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PNNL-36879

Device and Method for Parallel Measurement of Phosphoproteome and Proteome from Single Cells

September 2024

Liang Chen
Lye Meng Markillie

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
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Abstract

We present the development of an immobilized metal affinity chromatography (IMAC) chip designed to enable nanoscale phosphopeptide enrichment within microfabricated nanowells. This novel platform leverages surface chemistry to immobilize high-density Nickel-Nitrilotriacetic Acid (Ni-NTA) molecules on nanowells, followed by the application of Fe^{3+} for capturing and enriching phosphopeptides based on IMAC. The feasibility of the approach and system's efficiency were validated using a model protein, β-casein . The refinement of this technology should enable detection of hundreds of phosphopeptides from individual cells, offering exciting potential for parallel single-cell global proteomics and phosphoproteomics. Future efforts will explore the use of this technology to study phosphorylation dynamics in biological systems relevant to energy, environment and human health research.

Acknowledgments

This research was supported by the Strategic Investments Program, under the Laboratory Directed Research and Development (LDRD) Program at Pacific Northwest National Laboratory (PNNL). PNNL is a multi-program national laboratory operated for the U.S. Department of Energy (DOE) by Battelle Memorial Institute under Contract No. DE-AC05-76RL01830.

Acronyms and Abbreviations

(3-Aminopropyl)triethoxysilane	APTES
Gold nanoparticles	Au-NPs
IMAC	Immobilized metal affinity chromatography
Liquid chromatography	LC
Mass spectrometry	MS
NanoPOTS	Nanodroplet processing in one pot for trace samples
Ni-NTA	Nickel-Nitrilotriacetic acid

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1.0 Introduction

Protein phosphorylation is one of the most important post-translational modifications (PTMs) in living cells. It is involved in many regulatory functions, such as cell cycle control, receptor-mediated signal transduction, differentiation, proliferation, and metabolism (1-2). Recent studies indicate protein phosphorylation is fundamentally involved in many human diseases, including cancer, type-1 diabetes, and Alzheimer's disease. Phosphorylated proteins are potentially more reliable biomarkers for disease diagnosis and classification as well as drug development. However, current phosphoproteomic technologies require a large amount of tissue sample containing millions of cells (3) and cannot provide critical biological information on cell-type-specific phosphorylation dynamics and cell-to-cell heterogeneity.

The key challenge to measure phosphorylated proteins in small samples is efficient phosphopeptide enrichment (i.e., with minimal sample loss). To address this limitation, we developed a nanoscale phosphoproteomics platform leveraging PNNL's one pot for trace samples (nanoPOTS) (4-5) technology. This platform enables efficient phosphopeptide enrichment for subsequent liquid chromatography – mass spectrometry (LC-MS) characterization from low-input samples thus expanding PNNL's nanoPOTS capability from measuring global proteomes to gaining functional insights by characterizing protein phosphorylation with potential expansion to other post-translational modifications (PTMs).

2.0 Research Design and Methodology

We developed a nanoscale phosphoproteomics platform utilizing PNNL's nanoPOTS technology. As depicted in Figure 1, the glass surface was first treated with (3-Aminopropyl)triethoxysilane (APTES), generating a layer of cross-linked amines. Next, Ni-NTA was attached to the glass surface via NHS-amine chemistry. After introducing Fe^{3+}, the nanowell surface serves as a capture medium for phosphopeptide enrichment based on IMAC.

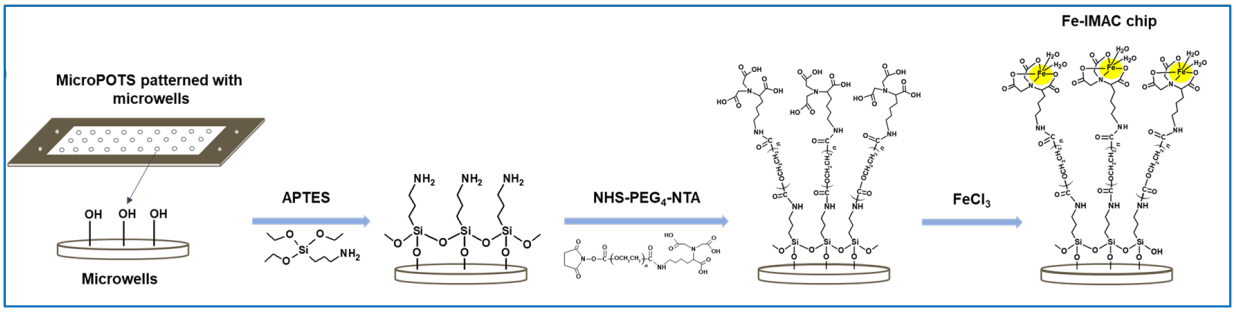


Figure 1. The fabrication of IMAC chips.

Next, we used a model protein (β-casein) digest to test the specificity and reproducibility of the modified nanowells for phosphoproteomics. We then loaded different quantities of β-casein peptides amounts (1-100 ng) onto the IMAC chip to evaluate the capture efficiency of the approach.

Additionally, we developed an integrated workflow for both, phosphopeptide enrichment and recovery of unmodified peptides (Figure 2). A conventional nanoPOTS workflow was used to perform cell isolation and protein digestion. The samples were then transferred from the nanoPOTS chip to the IMAC chip for phosphopeptide enrichment. After incubation, the IMAC chip was aligned with a 384-well plate, and a centrifugation-based sample transfer was carried out. The IMAC chip was then washed with an acidic solvent to remove any remaining unmodified peptides. Subsequently, the IMAC chip was loaded onto the nanoPOTS autosampler for LC-MS phosphoproteomics, and the 384-well plate, containing unmodified peptides, was submitted to global proteomics.

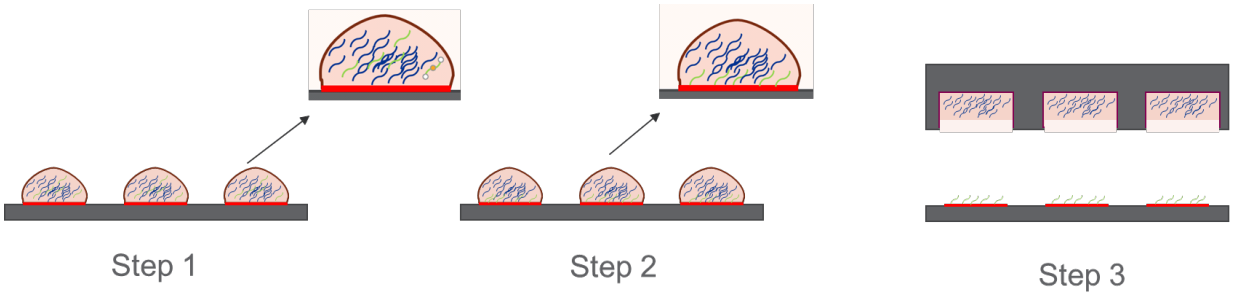


Figure 2. The schematic of the workflow for parallel enrichment of phosphopeptides and recovery of unmodified peptides from small samples prepared using nanPOTS. Step 1: Add the sample onto IMAC chip for selective enrichment of phosphopeptides. Step 2: Incubate the sample for one hour to allow the binding of phosphopeptides on well surface. Step 3: Spin the incubated sample to transfer it to a 384-well plate for parallel global proteomics and phosphoproteomics.

3.0 Outcomes

3.1 Design and Fabrication of the IMAC Chip

In this project, we first designed microwell chips and optimized the IMAC chip preparation procedure. To ensure sufficient capture surface and minimize sample loss due to absorption, we developed a microPOTS chip featuring an array of 3×9 microwells, each with a diameter of 2.2 mm (Figure 3a). For the IMAC chip preparation, we modified the microPOTS chip using (3-Aminopropyl)triethoxysilane (APTES) to introduce primary amine groups. We used a fluorescent labeling reagent, NHS-Cy3, which specifically reacts with primary amines, to measure the density of the amine groups on the chip surface. As shown in Figures 3b-c, a significant difference was observed between the APTES-coated microwells and the unmodified wells. A strong red fluorescence signal was detected in the APTES-coated microwells, while no signal was observed in the wells without APTES treatment. This suggested a successful and specific modification of the microwell surface with APTES, and confirmed the presence of primary amines essential for subsequent functionalization steps. Next, Ni-NTA was covalently linked to the glass surface via NHS-amine chemistry, followed by the introduction of Fe^{3+} ions. The resulting IMAC chip, functionalized with Fe^{3+}-NTA, serves as a capture medium for phosphopeptide enrichment based on immobilized metal affinity chromatography (IMAC).

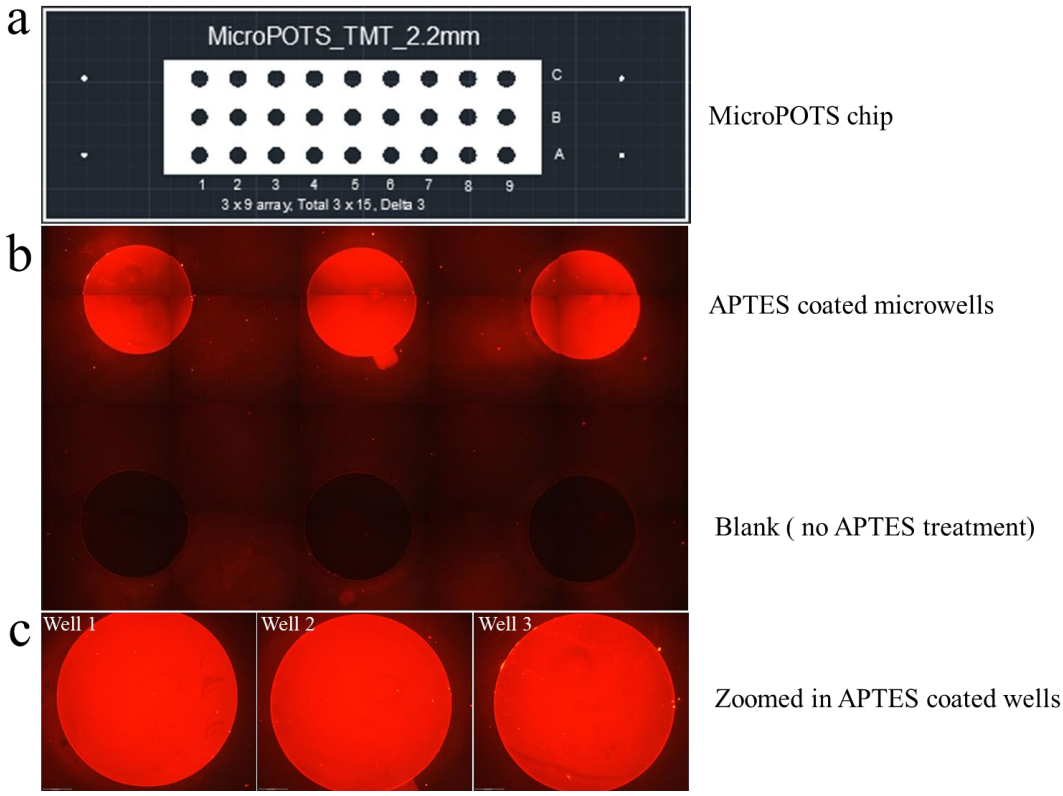
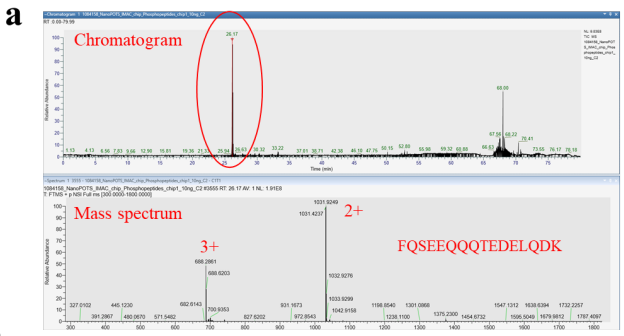


Figure 3. IMAC chip preparation. (a) AutoCAD design of the microPOTS chip. (b) Fluorescence images comparing microwells coated with APTES and microwells without APTES treatment. (c) Zoomed-in-view of the APTES coated microwells.

3.2 IMAX Chip Evaluation

To assess the phosphopeptide capturing efficiency and specificity of the IMAC chip, we prepared a series of β-casein digests and incubated them within the microwells. As shown in Figure 4a, clean phosphopeptide signal (corresponding to the sequence: FQSEEQQQTEDELQDK) was detected within the chromatogram (top) and mass spectrum (bottom). No significant interfering peptides were observed, demonstrating the high phosphopeptide specificity of the IMAC chip(Figure 4c). However, the capturing efficiency was lower than expected, with 10 ng of β-casein digest yielding a relatively low signal intensity (for phosphopeptide at *m/z*= 1031.42 (Figure 4b).



c

Peptide	Prev AA	Next AA	Peptide Le	Charges	Probabilit	Spectral Count	Intensity	Assigned	Observed	Protein	Protein ID	Entry Nam	Gene	Protein Descrip	Mapped G	Mapped Proteins
DIGSESTEDQAMEDIK	K	Q	16	2	0.9524	1	0	12M(15.9949), 4S(79		contam_s	P02662	CASA1_BC	CSN1S1	Alpha-S1-casein		
DMPIQAFLLYQEPVLGPVR	R	G	19	3	0.9836	1	0	2M(15.9949)		contam_s	P02666	CASB_BO	CSN2	Beta-casein		
FLDDDLTDDIMCVK	K	K	14	2	0.9524	1	0	11M(15.9949), 12C(5		contam_s	P00711	LALBA_BC	LALBA	Alpha-lactalbumin		
FQSEEQQQTEDELQDK	K	I	16	2, 3	0.9836	25	0	3S(79.9663)		contam_s	P02666	CASB_BO	CSN2	Beta-casein		
GVPQIEVTFDIDANGILNVSAVDK	R	S	24	2, 3	0.9524	3	0			sp|P19120	P19120	HSP7C_BC	HSPA8	Heat shock cognate 71 kDa protein		
GVPQIEVTFDIDANGIVHVSAK	R	D	22	3	0.9524	1	0			sp|Q3ZCH0	Q3ZCH0	GRP75_BC	HSPA9	Stress-70 protein, mitochondrial		
IEKFQSEEQQQTEDELQDK	K	I	19	2, 3	0.9524	4	0	6S(79.9663)		contam_s	P02666	CASB_BO	CSN2	Beta-casein		
LGEHNIDVLEGNEQFINAAK	R	I	20	3	0.9524	1	0			contam_s	P00761	TRYP_PIG		Trypsin		
MTQIMFETFNVPAMYVAIQAVLSLYASGR	K	T	29	3	0.9524	1	0	14M(15.9949), 1M(15		sp|P62739	P62739	ACTA_BO	ACTA2	Actin, aortic sm	ACTA1, A	sp|P68138|ACTS_BOVIN, sp|Q3ZCD7|ACTC_BOVIN, sp|Q5E9B5|ACTH_BOVIN
TPEVDDEALEKFDK	R	A	14	3	0.9524	1	0			sp|P02754	P02754	LACB_BO	LGB	Beta-lactoglobulin		
TVDMESTEVFTK	K	K	12	2	0.9524	1	0	4M(15.9949), 6S(79.9		contam_s	P02663	CASA2_BC	CSN1S2	Alpha-S2-casein		
VPQLEIVPNSAEER	K	L	14	2	0.9524	1	0	1S(79.9663)		contam_s	P02662	CASA1_BC	CSN1S1	Alpha-S1-casein		
YKVPQLEIVPNSAEER	K	L	16	3	0.9524	1	0	12S(79.9663)		contam_s	P02662	CASA1_BC	CSN1S1	Alpha-S1-casein		

Figure 4. Assessment of the efficiency and specificity of the IMAC chip using β-casein digest. (a) Chromatogram and mass spectrum of IMAC chip-enriched posphopeptide. (b) The peak area measured for a phosphopeptide at m/z= 1031.42 relates to the amount of β-casein digest added to the IMAC chip. (c) Peptide sequences identified by FragPipe from the LC-MS proteomics.

3.3 Strategies to Enhance Capture Efficiency

In an attempt to enhance the capture efficiency of the IMAC chip, we increased the surface roughness of the microPOTS chip through wet etching using 5M sodium hydroxide. Different incubation times and temperatures were tested. As shown in Figure 5a, etching for 2 hours at 90°C rendered the entire surface hydrophilic. Etching for 20 minutes at 70°C caused the microwells to expand, while the surrounding area remained hydrophobic. The optimal condition was found to be a 10-minute etching at 70°C, which preserved both the microwell structure and the surrounding hydrophobicity. This condition was used for further evaluations.

Next, the performance of the etched IMAC chip was compared to the non-etched chip. Both chips were modified with APTES to introduce primary amine groups onto the microwell surface, followed by incubation with NHS-Cy3. However, fluorescence results indicated that the etching process did not substantially enhance the surface roughness in a way that would improve the functionalization or capture efficiency of the IMAC chip.

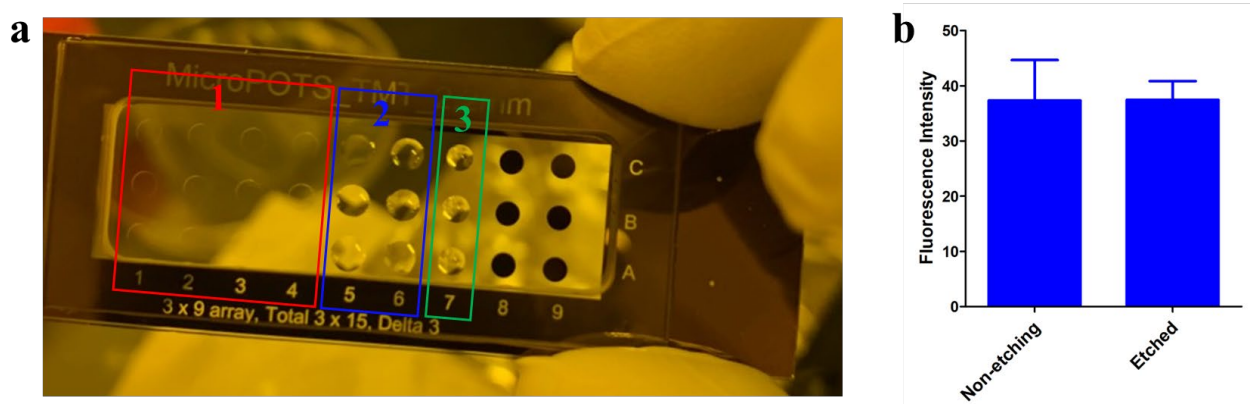


Figure 5. Wet etching of the microPOTS chip. (a) Image of the etched microPOTS chip, highlighting three areas subjected to different etching conditions (5M NaOH): area 1 (red) was etched for 2 hours at 90°C, area 2 (blue) was etched for 20 minutes at 70°C, and area 3 (yellow) was etched for 10 minutes at 70°C. (b) Fluorescence intensity comparison between etched and non-etched microPOTS chips.

The ability of the gold nanoparticles (Au-NPs) to increase the surface area for enhanced enrichment efficiency. As illustrated in Figure 6a, similar to the IMAC chip preparation, we first modified the microPOTS chip with (3-Aminopropyl)triethoxysilane (APTES) to introduce primary amine groups. We then introduced Ni-NTA functionalized Au-NPs to react with the amine groups on the chip surface (Figure 6b). Next, Fe^{3+} ions were introduced to generate Fe^{3+}-NTA, creating a capture medium for phosphopeptides. To assess the phosphopeptide capturing efficiency of the Ni-NTA Au-NPs coated chip, we prepared a series of β-casein digests and incubated them within the microwells. As shown in Figure 6c, the capturing efficiency of the Au-NPs coated chip was ~20% higher compared to the standard IMAC chip (Figure 4b). This improvement indicates that the increased surface area provided by the Au-NPs IMAC effectively enhanced the phosphopeptide enrichment capability of the chip. However, the layer of Au-NPs on the chip surface might introduce complications during sample injection, potentially leading to clogging of the sampling capillary and the trap column during LC-MS analysis.

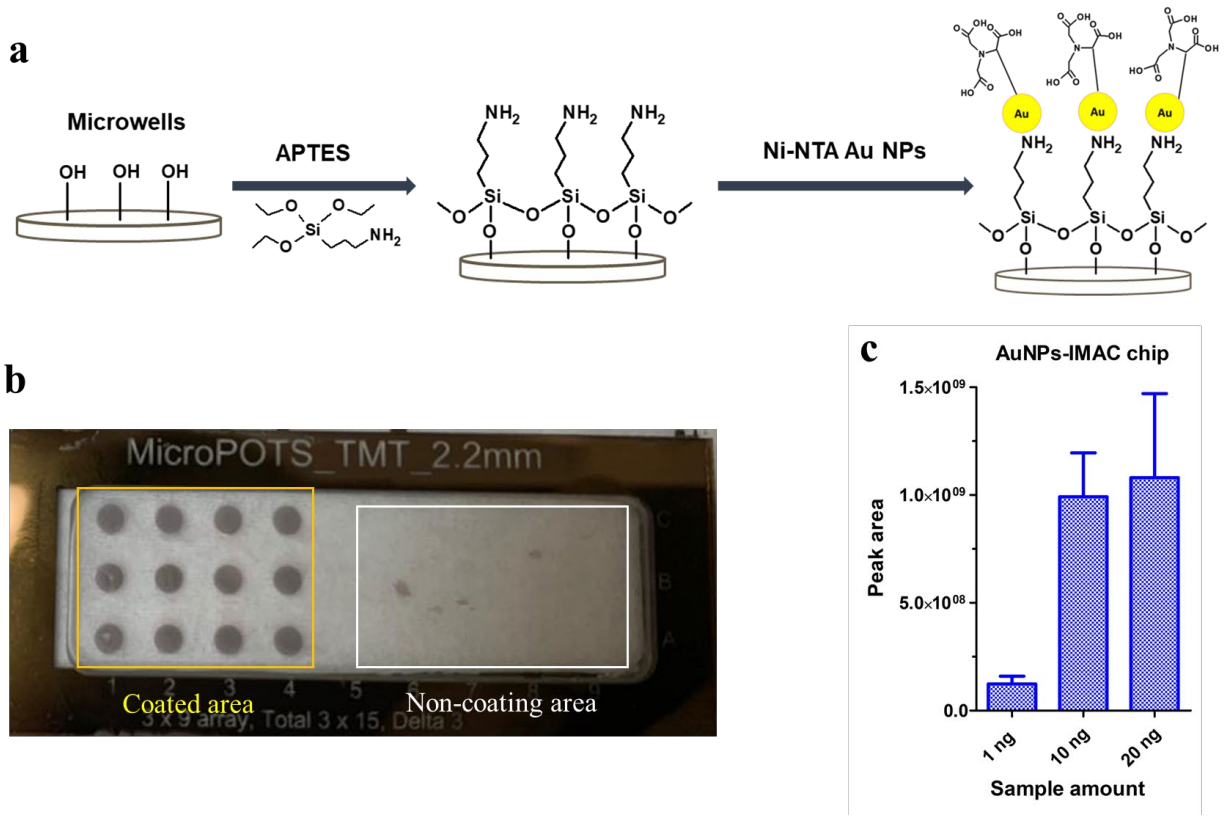


Figure 6. Fabrication of Au NPs-IMAC chip. (a) Au NPs-IMAC chip preparation workflow. (b) Image of the Ni-NTA Au NPs coated chip. (d) Peak area for m/z = 1031.42 relative to the amount of β-casein digest added to Au NPs-IMAC chip.

4.0 Conclusions

This project developed and validated a novel IMAC chip for the high-specificity enrichment of phosphopeptides. Integrating the IMAC chip with the nanoPOTS workflow enables the measurement of both enriched phosphoproteomes and unmodified proteins from the same single cells. This advancement represents a significant step forward in the field of single-cell proteomics.

Our efforts successfully demonstrated the feasibility of enriching phosphopeptides from minimal sample volumes, with initial tests using β-casein digest highlighting the chip's potential for high specificity in phosphopeptide capture. While the current capture efficiency of the chip requires further optimization, strategies such as using nanoparticles showed promising improvements. The Au-NPs-IMAC chip demonstrated 20% higher phosphopeptide capture efficiency compared to the standard IMAC chip, but it also introduced challenges due to potential clogging issues.

Evaluation of both the IMAC and Au-NPs-IMAC chips using more complex biological samples (e.g., cell lysates) appears to be the next logical step. This would help determine if the Au-NPs-IMAC chip's efficiency gains justify its operational complexities, or if the standard IMAC chip can provide adequate performance in low-input contexts, potentially eliminating the clogging issues associated with the Au-NPs coating. This evaluation will guide further development of the phosphopeptide enrichment methods for low-input and single-cell samples.

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