorbance was monitored at 280 nm. Key chromatography performance indicators such as plate number/m, resolution, and separation efficiency were tabulated.

2.5 Probe analytes

A mixture of three proteins with known isoelectric points—cytochrome c, hemoglobin, and phycocyanin—was used as a model analyte system. Each protein was prepared at a concentration of 50 µg/mL in deionized water, and the mixture was adjusted to a total concentration of approximately 1 ppm (1 µg/mL) for IEF experiments. NanoPak-C-IPG microbead arrays were prepared with three overlapping pH-gradient ranges: 4–6, 6–8, and 8–9, corresponding to the pI ranges of phycocyanin (pI 4–5), hemoglobin (pI 7–8), and cytochrome c (pI 8–9), respectively (**Table 3**).

2.6 Statistical analysis

Single-factor analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test, was used for statistical analysis. Sample sizes for extraction experiments were based on power calculations assuming statistical power above 80% and a minimum detectable effect size corresponding to a 20% difference. All tests were conducted at a 95% confidence level ($p < 0.05$).

3. Results and Discussion

3.1 Synthesis and characterization of NanoPak-C-IPG microbeads. Representative bright-field optical and scanning electron microscopy (SEM) images of 40 µm NanoPak-C-IPG microbeads are shown in **Figure 3a–b**, demonstrating that the beads are spherical and monodisperse within a standard deviation of approximately 25%. **Table 1** summarizes the technical specifications of the NanoPak-C-IPG microbeads. **Table 2** shows the pH gradients achieved as a function of ampholyte concentration. The microbeads are chemically stable and exhibit robust structural integrity. The

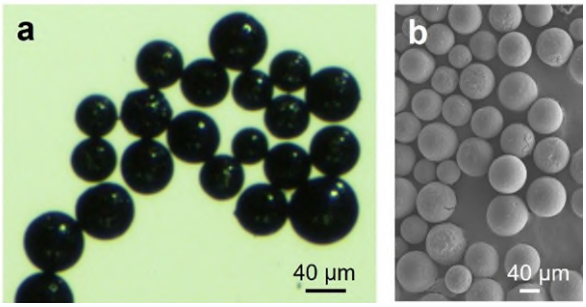

Figure 3. Representative (a) bright-field optical microscopy, and (b) scanning electron microscopy (SEM) images of microbeads. illustrating spherical morphology and size uniformity.

Table 1. Technical Specifications of NanoPak-C-IPG microbeads

Average Diameter	40-10000 µm (tunable)
Size Distribution	25% Standard Deviation
Surface area	200 m²/g
Porosity	10%
Pore Diameter (Median)	50 nm
Total moisture	0-5%
pH limit stability	0-14
Temperature limit stability	130 °C

Table 2. pH Gradient Achieved for Different Ampholyte Concentration

Ampholyte Concentration (Weight%)	pH Gradient Range
1	4-5
2	5-6
3	6-7
4	7-8
5	8-9

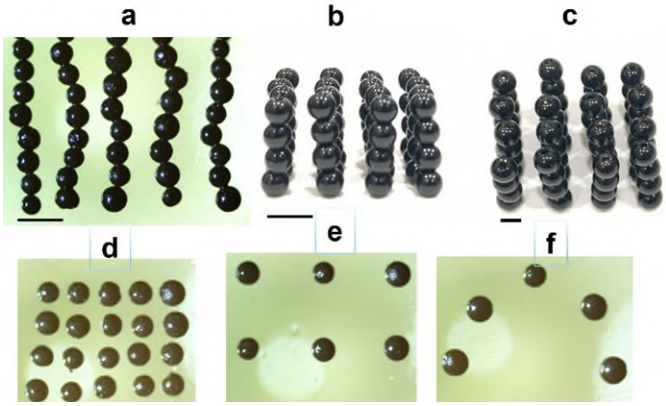

Figure 4. (a–c) Optical micrographs of 1D and 2D NanoPak-C-IPG microbead chains after UV curing, showing continuous bead–bead and bead–substrate connections. (d–f) Examples of 2D and 3D periodic microbead arrays with different spacings and geometries (square and circular), illustrating the design flexibility of the printing process.Size bar = 100 µm.

characterization results indicated that NanoPak-C-IPG microbeads are mechanically stable, chemically customizable support for immobilized pH gradients suitable for repeated handling and integration into printed microarray architectures.

3.2 Fabrication and characterization of 3D-printed microbead chains and arrays. **Figure 4** shows 3D-printed NanoPak-C-IPG microbead chains and arrays produced using the micro-extrusion bioprinter. UV-curing of the tetradecane/benzoyl peroxide-based microbead ink resulted in continuous 1D chains and ordered 2D and 3D periodic structures in which individual beads are joined to each other and to the substrate (**Figure 4a–c**). The fabrication protocol permits variation in bead spacing and arrangement, enabling square, circular, and other custom array formats (**Figure 4d–f**).

These configurations provide a flexible design space to explore 1D, 2D, and 3D architectures that are not accessible with conventional IPG or SDS–PAGE gels, nor with most capillary electrophoresis devices. Such architectures may ultimately support new classes of protein prefractionation devices tailored for single-cell and single-molecule proteomics, where microscale compartmentalization and minimized dead volume are critical.

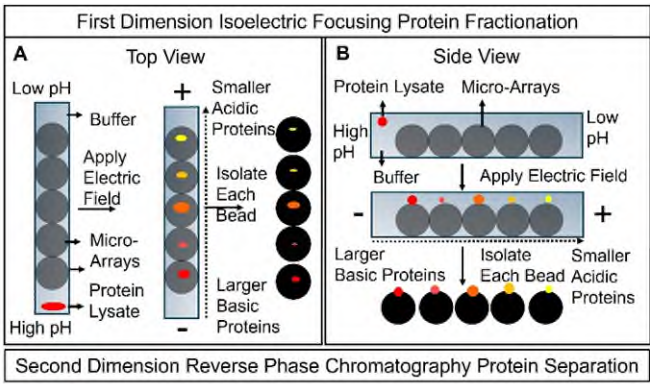

Figure 5. Depiction of the 2D protein separation workflow. (A) Top & (B) side view of the 2D microbead chain array that exploits isoelectric focusing (IEF) principles. Each microbead has a specific pH range and the chain array together exhibits a pH gradient. After IEF procedure, each microbead can be isolated and protein fractionated on its surface can be removed or interfaced with chromatography column that facilitates further second dimension reverse phase separation of the fractionated proteins.

3.3 Two-dimensional protein separation. **Figure 5** illustrates the two-dimensional protein separation setup employing NanoPak-C-IPG microbead arrays for first-dimension IEF and NanoPak-C all-carbon microbeads for second-dimension RP-HPLC. Each carbon microbead in the IPG array acts as a pH-tunable immobilized pH-gradient site with local buffering capacity and control over protein prefractionation reproducibility and resolution. The bead size, surface area, and ampholyte (comprising acrylamide monomers) together govern the immobilization and focusing of protein fractions at defined pH ranges.

After IEF, each microbead containing immobilized proteins can be isolated and its bound proteins eluted in a single step, significantly reducing handling time and complexity relative to traditional strip excision and elution workflows. The pre-fractionated proteins are then injected into HPLC columns packed with 6 µm All-Carbon microbeads, where they are separated by hydrophobicity and quantified by UV detection at 280 nm (**Figure 6a**).

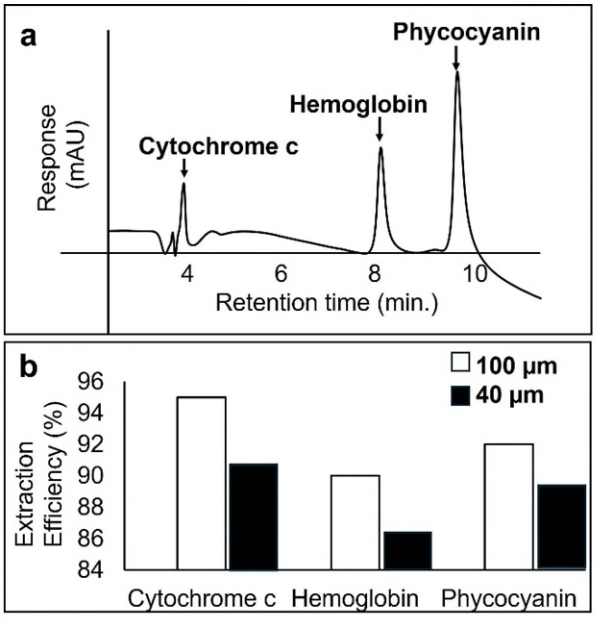

Figure 6. (a) Representative RP-HPLC chromatograms of the three-protein mixture (phycocyanin, hemoglobin, cytochrome, and (b) IEF extraction efficiencies (amount ratio of extracted protein: loaded protein) for 100 µm and 40 µm microbead arrays. Each experiment was repeated 3 times.

We performed IEF experiments with the three-protein mixture (phycocyanin, hemoglobin, and cytochrome c) across three overlapping pH ranges (4–6, 6–8, and 8–9) (**Table 3**) using NanoPak-C-IPG microbeads of 40 µm and 100 µm diameters.

Table 3. Isoelectric focusing (IEF) Standard Proteins

Proteins	*PI*
Cytochrome c	8.6-9
Human hemoglobin	7.1-7.5
Phycocyanin	4.4-4.8

We first determined the breakthrough concentration, a critical parameter defined as the sample concentration loaded onto the microbead substrates to obtain 99–95% retention [6]. The breakthrough concentration was the protein volume that does not lead to an increase in fractionated protein by IEF and separated protein by RP HPLC. It was determined by increasing protein standard volumes 10X at a time from 1 nL to 1 µL. Next, the extraction capacity, defined as the maximum extractable protein concentration, was determined. Based on the breakthrough concentration studies, sample volumes of 10 µL were used for 100 µm beads and 1 µL for 40 µm beads at a total protein concentration of 1 ppm. The extraction efficiency of the IEF step, defined as the fraction of loaded protein recovered in the eluted and chromatographically detected fractions, exceeded 90% for 100 µm beads and 85% for 40 µm beads (**Figure 6b**). These results indicate that NanoPak-C-IPG arrays can fractionate protein mixtures according to pI over different sample volumes without compromising extraction performance.

The NanoPak-C-IPG microbead arrays allow open format configurations. Such configurations address the macroscopic/microfluidic (“world-to-chip”) interface problem which often complicate the transfer of nanoliter-scale samples from enclosed microfluidic channels. By retaining a microwell plate–like form factor while reducing feature dimensions and enabling spatial organization, the arrays are compatible with single-cell and low-volume sample isolation methods, including cell suspensions, tissue biopsies, and nanopipette-based sampling.

The customizable microbead diameters and scalable array configurations should allow processing of both very small (sub-nanomolar) and larger lysate volumes. Smaller beads (e.g., 40 µm) can accommodate sub-nanomolar sample amounts, whereas larger beads (e.g., 400 µm) can hold higher-nanomolar lysates. Clustering multiple beads can further increase capacity as needed. The adoption of 3D printing technology enables rapid prototyping and fabrication of bespoke devices for biological sample processing, potentially enabling case-by-case device design and manufacturing in chromatography and related fields [7, 8].

A current workflow limitation is the manual transfer of microbeads between the IEF and chromatography steps, which adds labor and potential variability. A fully integrated setup incorporating 3D-printed NanoPak-C HPLC columns is planned. Its separation performance remains to be evaluated.

4. Conclusions

We have developed 3D printed NanoPak-C-IPG carbon microbead arrays as an alternative IEF prefractionation platform for protein analysis. The pH-tunable carbon microbead arrays act as spatially organized immobilized pH-gradient sites. Proof-of-principle experiments with a three-protein model mixture demonstrate that these arrays can perform pI-directed prefractionation across different sample volumes and that the focused proteins can be recovered and resolved in a second-dimension RP-HPLC step using NanoPak-C All-Carbon columns.

The IEF results (extraction efficiencies (>90% for 100 µm beads and >85% for 40 µm beads), along with open, microwell-compatible device format lay the foundation for further development

of the NanoPak-C-IPG arrays for low-input and single-cell proteomics workflows where sample handling losses and format compatibility are critical constraints. The combination of tunable bead size, adjustable pH gradients, and flexible 3D-printed architectures offers a design space that is difficult to access with conventional IPG strips, SDS–PAGE gels, or capillary IEF devices.

Our future efforts focus on quantitative benchmarking against commercial IPG strips, expansion to more complex protein mixtures, integration of automated bead handling, and on-chip RP-HPLC. These advancements aim to create a fully integrated, low-input 2D separation platform as an enabling technology for next-generation proteomics and other charge-based bioanalytical applications.

Acknowledgements. We thank Dr. Michael Parente for the original microbead array development, and methods lab team at Millennial Scientific. The National Institutes of Health (1R43TR005777-01A1) supported the work.

Note. A preprint manuscript version of this white paper is under preparation. It has not yet undergone peer review.

References

[1] H. Specht, N. Slavov, Transformative Opportunities for Single-Cell Proteomics, Journal of Proteome Research 17(8) (2018) 2565-2571.

[2] R.T. Kelly, Single-Cell Proteomics: Progress And Prospects, Molecular & Cellular Proteomics 19(11) (2020) 1739-1748.

[3] K. Chandramouli, P.-Y. Qian, Proteomics: Challenges, Techniques And Possibilities To Overcome Biological Sample Complexity, Human Genomics And Proteomics : Hgp 2009 (2009) 239204.

[4] K. Stejskal, J. Op De Beeck, G. Dürnberger, P. Jacobs, K. Mechtler, Ultrasensitive Nanolc-Ms Of Subnanogram Protein Samples Using Second Generation Micropillar Array Lc Technology With Orbitrap Exploris 480 And Faims Pro, Analytical Chemistry 93(25) (2021) 8704-8710.

[5] I. Rodríguez-Ruiz, V. Babenko, S. Martínez-Rodríguez, J.A. Gavira, Protein Separation Under A Microfluidic Regime, Analyst 143(3) (2018) 606-619.

[6] R. Ferrer, J. Beltran, J. Guiteras, Mathematical Procedure For The Determination Of The Breakthrough Volumes Of Polycyclic Aromatic Hydrocarbons (Pahs), Analytica Chimica Acta 346(2) (1997) 253-258.

[7] M.T.W. Hearn, Trends In Additive Manufacturing Of Chromatographic And Membrane Materials, Current Opinion In Chemical Engineering 18 (2017) 90-98.

[8] C. Fee, 3d-Printed Porous Bed Structures, Current Opinion In Chemical Engineering 18 (2017) 10-15.