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Contents

Phosphopeptide enrichment for Phosphoproteomic Analysis - A Tutorial and Review of Novel Materials

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Abbreviations used in the paper:

Post-translational modifications (PTMs), mass spectrometry (MS), liquid chromatography (LC), tandem MS (MS/MS), phosphopeptide (p-peptide), label-free quantification (LFQ), stable isotope labelling by amino acids in cell culture (SILAC), Tandem Mass Tags (TMT) isobaric tags for relative and absolute quantification (iTRAQ), phospho-serine (pSer), threonine (pThr), tyrosine (pTyr), immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), acetonitrile (ACN), sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), filter assisted sample preparation (FASP), solid phase extraction (SPE), high performance liquid chromatography (HPLC), reverse phase (RP), strong cation exchange (SCX), electrostatic repulsion hydrophilic interaction chromatography (ERLIC), solution isoelectric focusing (sIEF), strong anion exchange (SAX), histidine (pHis), arginine (Arg),

lysine (Lys), aspartate (Asp), glutamate (Glu), cysteine (Cys), ammonium hydroxide (NH_4OH), ammonium bicarbonate (NH_4HCO_3), ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid (TFA), dihydroxybenzoic acid (DHB), polydopamine (PDA), iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), phytic acid (PA), polyethyleneimine (PEI), polydopamine/poly(2-Aminoethyl methacrylate hydrochloride)/arginine (PAMA-Arg), bovine serum albumin (BSA), isoelectric point (IEP), molybdenum VI oxide (MoO_3), graphene oxide (GO), benzenetricarboxylic acid (H3btc), trimethyl-2-methacroyloxyethyl ammonium chloride (META), graphitized carbon black (GCB), fructose molecular with two phosphate groups (FDP), diphosphorylated fructose-modified dual-metal-centred zirconium (DZMOF), matrix-assisted laser desorption ionization (MALDI), graphene aerogel (GA), 1,4,7,10-tetraazacyclododecane N, N', N'', N'''-tetra-acetic acid (DOTA), polyacrylate (PAA), phosphate-binding molecular tag chromatography (Phos-tag), polymer-based metal ion affinity capture (PolyMAC), hydrazide functionalized monodispersed silica microspheres (HFMSM), formic acid (FA), adenosine tri-phosphate (ATP), molecularly imprinted polymers (MIP), sequential elution from IMAC (SIMAC), immunoaffinity precipitation (IAP), tandem IMAC (IMAC-IMAC), magnetic organic framework (MOF), sample-preparation (SP), ion mobility (IM), electrospray ionization (ESI), p-peptide paper-based analytical devices (phos-PAD), poly glycidyl methacrylate (PGMA).

Abstract

Significant technical advancements in phosphopeptide enrichment have enabled the identification of thousands of p-peptides (mono and multiply phosphorylated) in a single experiment. However, it is still not possible to enrich all p-peptide species in a single step. A range of new techniques and materials has been developed, with potential to provide a step-change in phosphopeptide enrichment. The first half of this review contains a tutorial for new potential phosphoproteomic researchers; discussing the key steps of a typical phosphoproteomic experiment used to investigate canonical phosphorylation sites (serine, threonine and tyrosine). The latter half then show-cases the latest developments in p-peptide enrichment including: i) Strategies to mitigate non-specific binding in immobilized metal ion affinity chromatography and metal oxide affinity chromatography protocols; ii) Techniques to separate multiply phosphorylated from monophosphorylated peptides (including canonical from non canonical phosphorylated peptides), or to simultaneously co-enrich other post-translational modifications; iii) New hybrid materials and methods directed towards enhanced selectivity and efficiency of metal-based enrichment; iv) Novel materials that hold promise for enhanced phosphotyrosine enrichment. A combination of well-understood techniques and materials is much more effective than any technique in isolation; but the field of phosphoproteomics currently requires benchmarking of novel materials against current methodologies fully evaluate their utility in peptide based proteoform analysis.

Key words: phosphoproteoform, canonical and non-canonical phosphorylation, enrichment, optimization, phosphoproteomics, phosphopeptide

1. Introduction

1.1 Biological significance of protein phosphorylation

Reversible phosphorylation is one of the most important post-translational modifications (PTMs) of proteins occurring in all domains of life [1]. A highly dynamic

and widespread process, phosphorylation regulates protein 'behaviour in almost every conceivable way' as noted by in 'The regulation of protein function by multisite phosphorylation - a 25 year update' by Professor Sir Philip Cohen in 2000 [2]. Phosphorylation essentially acts as a molecular switch, effecting temporal and spatial changes in protein function [3], with 30% proteins estimated to undergo phosphorylation [2]. Phosphorylation occurs at single (mono-) or multiple (multi-) sites and can co-occur with other PTM types to generate different 'proteoforms'. Phosphoproteoforms form a subset of the 'epiproteome': a term encompassing PTM, sequence and splice isoform variants encoded by a single gene [4]. Given the key role of phosphorylation in regulating protein function, global site-specific phosphorylation analysis provides a mechanistic understanding of cellular processes. The ideal method would be universal ie characterize all protein phosphoproteoforms within a given sample or sample set. This analytical approach is termed phosphoproteomic analysis: with focus on the subset of proteins in the proteome that are subject to phosphorylation.

Current approaches for the analysis of phosphosites predominately employ 'bottom up' mass spectrometry (MS) based techniques for p-peptide analysis. In this approach p-peptides are either present endogenously or derived by a proteolytic digestion, step during sample processing (see Tutorial, section 2.5). Complex mixtures of p-peptides are analysed in a discovery-focused 'bottom up' approach, which couples liquid chromatography (LC) with tandem MS (LC-MS/MS). The reader is referred to an article by Wilson et al, 2018 [5], which explains the principles and key steps of phosphoproteomic analysis in a way designed to engage readers without prior knowledge. Discovery phosphoproteomics can operate in quantitative mode for comparative sample analysis. This is achieved by coupling p-peptide enrichment with 'standard' quantitative proteomic LC-MS/MS workflow coupled to p-peptides enrichment methods, see Tutorial section 2.8. Quantitative proteomic workflows include label-free quantification (LFQ), stable isotope labelling by amino acids in cell culture

(SILAC) or the use of isobaric chemical tags such as Tandem Mass Tags (TMT) and isobaric tags for relative and absolute quantification (iTRAQ) [6]. To date, the identification of phospho-serine/threonine (pSer/pThr) sites using MS technique has improved, but the determination of tyrosine (pTyr) sites is challenging because the abundance of pTyr is significantly lower than that of pSer/pThr [3]. The field of phosphoproteomic applications is wide and publications are numerous and thus we select specific examples to illustrate key principles and developments, signposting to publications that provide step by step protocols to guide users in the application of established protocols (see sections 1.4 and 2.7).

1.2 Phosphoproteomics: Technical challenges and the need for p-peptide enrichment

The analysis of protein phosphorylation poses significant technical challenges both at the level of sample preparation, and during the subsequent MS analysis. Phosphoproteoforms are typically present in low abundance relative to their non-phosphorylated counterparts, due to the occurrence of phosphorylation at sub stoichiometric levels in biological samples. Phosphorylated peptides tend to have low ionisation efficiency due to (i) phosphate groups tending to lose protons to carry negative charges, and (ii) the background presence of large amounts of unphosphorylated peptides [6]. Selective phospho-enrichment is thus essential and critical to success, enabling large-scale phosphoproteomic analysis. P-peptide enrichment is the first step in phosphorylation site analysis (Fig 1a). Nanoflow LC-MS/MS and data processing generate both the amino acid sequence (thus protein identity) and characterization of phosphorylation sites [7]. Data processing can be performed using publicly or commercially available bioinformatic tools [8, 9]. To support assessing these datasets, a range of tools that allow visualization of quantitative PTM proteomic datasets have recently been catalogued and reviewed [10].

1.3 Overview of a phosphoproteomic workflow – key steps

It is currently possible to identify thousands of different phosphorylation sites within a single phosphoproteomic experiment. The experimental workflow involves 7 key steps: 1) protein extraction; 2) proteolytic digestion; 3) p-peptide enrichment; 4) fractionation; 5) LC-MS/MS; 6) data analysis; 7) biological inference, as explained in a review article by Riley and Coon in 2015 [11]. The analysis of protein phosphorylation poses significant technical challenges both at the level of sample preparation, and during the subsequent mass spectrometry analysis, as reviewed by Leitner *et al*., 2016 [7].

1.4 Current practice for p-peptide enrichment

Methods employing affinity-based p-peptide enrichment selectively bind the negatively charged phosphate groups (phosphorylated site) of the p-peptide to metal ions or metal oxide. These methods are termed Immobilized Metal Ion Affinity Chromatography (IMAC) and Metal Oxide Affinity Chromatography (MOAC), respectively. There are 3 main steps in the protocol: (1) Incubation: capture of negatively charged phosphate groups; (2) Washing: removal of non-specific binding. (3) Elution: release of bound p-peptides (Fig. 1c). Figures showing typical p-peptide enrichment strategies are included for both IMAC (Fig 2.) and MOAC (Fig 3.) Fe^{3+} and Ga^{3+} are the most common ions used for IMAC enrichment, although additional metal ions have emerged over time. In MOAC, TiO_2 still is the most commonly used metal oxide, and shows a strong binding efficiency for p-peptides [14]. The range of metal ions employed for IMAC/MOAC are detailed in supporting Tables S1A, S1B. Numerous efforts have been directed towards improving the specificity and sensitivity of IMAC from different aspects. These include the optimization of operating protocols and the development/testing of novel metal ions, for efficient binding and effective affinity resins [7, 12, 13].

Elution of p-peptide requires disruption of the binding between the phosphate group and substrate that is based on reversible Lewis acid-base interaction [12, 13, 19, 20]. Elution of p-peptides from IMAC and MOAC materials is typically achieved by displacing

the negatively charged phosphate with a high pH, basic buffer, or by incremental step-wise pH; however, highly acidic solutions have also been employed [18]. When considering these two approaches, IMAC studies generally result in higher detection of multi-p-peptides, while TiO_2 enrichment results in a high identification number of mono-p-peptides. This relative lack of multi-p-peptide enrichment from TiO_2 has been attributed to the dissociation difficulty and thus incomplete elution of multi-p-peptides [14].

1.5 Scope for improvement

Bioinformatic assessment of the available data indicates that current strategies have not yet captured all predicted phosphosites, so there is still scope for further improvements [15]. Protein phosphorylation analysis primarily identifies O-phosphorylated amino acids, where phosphate binds to the hydroxyl moiety in the γ-group, specifically: pSer, pThr and pTyr, termed the “canonical” phosphorylation sites. The relative abundance of these sites within biological samples is typically pSer>PThr>pTyr [2].

To ensure comprehensive analysis of biologically relevant phosphorylation events, two levels of improvements are required – data quantity and data quality. Firstly, the breadth and frequency of phosphoproteomic studies, whilst increasing, requires expansion to fill the data gap that will allow a more comprehensive understanding of phosphoproteome dynamics. To support this, a tutorial is included in section 2 of this manuscript. This is designed to aid experimental design, preparation, and execution for new researchers in the field, whilst also providing a reference guide for their own investigations based on the latest developments in the field described later. Secondly, the data quality gathered from these studies must be improved; to provide better quality phosphosite identifications/localizations during proteoform identification from MS data [16]. This review focuses on improving data quality by reducing the background sample noise (non p-peptides) through advanced techniques and materials to optimize the enrichment process. Optimization of the p-peptide enrichment processes can be

achieved by mitigating non-specific binding, whilst enhancing selectivity (high affinity towards p-peptides), sensitivity (low starting amounts), robustness (tolerance toward harsh working conditions), high-throughput (less-time consumption) and reproducibility. Steps that can be taken to improve the performance of IMAC and MOAC are outlined, such as working condition optimization, novel carriers or hybrid material synthesis, along with other new developments.

2. Tutorial for phosphoproteomic analysis-general workflow design

This tutorial aims to provide a 'how to guide' for designing a strategy to profile the phosphoproteome, specifically selecting the most appropriate workflow for a given sample. The focus is on recent protocols, providing step-by-step descriptions of procedures, including details of p-peptide enrichment strategy, catalogued in Table 2. (Table 2). Despite advances, the protocols are generally complex and multi-step, taking several days to complete – this is exemplified in the study of Mertins *et al.,* 2018 that describes in depth, parallel processing of the phosphoproteome and proteome using Ni-NTA IMAC for p-peptide enrichment from human tissue samples [17]. When considering p-peptide enrichment, improved sensitivity, dynamic range, processing time, and cost are all practical considerations in workflow adoption. Before beginning p-peptide analysis, a few key questions should be considered to enable effective planning and execution.

2.1 Starting with a question

As with all proteomic investigations, it should begin with a biological question. P-peptides present in a sample can be catalogued post enrichment; so combining p-peptide enrichment with quantitative proteomics enables sample comparison. This generates relative abundance data and a discovery type proteomics dataset. The new user should be aware that this dataset will typically be a list of p-peptides and their associated proteins, likely to contain information on several thousand phosphorylation sites, with many potential avenues of investigation at the protein level alone – without a

well-defined investigation this can make the data analysis challenging. It is thus advisable to define simple and disprovable null hypotheses, then use bioinformatics resources (section 2.9) to visualise the data and infer biological significance. Once identified, a p-peptide may be identified to be altered in amount between samples. They may reflect an increase or decrease in phosphorylation, but may simply reflect altered protein abundance. Be aware that p-peptide enrichment is accompanied by a loss of information about the non-phosphorylated proteome component, so studies that combine proteomic and phosphoproteomic datasets with a high overlap between protein and phosphoprotein quantifications can be helpful here. A key benefit is normalization of phosphorylation, which can help account for protein abundance differences [18].

2.2 Experimental/process design

As with other proteomic analyses, experimental design is key, particularly the inclusion of experimental replicates. It is typical to include 2 or 3 experimental replicates for proteomic analysis, but it has been noted that overlap between replicates of the p-peptide enrichment same method can require 4-5 replicates to approach asymptotical gains in downstream MS analysis [19]. P-peptides typically only make up a small proportion of total proteomic sample, so enrichment strategies enable and improve detection. As discussed, be aware that different enrichment strategies can produce a bias towards p-peptides that contain a single, or multiple, phosphorylation sites and can therefore create bias or blind-spots in the data. Determining the full range of phosphoproteins in a sample requires testing and combining of a range of complementary enrichment protocols.

2.3 Planning

With these questions considered, here are a few rules of thumb that will assist practical planning and execution of a phosphoproteome investigation. In terms of ‘how long will it take’: time will vary based on the specifics of the experimental/sample details and

associated technical challenges; however, a preliminary experimental run to the final data analysis stage will provide a clear estimate of the minimum requirements. Breaking this preliminary investigation into the following stages can help track progress towards the final experimental procedure: Extraction; Protein digestion; Peptide fractionation; Enrichment; Mass Spectrometry and Data Analysis [11]. A typical phosphoproteomic experiment can take several days work to prepare samples and the amount of LC-MS/MS time is dependent on the choice of p-peptide enrichment strategy, the use of sample fractionation (see sections 2.6, 2.7). The choice of quantitative proteomic analysis method influences MS run time, see section 2.8 [20].

2.4 Extraction

The first test to perform is absolute protein quantification from extraction. A trial of this is advisable even if the 'best' procedure for the organism/biological system has already been identified.

(1) Lysis. Chemical or physical lysis methods can effectively fracture cells, these can include one or a combination of sonication, liquid nitrogen grinding, bead-beating, or boiling in surfactant. Some cell types pose specific technical challenges, for which customised protocols are often available, for example for plant tissues [21]. Samples can be degraded rapidly at room temperature, so all treatment processes should be performed on ice. Protease and phosphatase inhibitors are critical for preventing protein and phosphorylation loss, so these must be included in the lysis buffer [22]. Phosphate-rich nucleic acids reduce sample quality, so inclusion of nucleases such as benzonase is beneficial [8].

(2) Contaminants. Be aware of potential contaminants: for example - phospholipids, photosynthetic pigments, and secondary metabolites in plant tissues produce interference that increases the sample complexity, reducing efficiency and specificity of p-peptide enrichment [21]. Sample clean-up is therefore important for improving the data quality, with the associated benefit of reducing maintenance of LC-MS/MS

equipment. As a general clean-up consideration, p-peptide enriched samples must ultimately be compatible with the downstream LC-MS/MS analysis ie. free of salts and detergents [4]. Total protein can be precipitated using acetone, acetonitrile (ACN) [5], or methanol/chloroform [8] for 2 h or overnight at -20 °C

(3) How much protein? In general, a larger amount of protein will assist in the detection of very low abundance p-peptides, and provide more flexibility in the enrichment process. Typical starting amounts are 0.1 - 5 mg total protein for enrichment [23], but protocols have been developed to enrich for sub-femtomole level p-peptides from cell line and human tissue samples where samples are amounts are limited. A protocol applicable to picograms of starting material, is that applied to human leukocyte antigen class I p-peptides [24]. Protein concentration can be determined using traditional protein assays, for example, the Bicinchoninic Acid assay which can be applied to analysis of either proteins or peptides, as required by the user [17]. Note that not all traditional protein assay reagents are compatible with common interferences such as detergents and buffer components used in sample preparation for phosphoproteomic analysis.

(4) Quality at a glance. Evaluation of protein sample quality, amount and complexity can be simply performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: 1D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D gel electrophoresis. This also provides a simple one step sample clean up and fractionation process for GeLC-MS, which can form a component of the SILAC quantitative proteomic workflow.

2.5 Protein digestion

(1) Proteolytic enzymes: Proteins are subject to enzymatic digestion using enzymes - typically trypsin, Lys-C, Glu-C or Lys-N. Trypsin, alone or in combination with Lys-C, is the most commonly used cleavage strategy. Trypsin is the most commonly used proteolytic

316 enzyme for proteomic and phosphoproteomic analysis, due to its high proteolytic
317 activity and cleavage specificity C terminal to lysine and arginine. Tryptic (or Lys-C plus
318 tryptic) peptides possess *m/z* values and ionisation characteristics that are well suited to
319 LC-MS/MS identification.

320 In some cases, digestion with trypsin can be incomplete, particularly for tightly folded
321 proteins. The presence of a phosphorylation site can also result in missed cleavage,
322 which impact quantitative accuracy. Use of sequential digestion protocol using Lys-C,
323 prior to trypsin, results in fewer missed cleavages [25]. Lys-C and Arg-C can also be used
324 in combination to generate 'tryptic' peptides, but this is not in routine use. It is
325 interesting to note, that our survey of novel materials (sections 3.2, 3.3), found that of
326 51 studies employed trypsin or Lys-C, trypsin for testing p-peptide enrichment using
327 novel enrichment formats. P-peptide discovery, is currently centred on tryptic peptides,
328 may also be enhanced by the use of alternative proteases. An optimized, robust protocol
329 suitable for proteolytic digestion by alternative enzymes to trypsin, namely
330 chymotrypsin, Lys-C, Lys-N, Asp-N, Glu-C or Arg-C has been established [25]. The key
331 benefits generation of p-peptides with different physico-chemical properties to tryptic
332 peptides, results in improves coverage (detectability) of the proteome and
333 phosphoproteome [26]. Sequential enzyme digests, combined with p-peptide
334 enrichment increase p-peptide coverage, for example Glu-C in combination with trypsin
335 [27]. There are clear benefits to using alternatives to tryptic digestion.

336 (2) Efficiency of proteolytic digestion: Strategies to assess proteolytic digestion efficiency
337 include use of 'spike' internal standards and isotope dilution techniques [28, 29]. This is
338 important because bottom-up proteomics relies on the efficiency and reproducibility of
339 protein enzymatic digestion. The presence of missed cleavages and nonspecific
340 cleavages are important sources of variation in protein quantitation. It is important to
341 note here that phosphorylation of amino acid residues close to trypsin cleavage sites can
342 influence cleavage efficiency to result in missed cleavage [30]. Missed cleavages are

sequence context specific [31]. For example, the presence of proline hinders proteolytic cleavage. A novel enzyme, EndoPro, a proline directed protease that cleaves with high C-terminal site of proline and alanine residues, with a broad pH range of activity mitigates this limitation of trypsin. EndoPro, unlike trypsin, has an added advantage of being able to cleave in the presence of a neighbouring phosphorylation site [32]. As such this enzyme represents a valuable addition to the protease 'toolkit' for p-peptide enrichment workflows.

(3) Modes of proteolytic digestion: This sample processing step can be performed in gel, in solution or alternatively using filter assisted sample preparation (FASP). In gel and FASP strategies offer the advantage of sample 'clean up' during the digestion protocol. As an example, FASP has been applied for removal of nucleic acids, phospholipids, photosynthetic pigments, and secondary metabolites from plant tissues as part of the universal plant phosphoproteomic workflow [21].

(4) Sample clean up: typically performed at the peptide level, both before and after p-peptide enrichment, to ensure compatibility with MS analyses. Materials such as C18, graphite carbon and Hexagonal boron nitride are typically used for solid phase extraction (SPE) for enriching and desalting of peptides with a range of physicochemical properties. The performance of boron nitride is comparable to combined C18 and graphite carbon material, as discussed in a review article discussing protocols used for sample clean up procedures in proteomics [33].

2.6 Fractionation

Sample complexity can be reduced by high performance liquid chromatography (HPLC) separation at the peptide level [14, 15]. Peptides derived from complex samples particularly benefit from an independent ie 'offline' pre-fractionation step, in addition to the fractionation 'online' (also termed 'hyphenated') or 'coupled' to LC-MS/MS. The 'offline' fractionation step should be orthogonal to the traditional 'online' nano flow

reverse phase (RP)-HPLC employed in LC-MS/MS to ensure that different physico-chemical properties are exploited for maximum reduction in sample complexity. Since reversed phase 'online' fractionation is based on hydrophobicity, 'offline' fractionation typically uses charge based separation. In terms of 'best practice', a comparison of strong cation exchange (SCX), electrostatic repulsion hydrophilic interaction chromatography (ERLIC), and solution isoelectric focusing (sIEF) fractionation upstream of RP-LC-MS/MS analysis (in terms of identified p-peptide numbers) indicated SCX-LC-MS/MS > sIEF-LC/MS-MS > ERLIC-LC-MS/MS. There was partial overlap in the type of p-peptide and phosphosites, identified between methods, but also populations of p-peptides unique to each fractionation type, indicating complementarity [9].

Chromatographic materials are available in both column (ml min^{-1} flow rates) and tip-based format. Dehghani and co-authors evaluated different fractionation strategies following TiO_2 treatment for p-peptide enrichment, including column-based SCX (the most commonly used SCX approach), pipette tip-based SCX, concatenated high-pH reversed-phase (basic-RP), and column-based strong anion exchange (SAX). SCX methods produced higher observations of p-peptides than either basic-RP or SAX; with SAX showing a greatly reduced number of phosphosite relative to basic-RP [8]. Fractionation of p-peptides using pipette tip-based columns leads to similar results as the common approach using liquid chromatography-based methods [8]. The number of observed p-peptides from the pipette tip-based SCX were comparable with the number detected using the column-SCX method, but with the advantage of greatly reduced cost, time and complexity. Basic-RP is popular due to high peak capacity, reproducible retention times, and orthogonality to low-pH RP based separation, and is applied for peptide fractionation either pre or post p-peptide enrichment [17, 21].

'Offline' fractionation reduces sample complexity, which is always beneficial; but it has limitations. Fractionation requires higher amounts of starting material, and increasing the number of fractions for LC-MS/MS can lead to diminishing returns on improving the

quality of the data, at the expense of a linear increase in MS time and associated costs. While offline fractionation is designed to achieve higher depth of p-peptide coverage ie more identifications, samples can be run without a pre-fractionation step as a 'single shot'. It should be noted that in this case that the number of p-peptides may exceed the analytical capacity (typically 1 microgram) of the nano flow RP-HPLC column [8].Trial runs of unfractionated sample can typically be trialled against 5 selected fractions, to determine the optimal output for the investigation and identify fractions that provide the highest number of peptides (the typical peptide amount is approximately 1 µg). There is a great diversity of 'offline' fractionation types and formats, and so it is recommended that the best methods should be determined empirically to meet requirements of the study.

Recent developments have enabled detection and profiling of p-peptides containing histidine (pHis) and other non-canonical phospho amino acids (aspartate (Asp), arginine (Arg), lysine (Lys), glutamate (Glu), cysteine (Cys)) based on selection of buffers that maintain the acid labile phosphate. This approach, termed UPAX leads to unbiased p-peptide enrichment strategy for both canonical and non-canonical phosphorylated peptides using SAX chromatography at near-neutral pH (pH 6.8). This study enabled identification of 1300 His, Arg, Lys, Asp, Glu and Cys phosphorylation sites [34]. The data clearly indicated that pHis, Lys and Arg are present at a similar order to the numbers observed for pTyr under basal conditions in human HeLa cell extracts [34].

2.7 P-peptide enrichment

A range of methods are available, of which IMAC, MOAC, PolyMAC and antibody-based enrichment of PTyr are well established (Fig. 1b). The mode of operation is sequential steps: peptide capture (including non-specific binding of non p-peptides), washing to remove non p-peptides and elution of p-peptides (Fig. 1c).

Methods for which there are step-by-step guides, with detailed protocols and information on theoretical and practical aspects are listed with starting amounts of

material and p-peptide data generated (Table 1). The protocol of Mertins *et al*., [17], in common with the other protocols lists, first resuspends proteolytically derived, tryptic peptides in buffer containing 0.1% trifluoroacetic acid at pH 2, to ensure that the carboxyl groups of the peptide C termini, glutamic and aspartic acid are protonated. The negatively charged p-peptides preferentially bind to the resin relative to non p-peptides. As a general note, performing a test run to establish p-peptide recovery aids establishment of a method and ensures efficient use of expensive reagents and samples.

The ideal method would efficiently capture all p-peptides in the sample and result in full recovery of p-peptides from the capture material. New developments in the area are summarised in Fig 1d and described in detail in section 3.

2.8 Mass Spectrometry

(1) A typical LC-MS/MS run time employs 1-3 hours gradients of RP-HPLC operating at nanoflow rates. A typical sample loading is up to 1 microgram of p-peptide on column. Evaluating sample loss during the different steps in the sample preparation workflow, allows the amount of starting materials to be estimated to ensure sufficient p-peptide is available for analysis.

(2) The physical run time of the MS analysis varies, dependent on how complex the sample is, and the exact running parameters used on the HPLC and MS, as has been reviewed in depth [11]. In general, label free quantification methods, comparing MS data between consecutive LC-MS/MS analysis of samples and replicates require more MS time than methods using multiplex sample analysis eg SILAC (2-3 plex), isobaric chemical labeling (4, 8 plex iTRAQ, up to 11 plex TMT). The choice of quantitative phosphoproteomic method also impacts run time, quantitative precision and accuracy [6].

2.9 MS Data Analysis

It is important to ensure that the correct proteomic database is available for database searching. Database files are typically in FASTA format although this is dependent on the search engine being used, and long-form database formats can provide additional information during analysis. These databases can be readily obtained from SwissProt and NCBI public data repositories for organisms with genome sequence data. Be aware, if the genome for the organism has not been sequenced and annotated, or if the study is a metaproteomic analysis, these are both considered advanced investigations with much more challenging data analysis [35], particularly at the level of PTM [36]. It is advisable to not perform a first-time phosphoproteomic analysis on these systems unless advanced bioinformatic support and researcher expertise in phosphoproteomics is available.

A range of computational tools and MS data analysis for p-peptide and site localisation are available, which are well catalogued and described, alongside proteomic data analysis pipelines by Paul et al., 2019 [37]. Locard-Paulet *et al* [38] compared 22 pipelines for bottom-up phosphoproteomics analysis involving data base search tools (MaxQuant, Proteome Discoverer, PeptideShaker), search engines (Andromeda, Comet, Mascot, MS Amanda, SequestHT, and X!Tandem), and localization scoring algorithms (delta score, D-score, PTM-score, phosphoRS, and Ascore) for a defined dataset. Variability in outcomes led to a recommendation to report search and phospho-localization parameters when publishing datasets, so as to enable accurate integration of phosphosite assignment from different analysis pipelines. Further processing for the data, using tools such as the open-access biological pathway knowledge base Reactome [39] becomes quite specific to the study in question; so practicing the bioinformatics analysis using a pre-generated dataset from a similar previous study can be valuable.

3. Strategies for improving p-peptide enrichment

This section highlights and discusses the recent improvements to the enrichment process. It broadly divides these into improvements that focus on the buffers, the separation matrix materials, and methods to provide enhanced coverage of the p-proteome.

3.1 Buffers

3.1.1 pH

As the interactions between p-peptides and IMAC/MOAC materials are Lewis acid-base reactions, pH is a major consideration during the enrichment process. Non-specific binding of acidic peptides containing aspartate (pI=2.9) and glutamate (pI=3.0) emerged as a major limitation, as reviewed by Fila and Honys, 2012 [22]. This is caused by the affinity between the carboxylate moiety on the amino acid γ-group and metal ions materials emulating the phosphate to metal ion affinity. This non-specific binding can be reduced by decreasing the pH of loading buffer below the pI of aspartate or glutamate to remove the negative of charge of acidic residues by protonation. The pH of loading buffer is a critical consideration, since the number of bound p-peptides will also decrease at low pH due to protonation of phosphate (pKa=2.12) groups. Thus, co-purification of acidic peptides and p-peptides is unavoidable [22]. Contamination with peptides rich in acidic residues is more pronounced in complex biological samples, and negatively impacts the enrichment efficiency. To prevent the non-specific binding issues caused by acidic peptides, *O*-methyl esterification can be performed on the acidic residues; however, the additional steps can result in sample loss through both increased sample interaction and side reactions [14].

The choice of elution buffer and elution method can have a significant impact. A number of strategies employ a step-wise pH or buffer elution methodology, some recent examples of these include: 1-5% ammonium solution (pH 10-11, step wise elution);

bis-Tris propane (pH 11.3); two-step elution (ammonium hydroxide (NH_4OH) and bis-Tris propane) [15]; 100 mM ammonium bicarbonate (NH_4HCO_3) (pH 9.2-11.3 step gradient and pH adjust by ammonia) [16]; and 10 mM ethylenediaminetetraacetic acid (EDTA) buffers [17]. Alternatively, highly acidic solutions such as 1% v/v trifluoroacetic acid (TFA), pH1.0 [18] have also been employed. The reader is referred to the review article by Fila and Honys for more theoretical information and a review of traditional IMAC and MOAC techniques, with a specific focus on optimization of elution buffers for IMAC [22].

3.1.2 Optimized buffers for IMAC elution

Obtaining an ideal elution buffer is imperative for efficiently eluting the bound p-peptides from IMAC resins, and therefore high p-peptide recovery. The ideal elution buffer should have good elution efficiency (ideally 100%) and should be compatible with subsequent MS analysis. IMAC-bound p-peptides can be eluted by phosphate containing buffers 10 mM EDTA, or ammonium hydroxide (pH 10-11) [40]. Optimal ratios of sample to IMAC material, and the most efficient eluent solution, should be determined empirically on a case by case basis, depending on small- or large- scale starting materials.

3.1.3 Optimized MOAC buffers

The chemisorption of p-peptides and non-specific binding of acidic peptides is problematic for MOAC using TiO_2. Previous efforts have been employed to investigate the optimized working conditions for buffers. For instance, the application of additive acids as non-p-peptide inhibitors to solve the strong surface Lewis acidity issues of metal oxides. Generally, the order of binding interactions with metal oxides of TiO_2 is: phosphate group > organic acid > carboxyl group [41]. Therefore, the addition of organic acids competes for binding sites with acidic residues, to reduce non-specific binding and thus enhance TiO_2 and ZrO_2 p-peptide enrichment specificity [41]. A number of ‘non-phosphopeptide- excluding compounds’ have been identified, including 2,5-DHB and phthalic acid [41]. Hydrophilic and soluble glycolic acid and lactic acid were shown

to be preferable because of better compatibility with LC-MS/MS than 2,5-DHB [42]. Mono p-peptide enrichment is less efficient through using 1M citric acid, which is possibly caused by its similar binding to TiO_2 beads as mono p-peptides [41]. In general, the effect of added acids as non-phospho peptides inhibitors has been demonstrated to vary with the utilized materials [43]. For instance, TiO_2, ZrO_2, In_2O_3 and Fe_2O_3 showed significantly improved performance for p-peptides enrichment with the addition of lactic acid in the loading buffer. In contrast, NiO and SnO_2 exhibited reduced specificity for p-peptide binding in the presence of lactic acid [43].

Furthermore, researchers noted that peptides containing multiple glutamine and asparagine residues (named N/Q-rich peptides) predominantly co-enriched with p-peptides by IMAC or TiO_2, as determined by examination of amino acids distribution patterns [44, 45]. Notably, the portions of poly-N/Q peptides of non-p-peptides varied depending on the examined species. Amide containing compounds as buffer modifiers mitigate N/Q-rich peptides for efficient TiO_2 enrichment. As a result, addition of 125 mM asparagine and glutamine amino acid amides in the wash buffer (70% ACN, 3% TFA) and an optimum peptide/TiO_2 ratio (408 μg/mg) [45] resulted in a 30% increase in detected p-peptides number, as well as a 5-fold decrease in the intensity of non-p-peptides, without an obvious change in p-peptides intensities [45]. Thus, modifications to buffers are of proven value in optimization of p-peptide protocols.

3.2 P-peptide enrichment – optimization strategies

3.2.1 Optimization of IMAC materials

3.2.1.1 Development of novel IMAC materials

Novel IMAC formats with high valence metal cation-IMAC such as Ti^{4+}, Zr^{4+} [46], Nb^{5+} [47, 48], Hf^{4+} [49], or Sn^{4+} [50] have attracted increasing interest due to their high enrichment efficiency, reusability and relative low detection limit relative to the traditionally used Fe^{3+} or Ga^{3+} (supporting Table S1A). Jiang and colleagues compared the enrichment

efficiency systematically, by coating Fe_3O_4@PDA (polydopamine) microspheres with eight different metal ions. Nb^{5+}, $Ti^{4+}$and Zr^{4+} showed better selectivity, while Nb^{5+}, Ti^{4+} and Ce^{4+} displayed higher sensitivity than the other tested materials on tryptic digests of nonfat milk or β-casein [46]. Thus, Sn^{4+} or Nb^{5+} were proposed to show similar performance as Ti^{4+} in terms of higher selectivity and enhanced sensitivity [46]. This agrees well with the previous finding that high valence metal cations present better p-peptide binding capacity due to a higher coordination number. In addition, the synthesis of binary metal ions shows promise. For instance, the novel synthesized Fe_3O_4@PDA-Ti/Nb outperforms either single usage or physical mixture format [48]. Notably, it is still unclear how applicable these findings apply to true complex biological samples, as they were tested on casein protein standards, which are arguably not representative of all p-peptide types in phosphoproteomic samples.

3.2.1.2 Development of chelating ligands for metal cations immobilization

In addition to testing higher valence metal ions, the method of IMAC can also be modified by testing different affinity substrate supports and alternative chelating ligands. Acidic chelating ligands (iminodiacetic acid (IDA), nitrilotriacetic acid (NTA) have mostly been applied to date. To improve on drawbacks: such as limited specificity and metal ions loss issues of IDA and NTA; a phosphate group was introduced to immobilize Ti^{4+} or Zr^{4+}, but the time-consuming nature of this process limits its more general application (metal ion immobilization, enrichment, elution) and limits the absorbent surface area. Moreover, to enhance the poor selectivity caused by the presence of carboxyl groups of IDA or NTA, alternate IMAC affinity substrates have been rapidly developed (see supporting Table S1B for representative formats).

Developments include:

(1) The application of graphene with large surface areas and the introduction of a **phytic acid** (PA) molecule provide a benefit due to the presence of six phosphate groups with

579 metal ion coordination ability resulting in more affinity sites for IMAC. Moreover, the
580 excellent hydrophilicity of PA can further reduce non-specific adsorption [51]. Example
581 formats include MagG@PEI@PA-Ti^{4+} [51] (Fig 2a) which yielded a total of 574 p-peptides
582 from 341 phosphoproteins were detected from 200 μg of HeLa cells using MagG@
583 @PA-Ti^{4+} [51]. The inclusion of polyethyleneimine (PEI), a water soluble polymer
584 conferred HILIC properties to the IMAC material, enabling binding of N glycopeptides in
585 addition to p-peptides (see section 3.3.8).
586 (2) Recently, the employment of guanidyl group to modify superparamagnetic SiO_2
587 spheres significantly enhance the p-peptide capture specificity, e.g. PAMA-Arg
588 (polydopamine/poly(2-Aminoethyl methacrylate hydrochloride)/arginine) nanospheres.
589 With abundant guanidyl and amino groups on these brushes, the newly synthesized
590 nanospheres exhibited superior selectivity, sensitivity (10^{-12} M) and prominent
591 recyclability (signal intensity of multi-p-peptide remains 80% after 5 cycle usage) for
592 β-casein as well as biological samples (egg white, non-fat milk and rat brain lysate) [52].
593 (3) **PDA** benefits from the abundant amine and catechol hydroxyl groups it contains,
594 enabling the coating on diverse surface of organic and inorganic materials through the
595 self-polymerization of dopamine under a weakly alkaline environment [53], as shown in
596 Fig 2a-2b. For instance, with high content of Ti^{4+} chelated by PDA, Ti^{4+}@PDA@GA was
597 proven to be highly efficient for enriching p-peptides from a mixture of model
598 phosphoproteins (β-casein) and non-phosphoprotein (bovine serum albumin, BSA), milk
599 and spiked human serum [53], as shown in Fig 2b.

600 (4) Finally, researchers have pursued development of an easy-to-conduct process to
601 cope with limited surface area, time consumption cost, and weak coordination.
602 Compared to the conventional IMAC technique, various micro/nanoparticles including
603 magnetic core-mesoporous shell variants, mesoporous SiO_2 supported nanocomposites
604 show promise [54]. On one hand, the synthesized novel sorbents mostly exhibit
605 excellent performances for p-peptide enrichment due to the intrinsic and robust
606 magnetic properties, which were beneficial for rapid enrichment and separation of

p-peptides. Examples include the development of Fe_3O_4@mSiO_2-Ti^{4+} [47], magG@PDA-Hf^{4+} [49] as indicated in Fig 2c, magG@PDA-Sn^{4+} [50], and Fe_3O_4@SiO_2@GMA@IDA@Ti^{4+} [55], all of which benefit from the utilization of magnetic microspheres. On the other hand, the loss of magnetization with the increased number of shells is not negligible, particularly for those nanostructures using magnetic core-shells. In addition, interference may be caused by interactions between the magnetic core and e.g. an acidic Ti^{4+} attachment medium [56]. The creation of MagSiO_2@SiO_2@PDA@Ti(IV) [56] mitigates this since the porous SiO_2 shell layer was introduced to protect magnetic core from the acidic medium of Ti^{4+} attachment. With stronger and stable magnetic responsiveness, these microspheres indicate good (detection limit of 50 fmol/mL) and stable reusability performance on five successive enrichment cycles for enriching p-peptides from the tested samples of tryptic digests of a β-casein/BSA mixture and human serum. Although, the drawback is that the synthesis of these solid supports has been shown to be time-consuming due to the separate covalent attachment of the functional groups into the sorbent structure, as well as a tedious post-derivatization process.

Efforts are developing toward the “green” synthesis of materials, which is easy-to-conduct and show excellent enrichment performance: sensitivity, selectivity and robustness.

3.2.2 Optimization of MOAC

One of the advantages of MOAC over IMAC is that the oxide form of the ion is more stable than the metal ion form and has better tolerance to salts, detergents and solvents, under operational pHs and temperatures, as well as good sensitivity and selectivity [57]. Intensive studies have been performed for p-peptide enrichment using metal oxides such as listed in supporting Table S2. Key factors affecting MOAC binding performance (Fig. 1b) towards p-peptides include properties of the material: surface area, pore size,

isoelectric point (IEP) and magnetic properties, and the presence of additives [43, 58, 59]. Among these, IEP is considered to be the most critical parameter for the enrichment performance of affinity materials. The widely used TiO_2, ZrO_2 and In_2O_3 have an IEP around of 6, which has been determined to be optimal for p-peptide enrichment [43]. TiO_2 -based approaches have been widely applied for p-peptide purification, due to higher selectivity and specificity (in terms of the number of identified p-peptides based on peptide counts) as well as robustness, amphoteric ion-exchange characteristics, and tolerance towards many regents (stable in wide pH ranges) [43, 57].

3.2.2.1 Novel Synthesized Metal Oxides

Commercially available TiO_2 is the most commonly used material for MOAC, but there are a number of new metal oxides that show promise. Such materials, include molybdenum (VI) oxide (MoO_3) nanocomposites coated on graphene oxide (MoO_3/GO) [60], In_2O_3 [43], and 1,3,5-benzenetricarboxylic acid (H3btc) [61]. These oxides can show greatly enhanced sensitivity to p-peptides, for example H3btc grafted on the PDA-coated Fe_3O_4 (Fe_3O_4@PDA@Er(btc)) achieved a detection limit of 20 amol/μL, and high efficiency when tested in human serum, making it a very promising candidate for phosphoproteome research [61].

3.2.2.2 Developments for MOAC -novel ligand supports

As with IMAC, specificity is still a bottleneck for MOAC enrichment, but improvements to the supporting sorbents can help to address this. A variety of micro/nanostructures have been developed aimed at high sensitivity and selectivity towards p-peptides. Further details are available in supporting Table S2. Whilst issues arising from the high Lewis acid strength of metal oxides can be addressed by application of diverse acids in loading and washing buffer; the mono p-peptide affinity and “shadow effects” in MOAC may be caused by the small and deep pores of metal oxide/s. These small crystallites can hinder the release of p-peptides, as reported for mesoporous TiO_2 [62], but can be mitigated by

using synthesized yolk-shell magnetic materials, as observed in Fe_3O_4@H-fTiO_2 (Fig. 3a) and Fe_3O_4@H-TiO_2@f-NiO [62, 63].

Supporting composites with high surface area have attracted interest due to their potential for greatly enhancing the loading capacity, which contributes improved p-peptide enrichment. Examples include coordination with GO, for GO-trimethyl-2-methacroyloxyethyl ammonium chloride-titania monolithic column (GO-META-TiO_2) [64] and F-TiO_2-GO (Fig. 3b) [65]. Supporting resins with high surface area also result in higher p-peptides coverage, favour the isolation of large p-peptides, and lead to the detection of high number p-peptides with higher abundance. For instance, the application of graphitized carbon black (mGCB@TiO_2) led to 48% enrichment coverage (p-peptides / total peptides), elution of 10% of the common p-peptides and favours detection of p-peptide >3 KDa, while commercial spin column resulted in the detection of 40% enrichment coverage but only 1% high abundant p-peptides, with p-peptide <2.5 KDa [66]. Another example is Fe_3O_4@H-fTiO_2 (yolk-shell magnetic nanoparticles modified with macro/mesoporous TiO_2 nanosheets). Compared to hollow magnetic mesoporous TiO_2 (Fe_3O_4@H-**m**TiO_2), the high p-peptide enrichment performance of hollow magnetic macro/mesoporous TiO_2 nanoparticles (Fe_3O_4@H-**f**TiO_2) is attributed to the high surface area and large pore volume owed by the porous nanostructure and large hollow space [62].

Improvements towards reducing the non-specific adsorption of acidic and other peptides during MOAC have been developed. One practically demonstrated example is the introduction of a functional group with high enrichment affinity toward p-peptides or interface to the metal oxide to eliminate the non-phosphorylated peptides. For instance, the introduction of a guanidyl-functionalized group into TiO_2 (F-TiO_2-GO) improved p-peptide binding for TiO_2 [65]. Another example is integration of a hydrophilic fructose-1,6- diphosphate (FDP), which acts as a modifier to regulate the surface properties of the diphosphorylated fructose-modified dual-metal-centred zirconium

687 (DZMOF) that inhibited non-specific binding from other peptides [67]. It is proposed to
688 benefit from the strong interaction between FDP and metal sites of DZMOF that
689 produces a high anti-interference performance for eliminating the non-phosphorylated
690 peptides [67]. This modification is thus an effective development for the enrichment of
691 p-peptides [67]. The negatively charged surface (pH 5-11) of nanocomposites
692 PI-Fe_xO_y-ZrO_2 was inferred to function (repulsive effect) to reduce the non-specific
693 adsorption of acidic peptides [68]. Through co-doping of magnetic Fe_xO_y partial and ZrO_2
694 nanoparticles on polyimide, PI-Fe_xO_y-ZrO_2 composite indicate uniform mesopore size of
695 ca. 3.9 nm, which blocks the entrance of protein but allowing the penetrance of
696 p-peptides into pore channels, efficiently reducing non-specific protein adsorption [68].
697 PI-Fe_xO_y-ZrO_2 exhibit better adsorption capacities towards the tryptic p-peptides from
698 human serum or BSA/β-casein mixture: p-peptide signal intensity was increased by
699 tenfold and a large number of p-peptides detected [68]. In summary, research in this
700 area has provided significant gains in performance.

701 For ease of flexibility in operation and application, efforts have been directed to provide
702 materials with wide pH range or elution conditions, highly acidic/alkaline or hydrophobic
703 conditions tolerance and more efficient p-peptide capture. An example format is
704 CIM-OH-TiO_2 column (CIMac™ hydroxyl-based analytical column with immobilized TiO_2
705 nanoparticles), which offers higher contact area with p-peptides than is achieved using
706 the traditional macroporous format [69]. Magnetic nanocomposites are attracting
707 interest from the proteomics researchers; a detailed analysis of their benefits and
708 limitations is provided by an overview article by Batalha et al [70].

709 **3.2.3 Physical configuration considerations**

710 A number of different configurations exist for operating MOAC-TiO_2, these include spin
711 column, analytical column, miniaturized column, batch format, nanoparticles, magnetic
712 beads and p-peptide-affinity MALDI (matrix-assisted laser desorption ionization) plates.
713 The column treated with manual pipetting is non-magnetic, and continuous mechanical

manual operation is needed during the enrichment procedure. This is very labour-intensive, and thus less time efficient. Spin columns are easier to operate than the manual column format, but are limited by both volume and column clogging issues. Magnetic format material is generally superior, because of the practical ease of magnetic separation, the avoidance of packing columns for material preparation, and much simpler manipulations for the loading, washing, and removal steps. Where applicable, nanoparticles are more effective than micro-particles due to their higher surface area. As an example, ZrO_2 packaged tips were shown to be superior to TiO_2 for mono p-peptide enrichment; however, these differences were found to be negligible once the material size was comparable [43].

3.2.4 Optimization of composition and structure of carriers and chelates

Efforts have focussed on exploring the diverse composition and structure of carriers and chelates of the support resins. These include different formats such as magnetic beads, MALDI plates, columns, tips and gels. In addition, researchers have improved and optimized diverse reaction conditions for different materials, for instance, the application of PDA-grafted hybrid magnetic particles for Fe_3O_4@PDA-Ti, Fe_3O_4@PDA-Nb or Fe_3O_4@PDA-Ti/Nb [48]. For highly efficient enrichment, batch- and tip-based approaches require further optimization. Developments of new supporting formats are necessary for enabling high-throughput approaches, due to the multiple manual handling steps required for these protocols. A few exciting new examples of these support formats include: the lab-in-syringe mode of Ti^{4+}@PDA@GA (graphene aerogel, GA)[53]; the TiO_2 nanoparticle packed channel array glass microchip [71]; instrument-free TiO_2-modified filter paper-based analytical device [72]; “green synthesised” Ti^{4+}-IMAC carbonaceous spheres using glucose, vinylphosphonic acid and water solvent [73] automated p-peptide enrichment and desalting tip set up [74]. All of these methods are time and cost effective, compared with the commonly used batch mode or micro-column format packed materials.

3.2.5 Co-doping of metal oxide/ion with metal oxide

The synthesis of hybrid materials based on the distinct selectivity of metal ions or metal dioxides to p-peptides is becoming an area of interest; with a typical diagram of the p-peptide enrichment strategy shown in Fig. 4.

Remarkably, composites containing different metal oxide/ions precursors demonstrated effective p-peptide enrichment capacity (supporting Table S3, for instance, TiO2/Bi/Fe/Zr [58], B0.15F0.15TNs [59], and MnFe2O4 MAMSs [75]).

Compared to the commercial available TiO_2, the co-doping of metal (ions Bi^{3+}, Fe^{3+} and Zr^{4+}) with TiO_2 nanocomposite results in an increased surface area, which leads to a lower detection threshold for casein/BSA, and a higher detection number of p-peptides (26% more for HeLa cells using TiO_2/Bi/Fe/Zr) [58] and phospho-sites (two-fold more for tissue protein extract from human liver using $B_{0.15}F_{0.15}$TNs) [59]. Similarly, the combined usage of ferric and manganous ions as precursors, in the novel synthesized MnFe2O4 MAMSs microspheres, showed a higher selectivity for p-peptides than Fe3O4 nanoparticles or MnOOH nanosheets individually [75]. Similarly, co-fabricating Zr and Ti simultaneously as a $(Zr\text{-}Ti)O_4$ composite [76], or Al_2O_3 with either La_2O_3, CeO_2, ZrO_2 or TiO_2 [77] proved to be superior to the individual metal oxides, demonstrated by their stronger specificity and higher selectivity for p-peptide enrichment.

Synthesized IMAC/MOAC hybrid materials also provide good adsorption capacity, which improves p-peptides enrichment capacity. This was demonstrated with the magnetic nanoparticle Fe_3O_4@nSiO_2@mSiO_2/TiO_2-Ti^{4+} (Fig. 4a), where β-casein p-peptide numbers were six times higher than Fe_3O_4@TiO_2; and more p-peptides were identified from human serum than IMAC (Fe_3O_4@nSiO_2@mSiO_2/Ti^{4+}) or MOAC (Fe_3O_4@nSiO_2@mSiO_2/TiO_2) alone, whilst providing equal detection for peptides with single and multiple phospho-sites [78]. These nanoparticles are constructed from a magnetic Fe_3O_4 core, which is stabilized by a supporting nonporous silica layer (@nSiO_2). A mesoporous silica layer (@mSiO_2) then provides large surface area, to which metal ions/oxides – in this case TiO_2 and Ti^{4+} (/TiO_2-Ti^{4+}) – are chelated by specific linkers.

3.2.6 Efficient coupling molecules for hybrid composites

Similar to the optimization on supporting ligands for IMAC or MOAC, enhanced p-peptide enrichment efficiency can be acquired through the incorporation of different coupling molecules for hybrid composites. These coupling molecules improve trapping affinity through improved covalence with metal ions, provision of charge-based selectivity, or provision of a porous binding environment with high surface area. Examples of these include: (1) DOTA (1,4,7,10-tetraazacyclododecane N, N', N'', N'''-tetra-acetic acid), which provides enhanced covalently binding with Zr^{4+}. The resulting TiO_2@DOTA-Zr (Fig. 4b) has demonstrated strong p-peptide trapping affinity [79]. (2) The coordination of polyacrylate (PAA) with Ti/TiO2 presents strongly hydrophilic carboxyl groups for PAA-Ti/TiO2, which prevents the non-specific binding from non-p-peptides [80] and resultant good selectivity. (3) Another example is the employment of TiO_2 as inner shell and flowerlike NiO as an outer shell for Fe_3O_4@H-TiO_2@f-NiO. The porous nanostructure and large hollow space endows it with a high surface area, large pore volume and a better enrichment performance than Fe_3O_4@H-TiO_2[63]. (4) In addition, contributions from superparamagnetism and ordered mesoporous channels of (magnetic) graphene into enhanced p-peptide affinities has been demonstrated for G@TiO_2@$mSiO_2$ [81] (as shown in Fig. 4c) and magG/PD/(Zr-Ti)O_4 [76].

Similar to the novel synthesized particles, the excellent enrichment performances of hybrid composites is inferred to be: (1) the combined advantages of various materials, for instance, the dual metal centres of DZMOF containing both inherent Zr-O clusters and immobilized Zr (IV) contribute greatly to its high selectivity [67]. (2) The increased surface area and pore volume. (3) The existence of abundant carboxylate groups in the synthesized materials compared to the single strategy. Disadvantages lie in the limited surface area and affinity sites caused by the irregular morphology, or single pore structure generated from the simultaneous reaction of the metal oxides.

3.2.7 Phosphate-binding molecular tag chromatography (Phos-tag)

The design of Phos-tag is based on the phosphate binding catalytic domain of alkaline phosphatase. Phos-tag molecules are anchored to a separation gel matrix that peptides are run through. P -peptide are trapped by the immobilized Phos-tag in the separation gel, and migrate more slowly than their non-phosphorylated counterparts, allowing p-peptides and non p-peptides to be separated due to their relative electrophoretic mobility [82, 83]. Interaction of phospho-sites with the Phos-tag reagent is similar to IMAC, with application to p-peptides and phosphoproteins [82-84]. Both techniques are based on the binding between the negative charge of phosphate and positive charge of ions. P-peptides are captured by the immobilized ions under acidic conditions and eluted by basic solutions such as ammonium. The major difference between the Phos-tag workflow and that for IMAC is in the pH aspect. For instance, the working pH for Phos-tag is alkaline or neutral, while the pH for IMAC is acidic (pH < 3). A benefit of the Phos-tag approach is the ability to capture both canonical, acid stable phosphorylated Ser/Thr/Tyr; and non- canonical, acid labile phosphorylation of His/Asp/Lys due to operation at neutral phosphate. Recently, a small number of p-peptide enrichment studies have been carried out to improve enrichment efficiency using the Phos-tag approach under different incubation and elution buffers, such as the exploitation of Phos-tag-based micropipette-tip format, and the incorporating of two zinc metal ions into the acrylamide-pendant Zn^{2+}-Phos-tag SDS-PAGE [82, 83]. This application of Phos-tag strategy suffers from limited separation ability and alkaline buffer instability. Using the Phos-tag as an additive in SDS-PAGE can also enable differentiation of phosphorylated and non-phosphorylated forms of a protein, as the electrophoretic mobility is altered due to the binding of the Phos-tag. This also enables separation of differently phosphorylated proteoforms in proteins with multiple p-sites [85].

3.2.8 Polymer-based metal ion affinity capture (PolyMAC)

For PolyMAC (polymer-based metal ion affinity capture) technique, p-peptides chelate to metal ion-functionalized soluble nanopolymer, and thus isolated in a homogeneous aqueous environment [86]. In brief, this protocol employed the following steps (1) the ions (e.g. Ti or Fe) were immobilized on soluble polymers for fast chelation; (2) then, the PolyMAC and p-peptide complexes were covalently coupled to the solid support (e.g. agarose beads); (3) finally, gel was washed and p-peptides were eluted by using ammonium hydroxide solution [86]. Compared to the commonly used solid-phase extraction method for p-peptide capture of IMAC or MOAC, PolyMAC utilize soluble functionalized dendrimers for p-peptide binding [86, 87]. Previously, a PolyMAC-Ti technique has been demonstrated to show superior reproducibility for p-peptides enrichment than IMAC or TiO_2 for cancer samples [11]. Later, PolyMAC enrichment using Fe demonstrated better selectivity and specificity than Ti, the combined PolyMAC-Ti and PolyMAC-Fe provided complementary information for B cell phosphoproteomic analyses [88]. There are also few reports on the use of hydroxyapatite chromatography for p-peptide enrichment [89]. Although these studies (using Phos-tag, PolyMAC and hydroxyapatite chromatography) offer some benefits, they have not been widely tested in complex samples; therefore, their superiorities to IMAC and MOAC at this stage have not been conclusively demonstrated.

3.3 Improving phosphoproteome coverage

3.3.1 Enhanced multi-p-peptide enrichment

Techniques have been developed for efficient enriching of proteins with high levels of phosphorylation, specifically peptides containing two or more phosphorylation sites, as a result of the biological importance of these peptides/proteins [14]. Capturing these multiple phosphosite peptides is challenging; due to both enrichment material capacity limits, and the high affinity between enrichment materials and phosphates that result in incomplete peptide elution [90]. An example of this high affinity, caused by electrostatic

interactions between Ti^{4+} and multiple phosphosite peptides, was seen in Fe_3O_4@SiO_2-PLP-Ti^{4+}, where low recovery rates of multiple phosphosite p-peptides were observed [91]. Multiple phosphosites on a peptide decrease the IEP; which can affect how the peptides are eluted. Furthermore, the MS detection and sequencing of these peptides is limited – particularly in a background of highly abundant non p-peptides and mono p-peptides. The additional phosphate residues reduce fragmentation efficiency, so the primary sequence of the peptide can be more difficult to determine; and the multiply charged peptides have severe suppression of ionization efficiency, which is caused by the co-existence of high abundance.

A selection of techniques has been employed to address multi-phosphorylated peptides issues, including:

(1) Improving operating conditions for conventionally used materials. Bae and colleagues noticed that selectivity of hydrazide functionalized monodispersed silica microspheres (HFMSM) [92] towards single or multiple p-peptides varied with the presence of different concentrations of formic acid (FA) in loading buffers. Lower pH/high acidity reduces the binding capacity of HFMSM towards both mono p-peptides and non-specific binding from acidic peptides. A higher concentration (1%, pH 2.4) of FA favored the enrichment of multiple p-peptides, whilst a lower concentration (0.02%, pH 3.2) promoted detection of both [92].

(2) The development of multi-p-peptides high binding capacity materials. Ti^{4+} ions have affinity towards single p-peptides, while mixed Ni^{2+}/Zn^{2+}, Ga^{3+} and Nb^{5+} ions show a preference for multi p-peptides. Compared to Ti^{4+}-ATP (adenosine tri-phosphate)-MNPs, Ga^{3+}-ATP-MNPs [93] was demonstrated to have higher selectivity and improved coverage (30% more) towards multi p-peptides from the tested rat liver mitochondria. It is speculated to be caused by the highly hydrophilic surface, where the immobilized Ga^{3+} provides suitable chelating strength for multi p-peptides, e.g. Fe^{3+}-IMAC gel *vs* Fe_3O_4@SiO_2-PLP-Ti^{4+} [91]. Interestingly, simultaneous analysis of mono- and multi-

p-peptides is possible through the use of cerium-based nanocomposites: P-CCF (PEG–Ce/CeO_2–Fe_3O_4) can be used to extract mono p-peptides, and a CSF (Ce/CeO_2–SO_4^{2-}/Fe_2O_3) probe can selectively enrich multiple p-peptides [94]. This technique benefits from the tight interactions between positively charged metal ions (Fe^{3+} and Ce^{4+}) and the negatively charged phosphate group; and de-phosphorylation catalysed by CeO_2 [94].

(3) Design and introduction of multiple phosphate recognition units. Multi p-peptide enrichment for Zn^{2+}-dipicolylamine complex-coated magnetic microspheres (ZnMMs) is enhanced due to both the large number of binuclear ZnDpa (the artificial receptor) binding sites, and a strong magnetic responsiveness on the microsphere's surface [95]. Another example is the introduction of hydrogen bonding smart copolymer, which can modulate the adsorption/desorption of multi-p-peptides on enrichment material [90]. Most of these techniques that have been applied have been limited to the determination of peptides containing 2 or 3 phosphosites [14].

(4) Decreasing nonspecific binding through hydrophobic interactions. This can be achieved through the introduction of phosphate ion pre-coordinated Ti^{4+}-IMAC [96]. The application of hydrazide, the amine-based functional group, aids the capture of p-peptides through electrostatic attraction and hydrogen bonding [92]; phosphoric acid-modified DZMOF showed improved selectivity toward multi p-peptides from both model proteins (α- and β-casein); and complex biological mixtures, where the identification number was increased from 605 (18.6%) to 1871 (70.1%) in HeLa cell lysate digests [67].

(5) Combined usage of different materials or approaches. This approach is unavoidable as a benchmarking technique, as the overlapping p-peptides ranged from 59% to 79% when using the same materials for independent experiment, and jointly identified p-peptides only ranged between 16% and 52% when different MOAC materials were applied [43]. Individually, TiO_2 shows higher affinity than phosphotyrosine selective

molecularly imprinted polymers (MIP) under identical experimental conditions [85]. However, TiO_2 has certain limitations; for instance, it shows a bias towards identification of mono p-peptides, which will result in the incomplete information for the multi p-peptide section of the data. In addition, the matrix components of MOAC may hinder the successful purification of p-peptides, as pointed in the study of human whole blood samples or acute myeloid leukemia samples, where Ti^{4+}-IMAC and Fe^{3+}-IMAC outperforms either TiO_2 beads, spin columns, or the graphitized carbon black-TiO_2 composite [97] toward multiple p-peptide detection. Thus, the combined application of IMAC and TiO_2 termed SIMAC (sequential elution from IMAC) was reasonable [14]. Briefly, to increase the identification coverage of multiple p-peptides, the acidic and IMAC flow-through elution, which contains non-p-peptides and mono-p-peptides, were pooled and submitted for further TiO_2 treatment, while the basic elution was analysed directly by LC-MS/MS [14]. Although SIMAC leads to more identification of multiple p-peptides [44], low enrichment efficiency from SIMAC (possibly due to the poor performance of MOAC) using an acute myeloid leukemia sample was found [44, 98].

In conclusion, combining the advantages of different techniques, e.g. SIMAC strategy [14] or the synthesis of hybrid materials, the binary materials are promising for providing complementary phosphoproteomic information because of the excellent affinity towards both single- and multiple- p-peptides.

3.3.2 Michael addition

Compared to affinity-based enrichment methods, chemical modifications have high specificity [7]. There are a range of chemical modifications available, including β-elimination, carbodi-imide condensation, oxidation-reduction condensation, and α-Diazo resin; which all functionally replace the phosphate group with another chemical group that can be targeted specifically. A typical example of this is β-elimination, in which the phosphate group of phospho -serine or -threonine is eliminated through the addition of basic solvents containing Ba^{2+} or Ca^{2+}, or cations of the lanthanide group [7].

This process is coupled with the addition of propanedithiol (the Michael addition), which covalently binds to thiols and introduce free SH groups for enrichment. Phosphate elimination during MS analysis prevents neutral loss, retains the intact peptide sequence-which is useful for identification - and increases the ionization efficiency for MS positive ion mode [99]; however, chemical treatments can result in both sample loss, due to multiple reaction steps, and the increase in sample complexity resulting from incomplete and side reactions [7].

3.3.3 Anti-tyrosine antibodies

As mentioned above, pTyr sites make up a smaller fraction of the p-proteome, and are often under-represented due to sampling bias. Commercially available anti-tyrosine antibodies have a high affinity for pTyr and can be used for selective enrichment of pTyr peptides; but poor reproducibility, low sensitivity, and limited availability/variability of antibody/bulk starting materials limit enrichment capacity - particularly for complex peptide mixtures [100], and high costs limit their wider application [101]. To solve the affinity specificity and quantification accuracy issue caused by sequence bias, pTyr antibody cocktails (combined different pTyr antibodies together) have been proposed [102]. A replacement biological-capture method has also been reported: where a pTyr super-binding protein domain, created by introducing 3 point mutations into the pTyr binding pocket of the pTyr binding SH2 domain of the Src protein, captures the pTyr peptides, which are then eluted using a competitive elution agent, biotin-pYEEI (pTyr-Glu-Glu-Ile) [101]. This technique, however, is limited by the biotin-pYEEI used to elute pTyr from the SH2 super-binder, as it must be removed from the sample before LC-MS/MS analysis. The additional purification step results in significant sample loss [103]. The low recovery of these biological enrichment methods limits their applications to untreated cells or tissue samples with much lower pTyr levels.

3.3.4 Incorporation of phosphate recognition artificial receptors

Compared to conventionally used ligands, the incorporation of the binuclear Zn^{2+}-dipicolylamine complex-coated ZnDpa as a phosphate-selective artificial receptor, greatly improved the p-peptide enriched efficiency [95]. As a result, the detection limit through using ZnDpa was 250 fmol for β-casein. Advances of ZnDpa are high affinity and specificity toward phosphate groups, contributing to the resolution of non-specific binding from acidic or basic residues issues [95]. The introduction of artificial receptors with the incorporation of materials with multiple binding sites may shine light on the interpretation of comprehensive phosphoproteome research. However, this enrichment testing was only carried out towards p-peptides from tryptic digests of standard proteins β-casein/BSA, and as mentioned above, requires further validation with true (more realistic) biological samples.

3.3.5 IMAC or MOAC with antibody-based treatment

Possemato et al. reported that only less than 5% overlapped phospho-sites were obtained upon the application of TiO_2 and immunoaffinity precipitation (IAP) with different antibodies (pY-, pAKT/AMPK-, pATM/R-, and pST) treatment, which indicate a pS:pT:pY ratio approximated at 90:10:<1 for TiO_2, but 51:29:20 for IAP data [104]. The low overlap between IMAC, MOAC and pTyr antibody has also been observed elsewhere [15], indicating the importance of a combined strategy. The SH2 super-binder enrichment strategy has been combined with IMAC with Ti^{4+}-IMAC [103], and an updated one-step SH2 super-binder method [105], as well as in a biphasic affinity chromatography approach, where Src SH2 super-binder was coupled with NeutrAvidin affinity chromatography, which resulted in enhanced specific selectivity [106].

3.3.6 Use of IMAC in combination with other techniques

Previously, the incorporation of TiO_2 with SIMAC has been found to improve the phosphoproteome coverage [107]. Further, the application of combining of different IMAC metals: (tandem IMAC: IMAC-IMAC) or the combining of IMAC with other

p-peptide enrichment techniques (e.g. Fe-IMAC with p-peptide precipitation by $CaCl_2$, pTyr phosphotyrosine immunoprecipitation and methyl esterification, as well as β-elimination) have been proven to be superior to the one step IMAC enrichment approach [14].

3.3.7 MOAC-TiO_2 with other techniques

The combination of phosphotyrosine-imprinted polymer with TiO_2 (pY-MIP-TiO_2) shed light on the study of pThr and pTyr, based on the finding that pY-MIP-TiO_2 protocol caused comparable identification numbers with TiO_2 alone, with enhanced ion signal intensities for pThr and pTyr, but not pSer [100]. Moreover, the incorporation of alternative β-elimination followed by the Michael approach is required for the precise assignment about the location of multisite-phosphorylated (especially higher than triply-phosphorylated peptides) Ser/Thr-rich regions after the MS detection of p-peptides using TiO_2 treatment [108], for which the presence of high number of phosphorylated residues on the same peptide decrease the IEP and thus challenge MS sequencing and detection. In addition, the combination of conventional TiO_2 with novel developed hydrogen bonding-based polymeric material was recommend for p-peptides enrichment [90], from which the latter may facilitate the discovery of high proportions of pThr and pTyr, particularly from multi-p-peptides.

3.3.8 Simultaneous detection of p-peptides and other PTMs

Phosphorylation and glycosylation are of the most ubiquitous PTMs, which are highly associated with various biological processes. Previous studies demonstrate crosstalk between the two PTMs [109], and so efforts have been made to improve the simultaneous detection of phosphorylation and other PTM, for example glycopeptides. A typical diagram of p-peptide and glycopeptide enrichment is shown in Fig. 5. Co-enrichment can be achieved either through using materials with various capabilities for different PTMs enrichment, or through fabricating nanomaterials that possess properties of both p-peptide enrichment (IMAC or MOAC) and glycopeptide enrichment by using affinity-based materials. These materials are shown in Table S4, and include

amino functioned [110], hydrophilic interaction liquid chromatograph for hydrophilic interaction [111], and boronic acid affinity for SPIOs@SiO_2@MOF (boronic acid-functionalized magnetic organic framework Zr-MOF nanocomposites, as shown in Fig. 5a) [112]. The good biocompatibility, excellent hydrophilic property and a large amount of Ti^{4+} endows the *de novo* synthesis of nanomaterials with great promise for the identification of low abundance N-glyco /p-peptides. The material can be 'tunable' for p-peptide or N-glyco peptides ie be used for simultaneous or independent selection steps using different enrichment conditions, exemplified by application MagG@PEI@PA-Ti4+ to HeLa cell extracts [51].

Other approaches utilize the affinity between glycan chains and TiO_2 or NH_2 hydrophilic interaction such as SiO_2-NH_2@TiO_2 hollow microspheres [113], TiO_2@SiO_2-$B(OH)_2$@Fe_3O_4@ TiO_2 sandwich-like nanosheets [114] (as shown in Fig. 5b), Fe_3O_4@Au-$B(OH)_2$@mTiO_2 core-shell microspheres [115], TiO_2-NH_2 modified MALDI plate (stability and reusability) [116] (Fig. 5c). These enhanced materials will help shed new light on phospho- and N-glyco- proteome research.

4. Summary and Perspectives

Challenges remain to establish the key goal of a single step enrichment that comprehensively captures all p-peptides in a sample, whilst also generating a sample that is compatible with LC-MS/MS with minimal processing. As the p-peptide/p-peptide plus other PTM enrichment efficiency varies between different techniques, it is strongly recommended that a chosen method is necessarily optimized for each new sample. It is important to decide on the focus or target of the enrichment in terms of the specific research question, since, in terms of current methodologies, "one size" may not fit all. The key conclusion from reviewing the latest literature in this field is that phosphoproteomic coverage is significantly improved by a combination of different methodologies. Specifically, the coverage of enrichments with different materials (ions or oxides) is higher than that for replicates with the same material. Hybrid materials synthesized from metal ions/oxides

materials synthesized from metal ions/oxides showed excellent enrichment performances compared to the component parts. Studies with novel formats are provided in Table format (Tables S1-S4) for 51 publications, with information on the material, test samples (tryptic or Lys-C, trypsin digests), reported sensitivity and selectivity, sample matrix and MS instrumentation. This finding appears to be the result of the synthesized composites possessing a relatively higher surface area, good aqueous dispersibility, and excellent magnetic responsiveness. Hybrid materials have been devised that utilise complementary features, addressing the inherent limitations that arise from intrinsic physiochemical properties of existing methods. For example, substrate ligands that have stability in strong alkaline and acid buffers, and exhibit hydrophilicity to minimize nonspecific binding. Materials using artificial receptors, for multiple phosphate-selective binding sites, also show promise.

Identifying the key factors affecting the p-peptide enrichment performance of affinity materials has led to optimization of: solvent tolerance, pore size, functional groups, and surface area within the materials. Furthermore, through the addition of various organic acids as non-p-peptide inhibitors, control of the peptide/enrichment material ratio, and use the optimal pH within the elution buffer as process parameters further improves enrichment efficiency. The identified parameters are not silver bullet solutions, and consideration should to be taken depending on the target of the study – for example, the specific affinity of different materials towards p-peptides varies in the presence of additives. Despite this, materials are gradually becoming available for unbiased sample preparation for protein analysis, such as solid-phase-enhanced sample-preparation (SP3) technology. SP3 is a paramagnetic bead-based approach for rapid, robust, and efficient processing of protein samples for proteomic analysis. This bead format utilizes hydrophilic interaction to mediate exchange or removal of components used for proteomic analysis [117]. Use of such materials have potential for widespread adoption, due to ease of handling and compatibility with p-peptide enrichment [114].

It is important to note that recent developments in MS analysis and peptide spectral matching algorithm aid phosphoproteome analysis. The use of ion mobility (IM) MS provides additional separation to mass-to-charge (m/z) based on structure/shape (collisional cross section). IM separations occur at a millisecond timescale and this couples effectively with LC-MS to increase separation of co-eluting peptide isomers with variant modified sites [99]. The recently developed method for structures for lossless ion manipulations ion mobility offers improved sensitivity and separation of p-peptides, in particular for resolution of p-peptide isomers, with application to discovery and targeted phosphoproteomic workflows [118]. Capillary Electrophoresis, which separates based on the size-to-charge ratio of the peptide – with sensitivity to charge state, is well suited to the study of PTMs, including phosphorylation. Coupling this approach to MS has brought gains in phosphoproteome coverage as exemplified by studies of mouse brain [119] and a colon cancer cell line [120].

Novel developed sorbents, especially micro/nanocomposites show great promise for phosphoproteomics; however, further exploration is needed before their large-scale practical application, due to the very limited data on true biological samples. Current data are generally limited towards a “standard” (simple) protein mixture (typically casein and BSA), with some limited reference to testing on samples representative of true biological samples - HeLa cell lysates or human body fluids (saliva, serum). Tests involve mixing of peptides derived from trypsin or Lys-C, trypsin digests of α and β isoforms of caseins/BSA ratio of 1:49 or 1:99 (w/w), selected as representative of the low stoichiometry of phosphoproteins typically seen in biological samples such as cell lysates. The limitation of this approach is that it lacks the complexity of the p-peptides derived from a cellular phosphoproteome. In addition, p-peptides derived from α and β casein generally contain acidic residues, are phosphorylated at stoichiometric levels, and have a multiple charge state, which limits how representative they are to p-peptides found in complex biological samples. Such studies are a good starting point, but are not useful for generating generally applicable “rules of thumb”, conclusions gained from

experience in different sample types. Many of the novel materials discussed in this paper were tested using MALDI sources (Supplementary Tables S1-4); however, ESI (electrospray ionization) is the primary ionisation source for many phosphoproteomic investigations. Unlike MALDI MS, LC-ESI MSMS is biased toward mono-phosphorylated peptides[121], so for representative results it is important that 'head to head' method comparisons are performed on the same LC-MS/MS system. Phosphoproteomic protocols can also benefit from developments in new materials, fractionation protocols such as UPAX for canonical and non-canonical p-peptide separation from non-phosphorylated peptides [108], widening the scope of phosphoproteomic analysis to include acid labile phosphorylation which have been under-reported due to lack of detection in pSer/PThr/pY directed workflows [122]. Ultimately, for the new materials and processes mentioned here to become widespread and provide true benefit to the field of phosphoproteomics, rigorous bench marking is needed against the widespread classical methods of p-peptide enrichment currently available; and the 'winners' of such tests must also be affordable and commercial available.

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Disclosure of interest

The authors have declared no conflicts of interest.

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Figure Legends

Graphical abstract: Schematic diagram for p-peptide enrichment strategies. Ti^{4+} lab-in-syringe polydopamine coated three-dimensional porous graphene aerogel sorbent carrying immobilized titanium (IV) ions (denoted as Ti^{4+}@PDA@GA [53]), Phos-PAD: p-peptide paper-based analytical devices [72], graphene oxide-trimethyl-2-methacroyloxyethyl ammonium chloride-titania (GO-META-TiO_2) [64]. PolyMAC: (polymer-based metal ion affinity capture) [86, 87]. Phosphotyrosine antibody cocktails [102]. Phosphotyrosine-imprinted polymer with TiO_2 [100]. IMAC-antibody (IMAC with phosphotyrosine antibody) [15]. High pH and pH step wise elution [15]. Gradient elution: 100 mM NH_4HCO_3 (pH 9.2-11.3 step gradient and pH adjust by ammonia [16]. Ti^{4+}@PDA@GA) Adapted with permission [53]. Copyright 2018, Springer Nature. Phos-PAD) Adapted with permission [72]. Copyright 2019, Elsevier. GO-META-TiO_2) Adapted with permission [64]. Copyright 2019, Elsevier.

Figure 1 Sample preparation workflows for phosphoproteomics (a). The key steps are "protein extraction", "proteolytic digestion", "p-peptide enrichment" and "fractionation" [11]. The latest developments for sample preparation workflows aim to minimise the number of processing steps, using robust and reproducible component methods. Common strategies for p-peptide enrichment (b) [88]. (c) Main steps for affinity-based p-peptide enrichment techniques. (d) Improvements for p-peptide enrichment. b) Adapted with permission [88]. Copyright 2017, the Royal Society of Chemistry.

Figure 2 Schemes for the creation and use of three novel enrichment materials: a) MagG@PEI@PA-Ti^{4+}, which can adsorb 53.5 μg mg^{-1} p-peptide with 90% recovery, and is sensitive to concentrations as low as 0.8 fmol.μl^{-1} [49], b) Ti^{4+}@PDA@GA, aerogel, formed from graphene oxide (GO), is used in a lab-in-syringe methodology that preferentially enriches p-proteins (1300-1345 μg mg^{-1} vs 4.8-160 μg mg^{-1}), can adsorb up to 1340 μg mg^{-1}, and is sensitive to concentrations as low as 2 fmol.μl^{-1} [37], and c)

magG@PD-Hf^{4+}, which showed sensitivity to concentrations as low as 0.08 fmol.μl^{-1} [47]. All figures adapted with permission: [49] Copyright 2018, American Chemical Society; [37] Copyright 2018, Springer Nature; [47] Copyright 2016, Elsevier.

Figure 3 Schemes for the creation and use of two novel MOAC formats: a) Fe_3O_4@H-fTiO_2 a functionalized TiO_2 layer nanoparticle, with larger pore volumes (0.52 cm^3g^{-1}) and higher surface area (144.71 m^2g^{-1}) than existing TiO2 magnetic spheres, showed a p-peptide:peptide selectivity ratio of up to 1:10000, and sensitivity down to 0.2 fmol.μl^{-1}, (α-casein, BSA, HeLa cell) [64]. b) GF-TiO_2-GO showed sensitivity down to 0.1 fmol.μl^{-1} and provided an unbiased mono-multi p-site peptide distribution in concentrations of up to 0.2 μg.mg^{-1}spheres (β-casein, BSA, non-fat milk and human serum) [67]. Figures adapted with permission: [64] Copyright 2018, Elsevier; [67] Copyright 2018, the Royal Society of Chemistry.

Figure 4 Schemes for the creation and use of 3 IMAC/MOAC hybrid materials: a) Fe_3O_4@n SiO_2@m SiO_2/TiO_2-Ti^{4+} is sensitive to p-peptides from concentrations as low as 40 fmol.μl^{-1}, has high surface area (179.3 m^2g^{-1}) and a selectivity ratio of 1:50, with an adsorption capacity of 133 mg/g (β-casein, BSA, non-fat milk) [76]; b) TiO_2@DOTA-Zr showed sensitivity of as low as 10 μmol.μl^{-1}, and a selectivity ratio of 1:10 (β-casein, BSA, nonfat milk, human serum) [77]; and c) G@TiO_2@mSiO_2 showed a sensitivity of 1 fmol.μl^{-1} and a selectivity ratio of 1 to 1000 (α- or β- casein, BSA, human serum) [79]. Adapted with permission: [76] Copyright 2017, Elsevier; [77] Copyright 2017, Elsevier; [79] Copyright 2016, Elsevier.

Figure 5 Typical workflow or schematic diagram of simultaneous N-glyco-peptide and p-peptide (a-c). Magnetic materials of (a) SPIOs@SiO_2@MOF (β-casein, BSA, IgG, rat brain, rat liver) [109], (b) TiO_2@SiO_2-$B(OH)_2$@Fe_3O_4@TiO_2 (β-casein, BSA, horseradish peroxidase, defatted milk) [111].Samples were incubated with materials for 30 min in

loading buffer (50% ACN, 0.1% or 0.25% TFA, v/v), washed 3 times, and then eluted by ammonia solutions (0.4 M or 10% wt%) after 30 min's treatment. (c) TiO_2-NH_2 modified MALDI plate (β-casein, BSA, horseradish peroxidase, human serum, human saliva) [113], for which 5 min of incubation (50% ACN, 1% TFA, or 95% ACN, 0.1% TFA, v/v), after 5 times washing, DHB was added for direct LC-MS/MS analysis. Reproduced from [109] Copyright 2019,American Chemical Society; [111] Copyright 2016, Springer Nature; [113]. Copyright 2017, Elsevier.

Supporting files

Tables

Table S1 Comparison of p-peptide enrichment by immobilized metal ion affinity chromatography (IMAC) for different metal ions (A) and different supporting ligands (B).

Table S2 Comparison of p-peptide enrichment by metal oxide affinity chromatography (MOAC) for different metal oxides (A) and different supporting ligands (B).

Table S3 Comparison of hybrid composites for p-peptide enrichment.

Table S4 Novel materials for simultaneous enrichment of p-peptide (P) and glycopeptide (G).

1568 **Table 1. Existing formats for metal based p-peptide enrichment.**

Technique/ Example format	Incubation	Washing	Elution	
MOAC TiO_2 magnetic beads	(1) TiO_2 material pre-condition according to manufacturer's instructions, eg. wash with condition buffer; (2) Desalted peptides incubate with magnetic TiO_2 beads for 30 min with the presence of 1M lactic acid or glycolic acid; (3) P-peptide was captured and retained by TiO_2.	non-p-peptide washed using high ACN buffer	3% ammonium solution	GE healthcare
IMAC (Phos-Select Iron Affinity Gel beads pack into microcolumn)	(1) Microcolumn preparation: gel beads were pipetting into special tips; the tips were then place into centrifuge adaptor; and conditioned before use. (2) Peptides were incubated with gel beads for 30 min.	Unbound peptides removed using wash buffer 250 mM acetic acid with 30% ACN	Bound peptides were released into 250 mM acetic acid pH 3.0 in 30% ACN	Sigma
Phos-tag micropipette	(1) Prepare Phos-tag agarose gel and phosphate-affinity micropipette-tip;	Wash three times with	Elute by syringe with buffer solution 0.10 M $Na_4P_2O_7$/0.10	[82, 83]

tip	(2) Condition the Phos-tag tip; Draw sample. gently into the micropipette-tip by using 1 ml syringe and agarose beads in suspension was kept for a few seconds, repeat 5 times;	washing buffer and water once using syringe	M CH_3COOH (pH 7.0) or 0.1 M aqueous HCl, 2% aqueous (v/v) TFA, 5% aqueous NH_3, or 0.10 M EDTA/NaOH (pH 7.0)	
PolyMAC reagent (-Ti or -Fe)	(1) Synthesis of PolyMAC reagent; (2) Peptides incubate with PolyMAC reagent for 5 min, and add capture buffer; (3) Transfer mixture to spin column with washed resin and incubate for 10 min with agitation; (4) Wash with loading buffer and incubate for 5 min with agitation;	Washed with washing buffer and water	400 mM ammonium hydroxide	[86, 87]

Table 2 Example p-peptide enrichment protocols published in the last five years, these can form user guides due to full and detailed protocols considering theoretical and practical aspects of workflow design and application

Sample and Study	p-peptide enrichment	Analyte/Starting amount	Fractionation	Number of p-peptides	Key Developments	Reference
Breast cancer subtypes from patient-derived mouse xenograft models (CPTAC) consortium Multiplex analysis using TMT 10 plex for comparative analysis	Ni-NTA IMAC	300 micrograms 5% directed to Proteomic analysis and 95% to phosphoproteomic analysis	High-pH reverse HPLC, prior to IMAC	35,000 p-peptides/ experiment, on average	Application to tissue blocks and mammalian samples. Multiplex analysis for higher throughput	[17]
Tomato Plants Quantitative proteomic analysis using dimethyl labelling	PolyMAC-Ti	200 microgram aliquots	High-pH reverse HPLC of enriched p-peptides	30,000 unique p-peptides from tomato leaves	Protocol for with universal application to plant samples	[21]
EGF stimulation of human U-87 glioblastoma cells Quantitative proteomic analysis by Label Free Quantification	MOAC Titansphere Phos-TiO	200 micrograms (originally developed for 1 mg)	None	~20,000 p-peptides, comprising 16,021 accurately localized phosphorylation sites	EasyPhos eliminates requirement for peptide desalting before p-peptide enrichment	[123]

HLA class I–associated p-peptides	IMAC Fe(III) ± NTA-Fe(III)	submicrogram levels of peptide material	None	161 p-peptides	Improved sensitivity Reduced nonspecific binding, improved peptide recoveries	[24]
Prostate cancer (xenograft tumours) Quantitative proteomic analysis by Label Free Quantification	pTyr immunoprecipitation and TiO_2 pSer.pThr MOAC-TiO_2	50-150 mg starting wet tissue	SCX to remove multiply charged peptides prior to enrichment of pSer, PThr MOAC-TiO_2		85% of the p-peptides identified are pTyr Demonstration of the value of experimental design, use of replicates in analysis of clinical samples	[36]

pY peptides using specific phosphotyrosine antibodies and TiO_2. We also describe the enrichment of phosphoserine/threonine (pST) peptides using strong cation exchange (SCX) followed by TiO_2.

Supporting Tables

Table S1A Comparison of p-peptide enrichment by immobilized metal ion affinity chromatography for different metal ions

Materials	Sensitivity	Selectivity β-casein:BSA digests (n:n)	Mono	Multi	Real sample matrix	Instrumentation	Ref
Fe_3O_4@PDA- Nb^{5+}	2 fmol	1:100	5	11	non-fat milk	MALDI-TOF/TOF (AB	[46]
Fe_3O_4@PDA-Ti^{4+}	2 fmol	1:100	5	9	non-fat milk	Sciex 5800)	
Fe_3O_4@PDA-Zr^{4+}	80 fmol	1:100	4	7	non-fat milk		
Fe_3O_4@PDA-Ce^{4+}	2 fmol	1:50	5	6	non-fat milk		
Fe_3O_4@PDA-Ga^{3+}	20 fmol	1:100	4	7	non-fat milk		
Fe_3O_4@PDA-Y^{3+}	80 fmol	1:50	2	5	non-fat milk		
Fe_3O_4@PDA-In^{3+}	200 fmol	1:50	4	5	non-fat milk		
Fe_3O_4@PDA-Fe^{3+}	200 fmol	1:50	5	6	non-fat milk		
Fe_3O_4@PDA- Nb/Ti binary composite	2 fmol	1:1000	8	11	human serum, nonfat milk	MALDI-TOF-MS	[48]
Urea-modified MIL-101 (Cr)-Amine based affinity	100 fmol/μL	1:200	-	-	human serum	MALDI-TOF-MS	[124]

1576 **Table S1B** Comparison of p-peptide enrichment by immobilized metal ion affinity chromatography for different supporting ligands

Materials	Sensitivity (fmol/μL)	Selectivity β-casein:BSA digests (n:n)	Real sample matrix	Instrumentation	Ref
Ti^{4+}@PDA@GA	30	1:200	milk, human serum	MALDI-TOF-MS	[53]
magG@PDA-Hf^{4+}	0.08	1:500	non-fat milk, human serum	MALDI-TOF-MS (AB Sciex 5800)	[49]
magG@PDA-Sn^{4+}	0.08	1:1000	human saliva	MALDI-TOF-MS and Orbitrap ESI-MS/MS (Q-Exactive)	[50]
DMSNs@PDA-Ti^{4+}	0.2 *	1:5000*	HeLa cell	MALDI-TOF-MS (AB Sciex 4800 plus)	[125]
magSiO_2@SiO_2@PDA@Ti(IV)	0.05	1:500	human serum	MALDI-TOF-MS (Voyager-DE PRO)	[56]
Fe_3O_4 @mSiO_2-Ti^{4+}	0.1	1:500	human serum, saliva	MALDI-TOF/TOF (AB Sciex 5800)	[47]
Fe_3O_4@SiO_2-PLP-Ti^{4+}	10 fmol,	1:500	non-fat milk, human serum	MALDI-TOF (Axima TOF^2), and RPLC-ESI–MS/MS (AB Sciex TripleTOF5600+)	[91]

	-	1:100	yeast	Orbitrap Elite hybrid ion trap-Orbitrap-MS	[55]
Fe_3O_4@silica@GMA@IDA@Ti^{4+}		1:100	non-fat milk, human serum, rat brain	MALDI-TOF-MS and LC-ESI–MS/MS	[126]
CF-NH_2-AZO-p(VPA-x)-Ti^{4+}	1×10^{-4}		human serum	MALDI-TOF-MS (Voyager‐DE PRO)	[127]
Ti (IV)@poly(VPA-co-EDMA) monolith	0.001	1:1500			
Ti-PA-MNPs	8×10^{-4}	1:2000	rat liver	MALDI-TOF-MS	[128]

*: data from α-casein : BSA; (1 M: 100 pmol/μL)

1579 **Table S2A** Comparison of p-peptide enrichment by metal oxide affinity chromatography (MOAC) for different metal oxides

Materials	**Sensitivity** (fmol/μL)	**Selectivity β-casein:BSA digests (n:n)**	**Real sample matrix**	**Instrumentation**	**Ref**
In_2O_3, SnO_2, NiO, Co_3O_4	-	-	human embryonic kidney cell line	Orbitrap XL or Orbitrap Elite	[43]
MoO_3/GO	1×10^{-3}	1:1000	nonfat milk, human serum	MALDI-TOF/TOF (AB Sciex 5800)	[60]
Fe_3O_4@PDA@Er(btc)	2×10^{-5}	1:500	human serum	MALDI-TOF/TOF (AB Sciex 5800)	[61]
zirconia/magnetic mesoporous composites	1.5	1:500	nonfat milk, human serum	MALDI-TOF-MS/MS (AB Sciex 4800 plus)	[129]
Zirconia/OMC	1.5	1:300	non-fat milk	MALDI-TOF-MS/MS (AB Sciex 4800 plus)	[130]
In-Tip La_2O_3 monolith	0.25	1:4500	egg yolk, human serum	MALDI-TOF/TOF-MS (Ultraflex-II)	[131]

1580

Table S2B Comparison of p-peptide enrichment by metal oxide affinity chromatography (MOAC) for different supporting ligands

Materials	Sensitivity	Selectivity β-casein:BSA digests (n:n)	Real sample matrix	Instrumentation	Ref
TMA-microchips (TiO_2)	0.4	1:100	egg white	MALDI-TOF-MS	[71]
Fe_3O_4@H-fTiO_2	0.2 *	1:10000*	HeLa cell	MALDI-TOF-MS (AB Sciex 4800 plus) and RPLC-ESI-MS/MS	[62]
mGCB@TiO_2	-	1:100	yeast	Orbitrap Elite hybrid ion trap-Orbitrap MS	[66]
GO-META-TiO_2	10	1:100	chicken egg white	MALDI-TOF-MS	[64]
CIM-OH-TiO_2	-	-	cytochrome C, lysozyme, human serum	LTQ Velos ion-trap MS	[69]
GF-TiO_2-GO	1×10^{-5}	1:100	non-fat milk, human serum	MALDI-TOF-MS	[65]
mesoporous TiO_2 in - tube solid - phase microextraction column	10	1:100	non-fat milk	MALDI-TOF-MS	[132]

*: data from α-casein : BSA

1584 **Table S3** Comparison of hybrid composites for p-peptide enrichment

Materials	Sensitivity (fmol/μL)	Selectivity β-casein:BSA digests (n:n)	Real sample matrix	Synthesis	Instrumentation	Ref
TiO_2/Bi/Fe/Zr	4×10^{-4}	1:1000	Hela cell	sol-gel method	MALDI-TOF/TOF (AB Sciex 5800)	[58]
$B_{0.15}F_{0.15}$TNs	2×10^{-3}	1:1200	human liver	sol-gel method	MALDI-TOF/TOF (AB Sciex 5800)	[59]
$MnFe_2O_4$ MAMSs	1	1:500	non-fat milk, human serum	solvothermal route	MALDI-TOF-MS (AB Sciex 4800)	[75]
$CuFeMnO_4$ nanospheres affinity probe	20	1:100	nonfat milk, A549 cells, human saliva, human serum	solvothermal route	MALDI-TOF-MS (AB Sciex 4800)	[133]
Fe_3O_4@PDA-Ti/Nb	2	1:1000	non-fat milk, human serum	-	MALDI-TOF-MS	[48]
Fe_3O_4@nSiO_2@mSiO_2/TiO_2-Ti^{4+}	4	1:50	nonfat milk	magnetic /mesoporous silica	MALDI-TOF/TOF (AB Sciex 5800)	[78]
Fe_3O_4@H-TiO_2@f-NiO	2×10^{-4}*	1:5000*	non-fat milk/human		MALDI-TOF	[63]

			serum/HeLa cell		(AB Sciex 4800 plus)	
PAA-Ti/TiO_2 composite	2	1:1000	human serum, mouse liver	-	MALDI-TOF/TOF (AB Sciex 5800)	[80]
TiO_2@DOTA-Zr	1×10^{-7}	1:10	nonfat milk, human serum	macrocyclic ligand	MALDI-TOF/TOF	[79]
magG/PD/(Zr-Ti)O_4	4.0×10^{-5}	1:8000 weight ratio	mouse brain tissue	magnetic/graphene	MALDI-TOF MS	[76]
G@TiO_2@mSiO_2	1	1:1000;	human serum	magnetic/graphene/ mesoporous silica	MALDI-TOF MS and MALDI-TOF/TOF	[81]
Al_2O_3 - TiO_2/ ZrO_2/CeO_2/ La_2O_3	10	1:1000;	human serum	co-precipitation	MALDI-TOF/TOF (Ultraflex-I)	[77]

*: data from α-casein : BSA

1586 **Table S4** Novel materials for simultaneous enrichment of p-peptide (P) and glycopeptide (G)

Materials	**Sensitivity** (fmol/μL)		**Selectivity** standard digests[a](n/n)		**Real sample matrix**	**Instrumentation**	**Ref**
	P	**G**	**P**	**G**			
						MALDI-TOF	[113]
SiO_2-NH_2@TiO_2	0.16	2	1/500	1/500	human serum albumin	(AB Sciex 5800)	
					human serum/saliva,	MALDI-TOF	[114]
TiO_2@SiO_2-$B(OH)_2$@Fe_3O_4@ TiO_2	0.8 - 8	2.5 - 25	1/1000	1/50	defatted milk	(AB Sciex 5800)	
	0.08 -				human serum/saliva,	MALDI-TOF	[115]
Fe_3O_4@Au-$B(OH)_2$@mTiO_2	0.8	2 - 20	1/1000	1/100	defatted milk	(AB Sciex 5800)	
						MALDI-TOF	[116]
TiO_2-NH_2 modified MALDI plate	8	20	1/200	1/100	human serum/saliva	(AB Sciex 5800)	
						MALDI-TOF	[134]
CS@PGMA@IDA-Ti^{4+}	0.1	0.1	1/500	1/100	human IgG, mouse liver	(AXIMA-CFP plus)	
						MALDI-TOF	[135]
Fe_3O_4@PDA@UiO-66-NH_2 (Zr^{3+})	0.02	0.2	1/500	1/100	human serum	(AB Sciex 5800)	
						MALDI-TOF (AB Sciex	[51]
MagG@PEI@PA-Ti^{4+}	0.1*	0.5[#]	1/5000*	1/1000[#]	human serum	4800 plus)	

						MALDI-TOF (AB Sciex	[111]
Mag-MSMs@PEI-PA-Ti^{4+}	0.2*	0.5 #	1/5000*	1/1000#	human serum, HeLa cell	4800 plus)	
						MALDI-TOF	[136]
Fe_3O_4@MIL-100(Fe)	0.1	0.1	1/50	1/20	human saliva	(AB Sciex 5800)	
						MALDI-TOF	[137]
co-PAN@Ti^{4+}	4	100	1/50	1/50	human serum	(AB Sciex 5800)	
						MALDI-TOF/TOF (AB	[138]
Fe_3O_4@mTiO_2-MSA	50	10^{-3}	1/800	1/100	human saliva	Sciex 5800)	
					rat brain, rat liver,	MALDI-TOF	[112]
SPIOs@SiO_2@MOF	10^{-4}	10^{-5}	1/400	1/500	mouse liver	(AB Sciex 5800)	

a: β-casein/BSA for P; horseradish peroxidase/BSA for G. *: data from α-casein/BSA; #: data from IgG/BSA.

PGMA: poly glycidyl methacrylate.