analytical chemistry

pubs.acs.org/ac Article

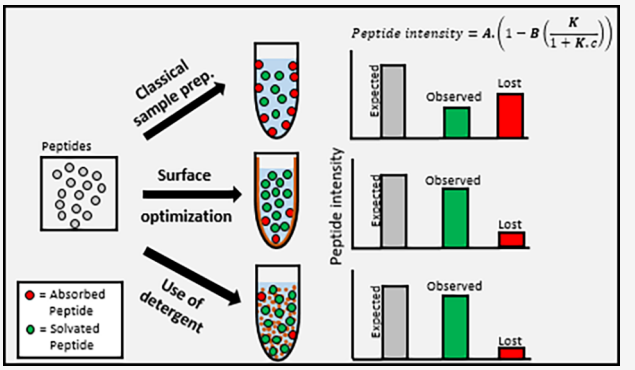
SIGNIFICANT IMPACT OF CONSUMABLE MATERIAL AND BUFFER COMPOSITION FOR LOW-CELL NUMBER PROTEOMIC SAMPLE PREPARATION

Christopher Kune,* Sylvia Tielens, Dominique Baiwir, Maximilien Fléron, Denis Vandormael, Gauthier Eppe, Laurent Nguyen, and Gabriel Mazzucchelli

Cite This: *Anal. Chem.* 2025, 97, 3836−3845 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Proteomics, essential for understanding gene and cell functions, faces challenges with peptide loss due to adsorption onto vial surfaces, especially in samples with low peptide quantities. Using HeLa tryptic digested standard solutions, we demonstrate preferential adsorption of peptides, particularly hydrophobic ones, onto polypropylene (PP) vials, leading to nonuniform signal loss. This phenomenon can alter protein quantification (e.g., Label-Free Quantification, LFQ) if no appropriate data processing is applied. Our study is based on understanding this adsorption phenomenon to establish recommendations for minimizing peptide loss. To address this issue, we evaluated the nature of surface material and buffer additives to reduce peptide-surface noncovalent binding. Here, we report that using vials made from polymer containing polar monomeric units such as poly(methyl methacrylate) (PMMA) or polyethylene terephthalate (PET) drastically reduces the hydrophobic peptide loss, increasing the global proteomics performance (4-fold increase in identified peptides for the single-cell equivalent peptide content range). Additionally, the incorporation of nonionic detergents like poly(ethylene oxide) (PEO) and n-Dodecyl-Beta-Maltoside (DDM) at optimized concentrations (0.0001% and 0.0075%, respectively) improves the overall proteomic performance and consistency, even across different vial materials. Implementing these recommendations on 0.2 ng/μL HeLa tryptic digest results in a 10-fold increase in terms of peptide signal. Application to True Single-Cell sample preparation without specialized instrumentation dramatically improves the performance, allowing for the identification of approximately 650 proteins, a stark contrast to none detected with classical protocols.



INTRODUCTION

Proteomics is a scientific discipline dedicated to the comprehensive study of proteins that are essential for understanding the functions of genes and cells. This interdisciplinary field encompasses a variety of techniques, such as imaging, array experiments, and genetic assays. Mass spectrometry (MS) has proven to be a powerful analytical tool for studying complex protein samples, with the current trend being the integration of liquid chromatography (LC) separation with electrospray ionization mass spectrometry.[1,2] Moreover, recent technological improvements in MS have significantly increased its sensitivity and ability to downsize proteomic workflows. These advancements have enabled analyses of samples containing a low amount of biological material (i.e., low amounts of proteins). Such capabilities are crucial for exploring specific areas like rare cell subpopulations,[3,4] conducting spatial-omics on biological tissues,[5,6] and investigating the proteome at the single-cell level.[7−10]

Scaling down biological material quantities for proteomic analysis often results in a significant decrease in the performance. This reduction is primarily attributed to limitations in the sensitivity of instruments (limits of detection and limits of quantification) and the loss of sample material during the preparation and purification processes.[11] The sensitivity of instruments being dependent on the equipment available on the market and the critical importance of sample preparation in enhancing the overall quality of the analysis necessitates more in-depth investigation. Optimized sample preparation approaches for samples containing low amounts of biological material, such as mPOP protocol, aim to eliminate sample cleaning, nonessential steps in the preparation process and sample transfer.[12] The mPOP protocol has been integrated

Received: July 17, 2024
Revised: February 3, 2025
Accepted: February 5, 2025
Published: February 11, 2025

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

into the Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS) workflow developed by the Slavov's group for single-cell proteomics analysis. This workflow is based on the use of Tandem Mass Tag (TMT) and a carrier channel to amplify peptide signals and counteract the detection limits of current instruments,[9,10] therefore allowing the identification and quantification of thousand proteins from a single-cell.

Even when these approaches are employed, material loss due to adsorption on surfaces can become critical when processing such extremely low amounts of starting biological material. Indeed, nonspecific protein or peptide adsorption is already known to occur on solid surfaces (e.g., pipet tips, vials and instrumentation parts) through noncovalent interactions (e.g., electrostatic, hydrophobic) and depend on experimental conditions (e.g., peptide properties, physical state of the surface and sample environmental properties).[13] If adsorption phenomenon becomes not negligible, peptide intensity signals in LC-MS analysis do not show a linear correlation with the initial concentration in vials.[14] As an example, Law and Shih reported that the calcitonin adsorption on soda lime silica glass is dependent on the concentration, with adsorption isotherms following the Langmuir and Freundlich models, depending on the solution pH.[15] The prediction of peptide adsorption on specific surfaces, based on their physicochemical properties is challenging since it results from a complex interplay of the properties of the surface (e.g., nature, shape, topology), the protein/peptide (e.g., hydrophobicity, charge, residue distribution, intramolecular and intermolecular interaction, conformation) and the buffer solvent (e.g., solvation force, composition, pH, temperature).[16−20] Therefore, selecting the appropriate vial material and sample preparation buffer is crucial when handling samples with low protein concentrations.[19] Various strategies have been developed to minimize the adsorption of proteins or peptides onto surfaces. These strategies mainly involve addition of exogenous protein-rich sample (e.g., bovine serum albumin, BSA),[19,21] organic solvent (e.g., dimethyl sulfoxide or acetonitrile),[22] or surfactant agents.[23−28] The use of organic solvents can influence the binding of hydrophilic peptides on the LC column[22,29] while incorporating external proteins into samples with low protein content is suboptimal, as it may lead to ionization competition. Therefore, the use of adapted surfactant agents is a promising approach for enhancing low-cell number proteomic performance. With the objective of eliminating cleaning steps, the surfactant agent must be compatible with LC-MS without interfering with the peptide separation and ionization. Among the compatible surfactant agents, n-Dodecyl-Beta-Maltoside (DDM)[27,30] and poly(ethylene oxide) (PEO)[25] have already been reported as effective additives reducing peptide adsorption on the vial surface.

Nowadays, minimizing peptide adsorption on surfaces has emerged as a priority for manufacturers of laboratory consumables for low-cell number proteomics as supported with innovations such as the QuanRecovery vials from Waters (UK) and the proteoCHIP from Cellenion instrument (France).[7] Another notable approach, called the nanoPot workflow,[31] has emerged from this need to reduce peptide loss due to surface adsorption. In that workflow, all sample preparation steps occur within a droplet, minimizing the number of molecular interactions with the surface and reducing the number of sample volumes. However, this sample preparation procedure requires dedicated instruments for precise submicroliter liquid dispensing.

In this study, we evaluated the effects of surface properties (i.e., the nature of the polymeric material) and the presence of surfactants on peptide loss due to surface adsorption. We report some recommendations and strategies to minimize peptide loss when scaling down protein starting amounts from standard proteomic analysis to low-cell-number and single-cell samples. These recommendations are based on (1) a one-pot strategy (designed to minimize sample transfers and contacts with laboratory consumables, including vials and tips),[32] (2) the use of adapted surface materials, and (3) the use of surfactant agents to reduce peptide affinity for surfaces.

MATERIAL AND METHOD

Single cell type sample: HeLa Tryptic digest peptides. HeLa tryptic digest standard solution from ThermoFisher Scientific (Pierce HeLa Protein Digest Standard, 88328) has been considered as a model for monitoring peptide binding on surfaces. HeLa peptide solutions from 0.2 ng/μL to 10 ng/μL have been prepared from a stock solution of 500 ng/μL using 0.1% TFA. Peptide solutions containing PEO or DDM have been prepared by spiking PEO or DDM stock solution at 0.1% (w/w) in Milli-Q water (see Supporting Information for detailed preparation protocol).

Cell Sample Preparation. HeLa cells (ATCC) were cultured to prepared real proteomics samples from one cell or ten cells using PEO, DDM or without buffer additives (see Supporting Information for detailed information and protocol)

Vial Design and Production. Vials have been molded by injection in different polymeric materials to investigate the impact of surface nature on proteomic performance. For this study, Poly(methyl methacrylate) (PMMA), polycarbonate (PC), polyethylene terephthalate (PET), Polyvinylidene fluoride (PVDF), cyclic olefin polymer and copolymer (COP and COC, respectively), and polypropylene (PP) were evaluated. These materials are all compatible with a 1% TFA solution and cover different polymer properties such as hydrophobicity and polarity. The compatibility with other commonly used solvents has also been investigated. It is important to note that some of these polymers, such as PMMA and PET, are not compatible with solvents containing acetonitrile (ACN) or methanol, which are primarily used in multiplex proteomic approaches. 0.3 mL PP vial from VWR (Cat. No. 548−0120), 1.5 mL Total Recovery Glass vial from Waters (Cat. No. 186005663CV), and 0.3 mL QuanRecovery PP vial from Waters (Cat. No. 186009186) have been evaluated as commercial vials. List and pictures of these vials are reported in Supporting Information, table S1 and Figure S1, respectively.

Liquid Chromatography−Mass Spectrometry. Peptide solutions were injected on an Acquity UPLC MClass liquid chromatography system from Waters (UK) connected to a timsTOF Pro2 or a timsTOF SCP instrument from Bruker (Bremen, Germany). Data dependent acquisition (DDA) analyses were performed on the timsTOF Pro2 for the analysis of 10 ng peptide samples, while data-independent acquisition (DIA) analyses were performed on the timsTOF SCP for the analysis of 0.2 ng peptide samples. Detailed LC-MS instruments configuration and settings are reported in Supporting Information.

Data Analysis. Identification and quantification of peptides and proteins have been computed on FragPipe 20.0 and DIA-NN 1.8.2 for DDA and DIA data, respectively, with match-between-run (MBR). Swiss-Prot reviewed human protein

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

database (FASTA file generated from UniProt) has been used for the protein identification on DDA data. A spectral library has been generated for DIA analysis from the analysis of 200 ng of HeLa tryptic digest by DDA. Python 3.11 scripts (involving Pandas, Scipy, Numpy, and Matplotlib libraries) have been developed to monitor peptide/protein intensities or counts in function of their properties (e.g., retention time, mass, and charge) or experimental conditions (e.g., the nature of the vial, concentration in vial, and buffer composition) and report 1D or 2D distribution plots (i.e., histogram or colored-based heatmaps). These scripts use the outputs generated by FragPipe 20.0 or DIAN-NN 1.8.2.

RESULTS AND DISCUSSION

Understanding Peptide Loss Attributable to Surface Binding. To evaluate the impact of nonspecific adsorption on the inner surface of vials and LC system, we monitored the total intensity of identified peptides from a consistent amount of injected peptide while varying the peptide concentration. This approach was selected because peptide loss due to surface adsorption depends on the concentration and should not be influenced by the injected quantity in the LC instruments. Therefore, various solutions of standard HeLa tryptic digest with concentrations varying from 1.1 to 20 ng/μL were prepared in commercially available polypropylene vials from VWR, which are widely used in LC-MS proteomics. To ensure constant surface exposure regardless of the peptide concentration, a fixed final volume of 20 μL was employed. For each sample, the injection volume was set to correspond to an expected peptide quantity of 10 ng (i.e., injection volumes ranging from 9 to 0.5 μL for peptide concentrations of 1.1 to 20 ng/μL, respectively). Notably, 10 ng of HeLa tryptic digest is roughly equivalent to the protein content of 50 HeLa cells, assuming an estimated protein content of approximately 0.2 ng per HeLa cell (as detailed in the Supporting Information). This quantity is aligned with the SCoPE-MS workflow and is compatible with the sensitivity of the Bruker timsTOF Pro2 instrument.

In the absence of peptide adsorption, consistent peptide intensity would be expected regardless of the peptide concentration as the same quantity of peptide is injected. However, as shown in Figure 1A, reducing the concentration of peptides while maintaining the same injected amount is associated with a decrease in peptide intensity. This finding indicates that the actual amount of peptide injected into the LC-MS system is less than the anticipated 10 ng, and this discrepancy relies on the effective concentration. This supports peptide loss due to adsorption phenomenon, as peptide adsorption on the surface reduces their effective concentration, subsequently reducing the injected peptide quantity. This phenomenon is particularly pronounced at lower concentrations, where a higher proportion of peptides is adsorbed. Interestingly, the observed decrease in total intensity with lower concentrations is also related to the peptide retention times, as shown in the color-coded heatmap in Figure 1B. This heatmap shows peptide intensities (log scale, represented by colors) as a function of peptide concentrations (*X*-axis) and retention times (ranging from 8 to 21 min on the *Y*-axis). In fact, the total peptide intensities significantly decline in the latter third of the LC gradient (particularly after 16 min), with a 5-fold decrease observed as the concentration drops from 20 to 1.1 ng/μL. Conversely, this decrease is approximately 3-fold in the middle third and minimal in the initial third. These

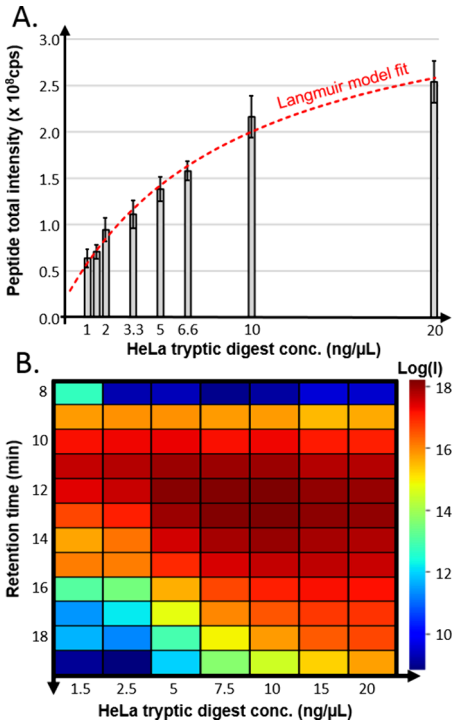


Figure 1. (A) Peptide total intensity (sum of intensities of identified peptide) from 10 ng of HeLa tryptic digest solutions analyses with concentration ranging from 1 to 20 ng/μL. Peptides were separated with a 15 min LC gradient and MS/MS spectra were acquired in DDA-PASEF mode (see Materials and Methods for more information). Peptides are identified and quantified by FragPipe with an FDR < 1% without any normalization on 3 replicates. Dash red line corresponds to a fit with a Langmuir model. (B) Distribution of peptide total intensity (expressed in Log_{10}) as a function of the retention time (*Y*-axis) and the HeLa tryptic digest concentration in PP vial.

observations suggest a potential correlation between peptide adsorption and peptide hydrophobicity. It appears that highly hydrophobic peptides are more prone to surface adsorption, particularly when using PP vials. This observation is consistent with the expected behavior for a PP surface, given the inherently hydrophobic nature of this material. Notably, polypropylene chains can engender van der Waals interactions with hydrophobic residues of peptides, which reduces their exposure to water. Consequently, hydrophobic peptides tend to be more readily adsorbed onto a hydrophobic surface. In contrast, hydrophilic peptides, being highly solubilized, exhibit a lower propensity for surface adsorption.

This discrepancy in adsorption behaviors leads to a nonuniform loss of peptides, which can have a significant impact on protein identification but also on protein quantification. Indeed, when aggregating peptides for protein quantification, such as in label-free quantification (LFQ), the intensities of hydrophobic peptides are considerably affected, resulting in greater signal variability. This could lead to misinterpretation in differential studies, even with the use of normalization algorithms, especially for low-abundance proteins containing the hydrophobic part (e.g., membrane proteins). This adsorption phenomenon seems to be especially critical in samples with low peptide concentrations (less than 20 ng/μL). The evolution of peptide total intensity as a function of peptide concentration aligns well with a simple implementation of the Langmuir adsorption model, modified

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

to establish a relationship between intensity and concentration (illustrated in eq 2). The Langmuir model equation used to establish this theoretical correlation is reported in eq 1, where $\%_{ads}$ is the equilibrium fractional occupancy, denoting the fraction of active sites occupied at equilibrium relative to the total number of available active sites, c is the molecule concentration, and K stands for the equilibrium constant governing the adsorption reaction. The adaptation of this equation to relate the measured total intensity of peptides (denoted ***Intensity***) with the concentration leads to eq 2 (explanation and demonstration of this equation are reported in Supporting Information); where α is the correlation factor between the infused quantity and the resulting measured intensity, which is related to a weighted-average ionization efficiency for the different peptides; $\boldsymbol{n_{expect\ peptide}}$ is the expected quantity of injected peptide (i.e., considering the peptide concentration multiplied by the injection volume) expressed in moles; $\boldsymbol{n_{site}}$ is the number of available adsorption site on the surface of the vial; and $\boldsymbol{V_{vial}}$ is the solution volume in the vial. It is important to note that $\boldsymbol{V_{vial}}$ was constant in this experiment. eq 2 can be parametrized to obtain the eq 4 that relates the measured intensities as a function of concentration while considering adsorption. This parametrization involves three parameters: A, B, and K. The definitions of A and B are reported in eq 3.

$$\%_{\text{ads}} = \frac{Kc}{1 + Kc} \tag{1}$$

$$\text{intensity} = \alpha n_{\text{expect peptide}}\left(1 - \frac{\frac{K}{1+Kc}\cdot n_{\text{site}}}{V_{\text{vial}}}\right) \tag{2}$$

$$\text{A} = \alpha n_{\text{expect peptide}} B = \frac{\text{n}_{\text{site}}}{V_{\text{vial}}} \tag{3}$$

$$\text{intensity} = \text{A}\left(1 - \text{B}\left(\frac{\text{K}}{1 + Kc}\right)\right) \tag{4}$$

The parameter A is related to the expected intensity in the absence of peptide adsorption, i.e., the expected total intensity of peptides from a 10 ng of HeLa tryptic digest injection in the case of this experiment. This parameter was constant for the HeLa solution series since the expected quantity of injected peptide was fixed at 10 ng. By definition, the parameter A is independent of the nature of the vial but depends on LC-MS instrumentation and methods (e.g., LC gradient, ionization source, ion optics in MS device) due to the factor α. The parameter B is directly related to the number of available adsorption sites and therefore strongly depends on the nature of the surface and the surface contact area. Here, parameter B is considered as constant for a given vial type and a given solution volume ($\boldsymbol{V_{vial}}$). Unlike parameter A, parameter B should be independent of the LC-MS instrument and method. Finally, parameter K represents a weighted-average equilibrium constant for all peptides since each peptide is defined with its own affinity for a given surface, depending, for example, on its hydrophobicity (among other factors), as discussed above. These three parameters were considered constant for each concentration range tested since the same LC-MS settings were used and all vials were filled with the same volume of the peptide solution (i.e., constant $\boldsymbol{V_{vial}}$). The strong correlation observed between experimental intensity and the Langmuir-type model (reported in Figure 1A) provides evidence that the signal loss is closely linked to peptide adsorption onto the surface.

Based on these observations, a key strategy to reduce peptide loss and increase the portion of injected peptide is to decrease the peptide's affinity for the vial surface and LC system, specifically by lowering the adsorption equilibrium constant (K). Indeed, K is a fundamental parameter that characterizes the extent of the adsorption of molecules on a solid surface. K is influenced by several factors, including the characteristics of the surface, the properties of the buffer, and the specific types of interactions involved (especially hydrophobic interactions in the case of PP vials). The nature of the surface where adsorption takes place is a key determinant of K. A surface with a strong affinity for the molecules will result in a higher K value, indicating a greater negative impact. Additionally, the composition of the buffer can substantially influence K. When the interactions between the molecules and buffer are highly favorable, this can lead to a situation where the liquid phase competes with the solid surface for interactions with the molecules. This competition may reduce adsorption on the solid surface because the buffer can effectively displace the molecules from the surface. There are therefore two strategies for reducing peptide affinity and improving the overall performance of the analyses by (1) adapting the nature of the vial surface to reduce its affinity for peptides; (2) modifying the solvent to increase its affinity for the peptide. These two strategies will be introduced and discussed in the following sections.

Evaluation of the Surface Nature Influence on Peptide Adsorption. Different vials with identical geometries but made from various raw materials were designed and manufactured to assess the influence of surface properties on peptide adsorption. In this experiment, a range of polymeric materials with varying degrees of hydrophobicity were examined to modulate hydrophobic interactions. These materials included Poly(methyl methacrylate) (PMMA), Polycarbonate (PC), polyethylene terephthalate (PET), Polyvinylidene fluoride (PVDF), cyclic olefin polymers and copolymers (COP and COC, respectively), and polypropylene (PP).

As for the PP commercial vial, the peptide losses have been evaluated on these polymeric materials by the evolution of the total peptide intensities as a function of the concentration for an expected injection of 10 ng of HeLa tryptic digest. Peptides were separated during a 15 min effective LC gradient and identified by DDA-PASEF on timsTOF PRO2. The results of this study are reported in Figure 2. The total peptide intensity evolution when using the PP vial from VWR is also reported in these results in white as a reference. Since the number of available sites of peptide adsorption could be influenced by the nature of the polymeric material, the peptide intensities reported in Figure 2A were normalized to the total peptide intensity of the 20 ng/μL samples for each polymeric material, respectively (i.e., total peptide intensities for the 20 ng/μL samples were set to 100% for each polymeric material, as shown for the 20 ng/μL results in Figure 2A). This allows evaluating the peptide resulting from the adsorption equilibrium constant (K) for the different polymeric material removing the contribution of the other material properties. As an initial observation from these results, the more hydrophobic polymers, such as COC, COP, and PP, result in a significant decrease in total peptide intensity with concentration compared to the more hydrophilic polymeric materials such

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

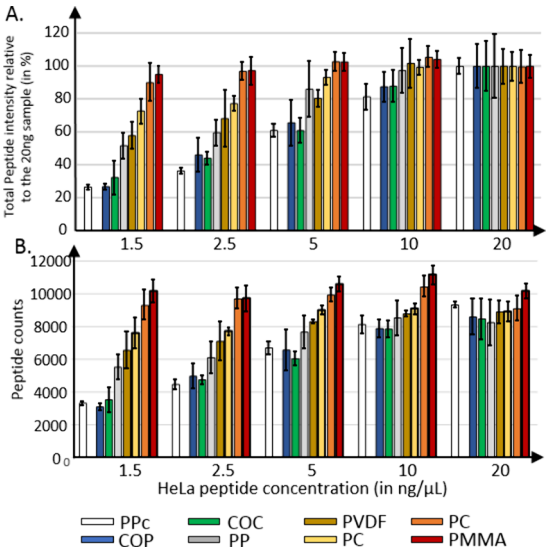


Figure 2. (A) Total peptide intensities expressed as percentages relative to the 20 ng samples, and (B) the peptide counts as a function of both the polymeric material nature of the vial and its concentration in LC-MS analysis, from a 10 ng injection. A range of polymeric materials, including COP (blue), COC (green), PP (gray), PVDF (brown), PC (yellow), PET (orange), and PMMA (red), was examined. Additionally, a PP commercial vial from VWR (PPc) was included, represented in white. Peptides were identified and quantified using FragPipe 20.0 software, with a false discovery rate (FDR) less than 1% and match-between-run (MBR). Relative total peptide intensities were calculated using MaxLFQ intensities provided by the FragPipe software. The reported values and error bars represent the averages and uncertainties derived from triplicate experiments. This supports that the use of low hydrophobic polymeric materials (i.e., more "polar" monomeric units) should enable the recovery of hydrophobic peptide signals when decreasing peptide concentration, thereby reducing the hydrophobic interactions with the surface.

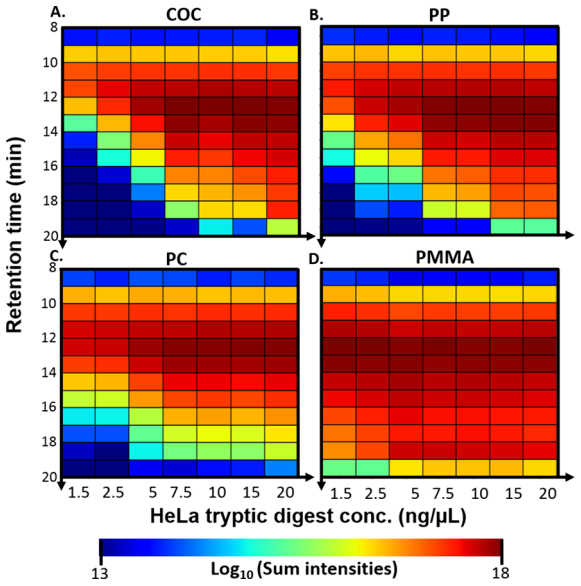


Figure 3. Peptide intensity distribution as a function of the retention time (*Y*-axis, in minutes) and the HeLa tryptic digest peptide solution concentration (*X*-axis, in ng/μL) for vials molded in COC (A), PP (B), PC (C) and PMMA (D). Peptide intensity values are logarithmically scaled ($\log_{10}$) and visualized using a color gradient, ranging from blue to red for, respectively, $\log_{10}$(intensity) of 13 to 18.

as polycarbonate (PC) and poly(methyl methacrylate) (PMMA). This suggests that using hydrophilic polymeric material allows for reducing hydrophobic interactions between hydrophobic peptides and the surface. Figure 2B reported the counts of peptide identification with FDR < 1%, with the concentration for the different nature of the polymeric material. As expected, the peptide counts are strongly correlated to the peptide total intensities. Figure 3 reports the peptide intensity heatmap for COC, PP, PC and PMMA as a function of the retention time (on the *Y*-axis) and the concentration (on the *X*-axis). These graphs show that the total intensities of hydrophobic peptides (i.e., higher retention times) gradually increase from the two most hydrophobic evaluated polymeric materials (i.e., COC and PP) to the two least hydrophobic (i.e., PC and PMMA) at low peptide concentration.

In the literature, it is well established that predicting peptide adsorption on specific surfaces based solely on their biochemical characteristics is barely reliable.[19,20] However, in the context of bottom-up proteomic samples, it becomes evident that the hydrophobicity of the peptide and the exposed surface are critical parameters that explain peptide loss. One might speculate that the primary challenge in predicting peptide affinity for a given polymeric surface lies in accurately determining its hydrophobicity. Indeed, the prediction of the hydrophobicity of a polymeric material solely based on its chemical composition could not be sufficient as the polymeric chains, structure, and geometry of the material can also influence its hydrophobic properties. Unfortunately, such detailed information is not always readily available from polymer or vial suppliers. To illustrate this point, even though the chemical composition of the monomeric units in PC suggests that this material should be more hydrophilic than PMMA, PC polymeric material exhibits slightly more pronounced hydrophobic interactions than PMMA, resulting in a slight decrease in total peptide intensities. As a recommendation, the chemical nature can be considered as a main criterion for a large selection of potential candidates, but experimental screening of the candidates must be performed to identify the best polymeric material for proteomic analysis on low-concentrate samples.

For practical applications, vial manufacturers typically limit their choices to some versatile polymer materials, such as PP or glass material. However, in situations where the resources for crafting custom vials are unavailable, researchers are compelled to work with materials that are readily accessible. It is essential, however, to be able to compare these commercial vials to select the most suitable option based on the proteomic applications. As an illustration, an experimental screening of the in-house manufactured vials with three commercially available vials: a PP vial from VWR (PPc), a PP QuanRecovery vial from Waters (QR), and a glass Total Recovery vial from Waters (TR) was conducted with the injection of 0.2 ng of HeLa tryptic digest peptide standard solution (concentration: 0.2 ng/μL) representing the expected single-cell equivalent tryptic digest peptides. This solution is used as a model to evaluate the influence of the vial nature on "true" single-cell proteomic performance. Peptides were separated during a 25 min effective LC gradient and identified by DIA-PASEF on timsTOF SCP. The outcomes of this study are depicted in Figure 4 and are compared to the PMMA vials. The proteomics performance was monitored based on the total

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

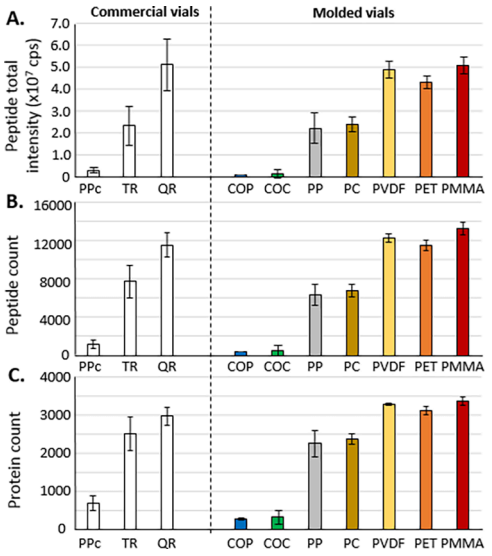


Figure 4. (A) Absolute total peptide intensities, (B) peptide counts, and (C) protein counts as a function of the commercial vial: PPc, TR, QR (white) and in-house manufactured vial: COP (blue), COC (green), PP (gray), PVDF (brown), PC (yellow), PET (orange), and PMMA (red) for 0.2 ng HeLa tryptic digest standard solution (0.2 ng/μL in 0.1% TFA). Data were acquired on the timsTOF SCP instrument in DIA-PASEF mode. Peptides were identified and quantified using DIA-NN 1.8.2 software, using an in-house spectral library with FDR < 1% and without MBR. The reported values and error bars are the average values and uncertainties derived from triplicate experiments.

peptide intensity expressed in counts. The values presented in these figures are averaged from triplicate analyses. Regarding total ion intensity, the PP vials from VWR yielded the lowest total peptide intensity, followed by the glass vial from Waters. Remarkably, the QuanRecovery vial demonstrated impressive results, surpassing even the performance of the custom PMMA vial, despite being categorized as an PP vial. The QuanRecovery vial is treated with MaxPeak HPS Technology (Waters), which helps to reduce peptide adsorption and could explain the observed gain in peptide total intensity compared to the other PP vial. As discussed above, the peptide count is strongly correlated to the total peptide intensities.

Using PEO or DDM to reduce Peptide Loss. A second option for reducing the surface affinity for peptide in solution is to add nonionic detergent directly to the solvent. The use of nonionic detergents in peptide solutions could reduce adsorption by forming micelles, decreasing adhesion forces, and/or changing vial surface properties by passivation. One can suspect that the adsorption of detergent on "apolar" surfaces such as COC, COP, and PP could functionalize these surfaces, reducing their hydrophobicity. However, it is important to note that the inverse phenomenon can occur on more "polar" surfaces leading to an increase in hydrophobicity by masking polar moieties. As for estimating surface hydrophobicity, predicting the efficiency of detergents in reducing peptide adsorption is challenging. Therefore, experimental screenings appear to be the best approach for evaluating their influence. In this study, we explored the use of PEO and DDM as nonionic detergents compatible with the one-pot strategy requiring minimal preparation steps for low-cell number sample. Their advantage lies in their compatibility with MS analysis, eliminating the need for a cleaning procedure before injection into LC-MS. The minimum concentration required to observe the beneficial effects of DDM and PEO was estimated by downscaling their concentration from 0.1%

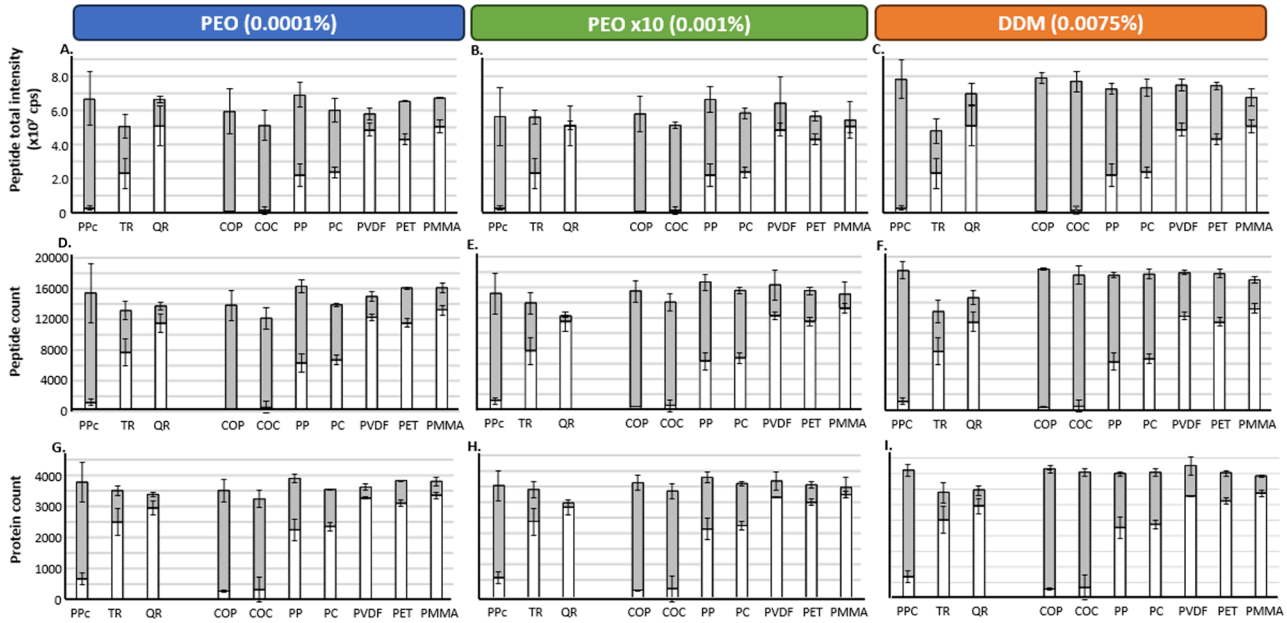


Figure 5. (A-C) Total peptide intensities (expressed in 10^8 counts) as a function of the vial used for different buffer compositions: (A) PEO 0.0001%, (B) PEO 0.001%, and (C) DDM 0.0075%. (D-F). Number of identified peptides with FDR < 1% as a function of the vial used for different buffer compositions: (D) PEO 0.0001%, (E) PEO 0.001%, and (F) DDM 0.0075%. (G-I). Number of identified proteins with FDR < 1% and with a unique peptide as minimum as a function of the used vials for different buffer compositions: (G) PEO 0.0001%, (H) PEO 0.001%, and (I) DDM 0.0075%. No MBR has been applied. White bars correspond to the results in absence of detergent (PEO or DDM) while gray bars are the observed increase using the corresponding buffer composition. All three buffer compositions contained 0.1% of TFA. The reported values and error bars are the average values and uncertainties derived from triplicate experiments.

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

to 0.001% and 0.001% to 0.00001% (weight-to-weight percentages), respectively, on 0.2 ng HeLa tryptic digest (see Supporting Information, Figure S2). The minimum concentrations of DDM and PEO required for 0.2 ng of HeLa were determined to be 0.0075% and 0.0001%, respectively, for PP vials, in weight-to-weight ratio. We therefore used these concentrations to evaluate the gain in proteomic performance using DDM and PEO on the different vials discussed in the preceding section. For PEO, we also assessed a 10-fold increase in the minimal concentration, as the latter appears to be insufficient for certain vial types such as COC and COP. These additives have been evaluated on the injection of 0.2 ng of HeLa tryptic solution (0.2 ng/μL, injected volume = 1 μL) as a single-cell model. The labels "noAdd", "DDM", "PEO", and 'PEOx10' correspond to 0.2 ng of HeLa tryptic digestion without additives, with 0.0075% DDM, with 0.0001% PEO, and with 0.001% PEO, respectively. Peptides were separated during a 25 min effective LC gradient and identified by DIA-PASEF on timsTOF SCP. The total peptide intensity gains (expressed in counts and represented in gray) using PEO, PEOx10 and DDM additives are reported in Figure 5.A, 5.B, and 5.C, respectively. The peptide identification gains (represented in gray) are also reported for PEO, PEOx10 and DDM in Figure 5.D, 5.E, and 5.F, respectively. The protein count gains are also reported in gray for PEO, PEOx10 and DDM in Figure 5.G, 5.H, and 5.I, respectively. These gains are represented relative to the results obtained for the same solution without additive ("noAdd", represented in white).

As for the modification of the vial material, the peptide distributions based on retention times have been monitored (box plots in the Supporting Information, Figure S3 for all in-house manufactured vials and PPc as reference). As already discussed, fewer hydrophobic peptides are detected in PP, COP, and COC vials without additives. Indeed, the mean retention times for peptides identified in COC, COP, and PP are approximately 21, 21, and 23 min, respectively, while the mean retention time when using a PMMA vial is close to 26 min. These results are in perfect agreement with previous observations. In the presence of 0.0001% of PEO, the peptide distribution observed for the COC and COP vials is shifted to the right to 24 min, while all the other vials exhibit similar distributions. This supports that hydrophobic peptides are recovered with PEO and suggests that PEO reduces the surface affinity difference between the different materials investigated in this study. At higher PEO concentrations, the difference between all polymeric materials is reduced, suggesting that more PEO was required for the COP and COC vials to reduce peptide affinity for the surface. It is interesting to note that with 0.001% PEO, all peptide distributions are similar regardless of the polymeric material. This leveling effect on surface peptide adsorption becomes more pronounced when employing DDM, as it ensures uniform distribution of all peptides, no matter the vial material used. In the presence of these additives, hydrophobic materials (e.g., COC and COP vials) seem to be as much adapted for single-cell proteomics as more hydrophilic materials (e.g., PET and PMMA vials). Interestingly, while the use of PEO or DDM enhances proteomic performance across all commercial vial types (e.g., PPc, TR, and QR), the improvement is notably less pronounced in TR and QR vials compared to that of PPc. This suggests that the performance gains associated with PEO or DDM are more substantial in PPc vials, potentially leading to a comparatively lower overall performance in TR and QR vials. Moreover, the UpSet plot representation of these data (see the Supporting Information, Figure S4 for all in-house manufactured vials and PPc as reference) shows that the majority of peptides is commonly detected in all the investigated vials supporting that addition of PEO or DDM minimizes the influence of the vial nature on peptide adsorption and thus on proteomic performance.

It is important to consider that PEO or DDM is detected in the MS instrument and could contaminate the LC-MS system as it is detected in its corresponding retention time (see the Supporting Information, Figure S5 and Figure S6). DDM contamination is detected after the peptide elution (i.e., higher retention time) without overlapping peptides signals. Interestingly, DDM and cluster ions are detected with ammonium adduct ($[C_{24}H_{46}O_{11} + NH_4]^+$, $[(C_{24}H_{46}O_{11})_2 + NH_4]^+$, $[(C_{24}H_{46}O_{11})_3 + NH_4]^+$ = 528.3384, 1038.6424, 1548.9464) at the same retention time suggesting that even if the concentration in solution is maintained below the micellar concentration threshold, some clusters can be formed during the LC separation (see the Supporting Information S4). This can explain its elution at a higher retention time. Concerning PEO, polymers with high molecular weights were selected to avoid contamination in the retention range of peptides. Nonetheless, some PEO fragments were detected within the peptide retention time distribution. These signals, however, did not compromise the MS/MS peptide analysis, as they could be readily excluded from precursor selection in the DIA-PASEF method due to their characteristic ion mobility (See the Supporting Information S5). These contaminations are therefore not critical for bottom-up proteomic analyses. However, we suggest adapting the LC method to ensure a sufficient cleaning step at a higher acetonitrile content solvent after the elution of PEO or DDM to avoid their accumulation in the LC system (e.g., columns and tubing). It is important to note that the PEO and DDM contaminations are dependent on the injected quantity and should be different as a function of the injected volume in the LC system.

Recommendation for single-cell proteomic experiments. All of the previous results have been obtained from protein digest samples equivalent to a low-cell number sample, i.e., a diluted standard solution of HeLa tryptic digest. The compatibility of PEO or DDM with proteomic sample preparation was assessed using 10 FACS-sorted HeLa cell samples, representing approximately 2 ng of proteins. These cells were collected in a PP 96-well PCR plate and were prepared for proteomic analysis directly within the wells, employing a one-pot strategy. The final volume of the sample preparation was 12 μL, leading to a protein concentration of $\pm$0.17 ng/μL. Nine μL of the sample was finally injected for LC-MS analysis, corresponding to an expected injected quantity of 1.5 ng.

Sample preparation with PEO or DDM addition during the cell lysis (i.e., at the beginning of the proteomic sample preparation) has been compared to the addition of PEO or DDM after the protein digestion (i.e., at the end of the proteomic sample preparation). In both cases, the final concentrations (w/w) of PEO or DDM were 0.001% and 0.0075%, respectively. The proteomic performance are reported in Figure 6 in terms of total peptide intensities (Figure 6.A, in log scale), identified peptide counts with 1% FDR (Figure 6.B), and identified protein counts (Figure 6.C) for 5 replicates. A first observation is that the total peptide intensities are far lower than the peptide intensity expected

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

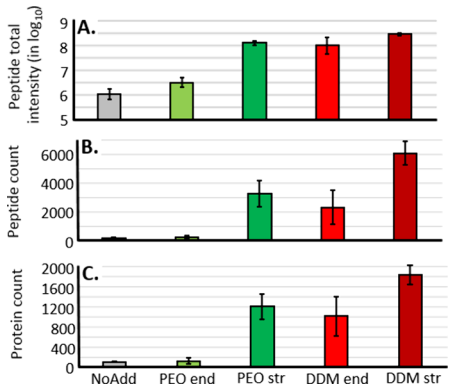


Figure 6. Proteomics performance obtained from 10 HeLa cells sample collected by FACS in a commercial PP 96-well plate from ThermoFisher as a function of the addition of detergent. "NoAdd" (gray bars) corresponds to the sample preparation without detergent. "PEO end" and "DDM end" (light green and light red, respectively) correspond to the sample preparation protocol involving the addition of PEO and DDM, respectively, before the LC-MS injection. "PEO str" and "DDM str" (dark green and dark red, respectively) correspond to the sample preparation protocol involving the addition of PEO and DDM, respectively, as the first step during cell lysis. The proteomic performance is evaluated in terms of (A) total peptide intensity (expressed in $\log_{10}$), (B) count of identified peptides with FDR < 1% and (C) count of identified protein with an FDR < 1% and with at least one unique peptide.

from a standard solution of HeLa tryptic digests at an equivalent quantity (i.e., 2 ng). This can be explained by (1) a significant loss of material during the sample preparation or (2) a low sample preparation efficiency (e.g., cell lysis efficiency and trypsin digestion yield). One can also observe that error bars reported for the 10-HeLa cell samples are higher than those with an equivalent standard solution of HeLa, but this can be explained by the heterogeneity of HeLa cells. From this comparative study, it clearly appears that there is an advantage to perform the entire sample preparation in the presence of nonionic detergent as the proteomic performance are better when PEO or DDM are added during the cell lysis. Moreover, DDM seems to lead to better performance than PEO for this precise case involving 10 HeLa cells and a PP 96-well plate.

From these observations, we recommend adding DDM directly after cell collection or collecting cells directly in a solution containing DDM. The interest in adding PEO or DDM for the sample preparation has also been evaluated on true single-cell experiments (see the Supporting Information, Figure S7). These single cells have been collected in a PP 96-well plate using a FACS sorter. No signal was detected in the absence of nonionic detergent. Conversely, an average of approximately 300 proteins and 630 proteins were observed with, respectively 0.0001% PEO and 0.0075% DDM, across five replicates.

CONCLUSION

This study highlights that a proportion of peptides can be adsorbed onto the surface of vials, thereby reducing proteomics performance, especially for samples containing low amounts of proteins. Standard solutions of HeLa tryptic digest peptide at different concentrations have been used as a model to evaluate this effect. The relationship between concentration and peptide intensities fits a Langmuir-type adsorption model, supporting peptide adsorption on surfaces. On commonly used vials made of PP, signal loss is predominant for peptides eluting at high retention times, suggesting that hydrophobic peptides are more impacted than hydrophilic peptides. This could lead to significant misinterpretations in differential studies based on Label-Free Quantification (LFQ), even with the use of normalization algorithms. Based on the established equation relating intensity to peptide concentration, some recommendations have been suggested: (1) Reducing the exposure to the surface by prioritizing one-pot strategies and minimizing sample preparation steps. (2) Reducing peptide affinity for the surface by adapting the vial surface or the peptide buffer. (3) Reducing the working volume to increase concentration and reduce exposition to the surface. We demonstrate that the surface nature of vial directly influences peptide adsorption. The use of custom vials molded with more polar polymers, such as poly(methyl methacrylate) (PMMA), allows a 15-fold increase of total peptide intensity for very low-concentration peptide solution (i.e., < 2 ng/μL) compared to commercial PP vials by reducing the adsorption of peptides, especially hydrophobic ones. On the other hand, the use of more hydrophobic polymeric material drastically increases the peptide loss supporting the influence of the surface nature on the proteomics performance. The use of nonionic detergents such as PEO and DDM at adapted concentrations reduces performance heterogeneity between the different investigated vial materials. For the investigated vials, the recommended concentration of PEO or DDM was estimated at 0.0001% or 0.0075%, respectively, which is below the currently reported values in the literature. However, refining these concentrations is recommended when using different vials. Using these compounds makes it possible to achieve similar results with commercial PP vials as those observed with PMMA vials. Advantages of PEO and DDM for the sample preparation of 10 HeLa cells or a single HeLa cell sorted in a PP 96-well plate have also been evaluated. From these results, we demonstrate that the use of PEO and DDM strongly increases the detection of peptides and proteins, especially when added at the beginning of the sample preparation, i.e., during cell lysis, as already observed in the literature.[25,27,30] For HeLa cell samples, DDM leads to better results than PEO. In the case of single-cell sample preparation with a classical workflow in a PP 96-well plate (i.e., without adapted lab consumables and equipment), we report that no protein was identified without the addition of PEO or DDM. However, when DDM is added during the cell lysis, we identified more than 600 proteins with an FDR < 1% without using match-between-runs (MBR) algorithm. Based on these results, using a one-pot protocol with an appropriate additive appears to be crucial for enhancing proteomic analysis of samples with a low protein content, such as single-cell proteomics, especially when using standard sample preparation equipment. Furthermore, employing MBR for matching protein identifications across acquisition data, along with the addition of isobaric or isotopic cell carriers, is a strategy that should be considered to further improve proteome coverage.

ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data (raw data and search files) have been deposited to the ProteomXchange Consortium via the PRIDE[33] partner repository with the data set identifiers

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845

PXD057621, PXD057638, PXD057609 for respectively HeLa tryptic digest DDA, HeLa tryptic digest DIA and 1 to 10 HeLa cell(s) DIA analysis.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c03709.

Detailed sample preparation and LC-MS instruments settings; demonstration of peptide intensity-concentration relationship; Table S1: list of vials with abbreviations, names, suppliers; Figure S1: pictures of vials used in the study; Figure S2: proteomics performance as a function of PEO or DDM concentration; Figure S3: box plots of peptide distributions without additive, DDM, or PEO; Figure S4: UPSET plot of differential peptide analysis by polymeric material and surfactant; Figures S5 and S6: PEO and DDM signal contamination; Figure S7: proteomics performance from single HeLa cell samples using DDM PEO (PDF)

AUTHOR INFORMATION

Corresponding Author

Christopher Kune − *Mass Spectrometry Laboratory, MolSys Research Unit, University of Liege, Liege B-4000, Belgium;* orcid.org/0000-0002-3010-8173; Email: C.Kune@uliege.be

Authors

Sylvia Tielens − *Laboratory of Molecular Regulation of Neurogenesis, GIGA-Stem Cells, University of Liège, Liège B-4000, Belgium*

Dominique Baiwir − *GIGA Proteomics Facility, University of Liège, Liège B-4000, Belgium;* orcid.org/0000-0002-1622-0118

Maximilien Fléron − *GIGA Proteomics Facility, University of Liège, Liège B-4000, Belgium*

Denis Vandormael − *Sirris, Liege B-4000, Belgium*

Gauthier Eppe − *Mass Spectrometry Laboratory, MolSys Research Unit, University of Liege, Liege B-4000, Belgium;* orcid.org/0000-0002-4821-3115

Laurent Nguyen − *Laboratory of Molecular Regulation of Neurogenesis, GIGA-Stem Cells, University of Liège, Liège B-4000, Belgium*

Gabriel Mazzucchelli − *Mass Spectrometry Laboratory, MolSys Research Unit, University of Liege, Liege B-4000, Belgium; GIGA Proteomics Facility, University of Liège, Liège B-4000, Belgium;* orcid.org/0000-0002-8757-8133

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.4c03709

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by a Win2Wal grant from the Wallonie Recherche SPW (ChipOmics Project, Grant 201012). The authors gratefully acknowledge the GIGA Flow Cytometry platform for their invaluable assistance with cell sorting. Additionally, the authors express their appreciation to Nancy Rosière and Lisette Trzpiot from the GIGA Proteomics platform and MSLab for their expertise in cell sample preparation and proteomic quantification analysis. The TimsTOF SCP was funded thanks to the Belgian Foundation against Cancer (agreement 2022-040A). L.N. is research director at the Fonds de la Recherche Scientifique (FRS) − Fonds National de la Recherche Scientifique (FNRS).

REFERENCES

(1) Aebersold, R.; Mann, M. *Nature* **2003**, *422* (6928), 198−207.
(2) Cravatt, B. F.; Simon, G. M.; Yates, J. R., III *Nature* **2007**, *450* (7172), 991−1000.
(3) Wang, N.; Xu, M.; Wang, P.; Li, L. *Anal. Chem.* **2010**, *82* (6), 2262−2271.
(4) Drissi, R.; Dubois, M.-L.; Boisvert, F.-M. *FEBS J.* **2013**, *280* (22), 5626−5634.
(5) Mezger, S. T. P.; Mingels, A. M. A.; Bekers, O.; Heeren, R. M. A.; Cillero-Pastor, B. *Anal. Chem.* **2021**, *93* (4), 2527−2533.
(6) Taylor, M. J.; Lukowski, J. K.; Anderton, C. R. *J. Am. Soc. Mass Spectrom.* **2021**, *32* (4), 872−894.
(7) Ctortecka, C.; Hartlmayr, D.; Seth, A.; Mendjan, S.; Tourniaire, G.; Mechtler, K. An Automated Workflow for Multiplexed Single-Cell Proteomics Sample Preparation at Unprecedented Sensitivity. *bioRxiv* **2022**, 2021.04.14.439828. .
(8) Bennett, H. M.; Stephenson, W.; Rose, C. M.; Darmanis, S. *Nat. Methods* **2023**, *20* (3), 363−374.
(9) Budnik, B.; Levy, E.; Harmange, G.; Slavov, N. *Genome Biol.* **2018**, *19* (1), 161.
(10) Petelski, A. A.; Emmott, E.; Leduc, A.; Huffman, R. G.; Specht, H.; Perlman, D. H.; Slavov, N. *Nat. Protoc.* **2021**, *16* (12), 5398−5425.
(11) Blankenburg, S.; Hentschker, C.; Nagel, A.; Hildebrandt, P.; Michalik, S.; Dittmar, D.; Surmann, K.; Völker, U. *PROTEOMICS* **2019**, *19* (23), No. 1900192.
(12) Specht, H.; Harmange, G.; Perlman, D. H.; Emmott, E.; Niziolek, Z.; Budnik, B.; Slavov, N. *bioRxiv* **2018**, No. 399774.
(13) Pezeshki, A.; Vergote, V.; Van Dorpe, S.; Baert, B.; Burvenich, C.; Popkov, A.; De Spiegeleer, B. *J. Pharm. Biomed. Anal.* **2009**, *49* (3), 607−612.
(14) Warwood, S.; Byron, A.; Humphries, M. J.; Knight, D. *J. Proteomics* **2013**, *85*, 160−164.
(15) Law, S. L.; Shih, C. L. *Drug Dev. Ind. Pharm.* **1999**, *25* (2), 253−256.
(16) Krause, K. D.; Roy, S.; Hore, D. K. *Biointerphases* **2017**, *12* (2), No. 02D407.
(17) Skelton, A. A.; Liang, T.; Walsh, T. R. *ACS Appl. Mater. Interfaces* **2009**, *1* (7), 1482−1491.
(18) Horinek, D.; Serr, A.; Geisler, M.; Pirzer, T.; Slotta, U.; Lud, S. Q.; Garrido, J. A.; Scheibel, T.; Hugel, T.; Netz, R. R. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (8), 2842−2847.
(19) Goebel-Stengel, M.; Stengel, A.; Taché, Y.; Reeve, J. R. *Anal. Biochem.* **2011**, *414* (1), 38−46.
(20) Ovesen, R. G.; Göransson, U.; Hansen, S. H.; Nielsen, J.; Hansen, H. C. B. *J. Chromatogr. A* **2011**, *1218* (44), 7964−7970.
(21) Dixit, C. K.; Vashist, S. K.; MacCraith, B. D.; O'Kennedy, R. *Analyst* **2011**, *136* (7), 1406−1411.
(22) van Midwoud, P. M.; Rieux, L.; Bischoff, R.; Verpoorte, E.; Niederländer, H. A. G. *J. Proteome Res.* **2007**, *6* (2), 781−791.
(23) Gan, G.; Xu, X.; Chen, X.; Zhang, X.-F.; Wang, J.; Zhong, C.-Q. *Mol. Cell. Proteomics* **2021**, *20*, No. 100051.
(24) Li, W.; Luo, S.; Smith, H. T.; Tse, F. L. S. *J. Chromatogr. B* **2010**, *878* (5), 583−589.
(25) Stejskal, K.; Potěšil, D.; Zdráhal, Z. *J. Proteome Res.* **2013**, *12* (6), 3057−3062.
(26) Kawashima, Y.; Takahashi, N.; Satoh, M.; Saito, T.; Kado, S.; Nomura, F.; Matsumoto, H.; Kodera, Y. *PROTEOMICS* **2013**, *13* (5), 751−755.
(27) Nie, S.; O'Brien Johnson, R.; Livson, Y.; Greer, T.; Zheng, X.; Li, N. *Anal. Biochem.* **2022**, *658*, No. 114924.
(28) Waas, M.; Bhattacharya, S.; Chuppa, S.; Wu, X.; Jensen, D. R.; Omasits, U.; Wollscheid, B.; Volkman, B. F.; Noon, K. R.; Gundry, R. L. *Anal. Chem.* **2014**, *86* (3), 1551−1559.

(29) Vatansever, B.; Lahrichi, S. L.; Thiocone, A.; Salluce, N.; Mathieu, M.; Grouzmann, E.; Rochat, B. *J. Sep. Sci.* **2010**, *33* (16), 2478−2488.
(30) Liu, J.; Wang, F.; Mao, J.; Zhang, Z.; Liu, Z.; Huang, G.; Cheng, K.; Zou, H. *Anal. Chem.* **2015**, *87* (4), 2054−2057.
(31) Zhu, Y.; Piehowski, P. D.; Zhao, R.; Chen, J.; Shen, Y.; Moore, R. J.; Shukla, A. K.; Petyuk, V. A.; Campbell-Thompson, M.; Mathews, C. E.; Smith, R. D.; Qian, W.-J.; Kelly, R. T. *Nat. Commun.* **2018**, *9* (1), 882.
(32) Tsai, C.-F.; Zhang, P.; Scholten, D.; Martin, K.; Wang, Y.-T.; Zhao, R.; Chrisler, W. B.; Patel, D. B.; Dou, M.; Jia, Y.; Reduzzi, C.; Liu, X.; Moore, R. J.; Burnum-Johnson, K. E.; Lin, M.-H.; Hsu, C.-C.; Jacobs, J. M.; Kagan, J.; Srivastava, S.; Rodland, K. D.; Steven Wiley, H.; Qian, W.-J.; Smith, R. D.; Zhu, Y.; Cristofanilli, M.; Liu, T.; Liu, H.; Shi, T. *Commun. Biol.* **2021**, *4* (1), 265.
(33) Perez-Riverol, Y.; Bai, J.; Bandla, C.; García-Seisdedos, D.; Hewapathirana, S.; Kamatchinathan, S.; Kundu, D. J.; Prakash, A.; Frericks-Zipper, A.; Eisenacher, M.; Walzer, M.; Wang, S.; Brazma, A.; Vizcaíno, J. A. *Nucleic Acids Res.* **2022**, *50* (D1), D543−D552.

https://doi.org/10.1021/acs.analchem.4c03709
Anal. Chem. 2025, 97, 3836−3845