Development of fully automated pipeline for phosphoproteome profiling: optimisation of phosphopeptide enrichment

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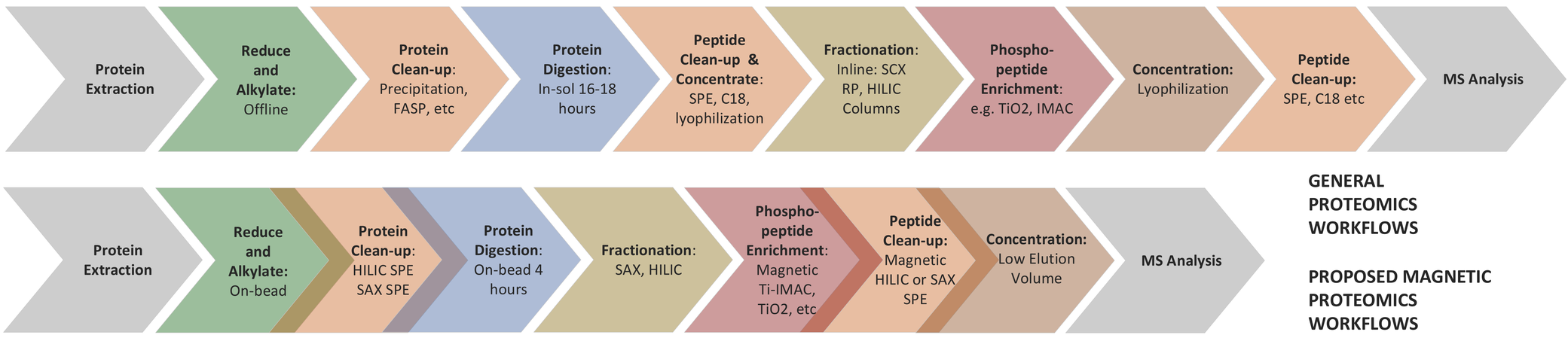
GRAPHICAL ABSTRACT

RESEARCH AIMS
- HT COMPATIBLE
- AUTOMATED
- VERSATILE WORKFLOWS
- REAGENT COMPATIBILITY
- VENDOR INDEPENDENT
- MODULAR

CHALLENGES
- AUTOMATION
- INTEGRATION
- REPRODUCIBILITY
- DATA QUALITY

ADVANTAGES
- HIGHLY REPRODUCIBLE
- LINEARLY SCALABLE
- 96 SAMPLES in <8 HRS
- 5 MIN PER SAMPLE



Previous Work:
- Optimisation of HILIC and SAX for automated protein and peptide clean-up
- On and off-bead reduction and alkylation

Future Work:
- Use of LysC & Trypsin for on-bead digestion.
- In-depth evaluation of SAX for sample clean-up

Tools Available:
HILIC, SAX, Hydrazide, IP

Future Work:
- Evaluate tools
- Integrate and automate fractionation strategies
- Deep proteome profiling

Previous Work:
- Integration of HILIC & Ti-IMAC workflow under range of digest conditions & Comparison to FASP & Ti-IMAC workflow
- Evaluation of HILIC and SAX for peptide desalting prior to MS

Future Work:
- Evaluation of workflow for complex samples
- Optimisation of SAX for peptide desalting

Aims of Current Work:
- Optimization of Phospho-enrichment strategy
- Evaluate tools for phospho-enrichment with respect to recovery and specificity
- Evaluation of buffers on phospho-enrichment
- Identify best conditions for enrichment
- Identify overlapping buffers for possible mixing of chemistries to evaluate for improved coverage

INTRODUCTION

As clinical proteomics applications start to reach maturity, this necessitates the requirement for robust and routine high throughput sample preparation workflows that allow processing of large sample cohorts. However, efficient sample preparation remains the *Achilles Heel* for mass spectrometry analysis, with current methods lacking the throughput, transferability and reproducibility required to deal with these large clinical sample numbers in a routinized laboratory setting. To address these we focus on the implementation of versatile and automatable magnetic bead based sample preparation. Magnetic beads are considered desirable since these are easy to handle, linearly scalable, and high throughput compatible with the relatively simple integration of a magnetic stand in a variety of liquid handling stations making it fairly agnostic for the liquid handling or magnetic bead handling station that may be present in the sample preparation laboratory. We have previously demonstrated protein and peptide clean-up workflows using magnetic HILIC for Solid Phase Extraction (HILIC SPE) from a broad range of common contaminants and the coupling of this protocol to magnetic-based Ti-IMAC based phosphopeptide enrichment (https://www.resynbio.com/index_htm_files/LS2_Zurich_2019.pdf). The use of magnetic beads for highly efficient phosphopeptide enrichment has previously been demonstrated (Tape *et al*. 2014, Baath *et al.* 2018), and in the current work we evaluate the buffer composition (14 buffers) on recovery and specificity for phosphopeptide enrichment from 6 different enrichment chemistries, Ti-IMAC, Zr-IMAC, TiO_2, ZrO_2, Fe-NTA, Fe-IMAC.

Although our aim is to fully automate mass spectrometry workflows, the protocols are also suitable for manual preparation with the ability to perform parallel sample processing using a magnetic stand. The protocols can be transferred to a variety of liquid or bead handling systems. All experiments were performed on a KingFisher™ Duo (ThermoFisher) magnetic bead handling station (protocols available on request).

METHODS

We evaluated a range of phosphopeptide enrichment tools with particular interest on how buffer composition affects phosphopeptide enrichment efficiency and recovery. To this end we assessed a range of commercially available magnetic bead chemistries (ReSyn Biosciences), including Ti-IMAC (chelated Ti^{4+} ions), Zr-IMAC (Zr^{4+} chelated ions), TiO_2 and ZrO_2 (titanium and zirconium dioxide nanoparticles), and two prototype magnetic beads containing Fe^{3+} ions chelated to two different supports (Fe-IMAC and Fe-NTA, ReSyn Biosciences). For initial evaluation of buffer composition a relatively simple mixture was generated from a tryptic digest of Casein and BSA. The optimal conditions will be applied to complex lysates (work currently in progress). For all experiments 1 mg of beads was used, except ZrO_2 where 10 mg was evaluated (high density nanoparticles attached to the beads).

A Casein & BSA tryptic digest was enriched in an automated manner on a KingFisher Duo magnetic handling station (protocols available on request) using the range of magnetic beads. Up to 14 different buffer combinations were evaluated for binding and washing in the phosphopeptide enrichment protocol (Refer Table, Right). Post-enrichment, samples were spiked with 3 isotopically labelled peptide standards for casein phosphopeptides, i.e. VPQLEIVPN[pSer]AEER, YKVPQLEIVPN[pSer]AEER and FQ[pSer]EEQQQTEDEQDK. MALDI-TOF MS1 spectra were generated and the signal for each phosphopeptide was normalised against the highest responding heavy internal standard (YKVPQLE[I_C13N15]VPN[pSer]AEER) in order to quantify enrichment efficiency for each buffer composition. Experiments were analysed in duplicate.

Experiment 1

BC	Binding buffer	Wash buffer 1	Wash buffer 2
1	80% ACN, 5% TFA, 1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
2	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic
3	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
4	50%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA

Experiment 2

3	50%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
4	50%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA
5	50%ACN/ 0.1% Acetic, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
6	50%ACN/ 0.1% Acetic, 0.5M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
7	80%ACN/ 0.1% Acetic	80% ACN, 1% TFA	10% ACN, 0.2% TFA
8	50%ACN/ 0.1% Acetic, 0.1M GA	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
9	50%ACN/ 0.1% Acetic, 0.5M GA	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA
10	80%ACN/ 0.1% Acetic	50%ACN/ 0.1% Acetic	10% ACN, 0.2% TFA

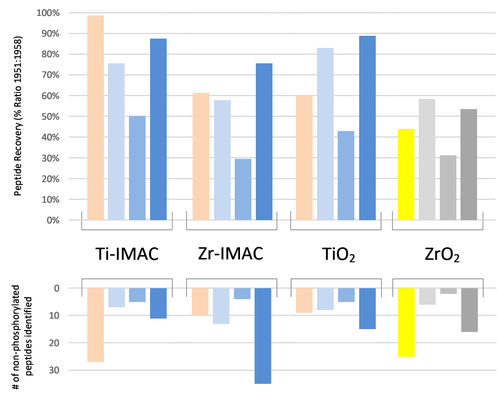
Experiment 3

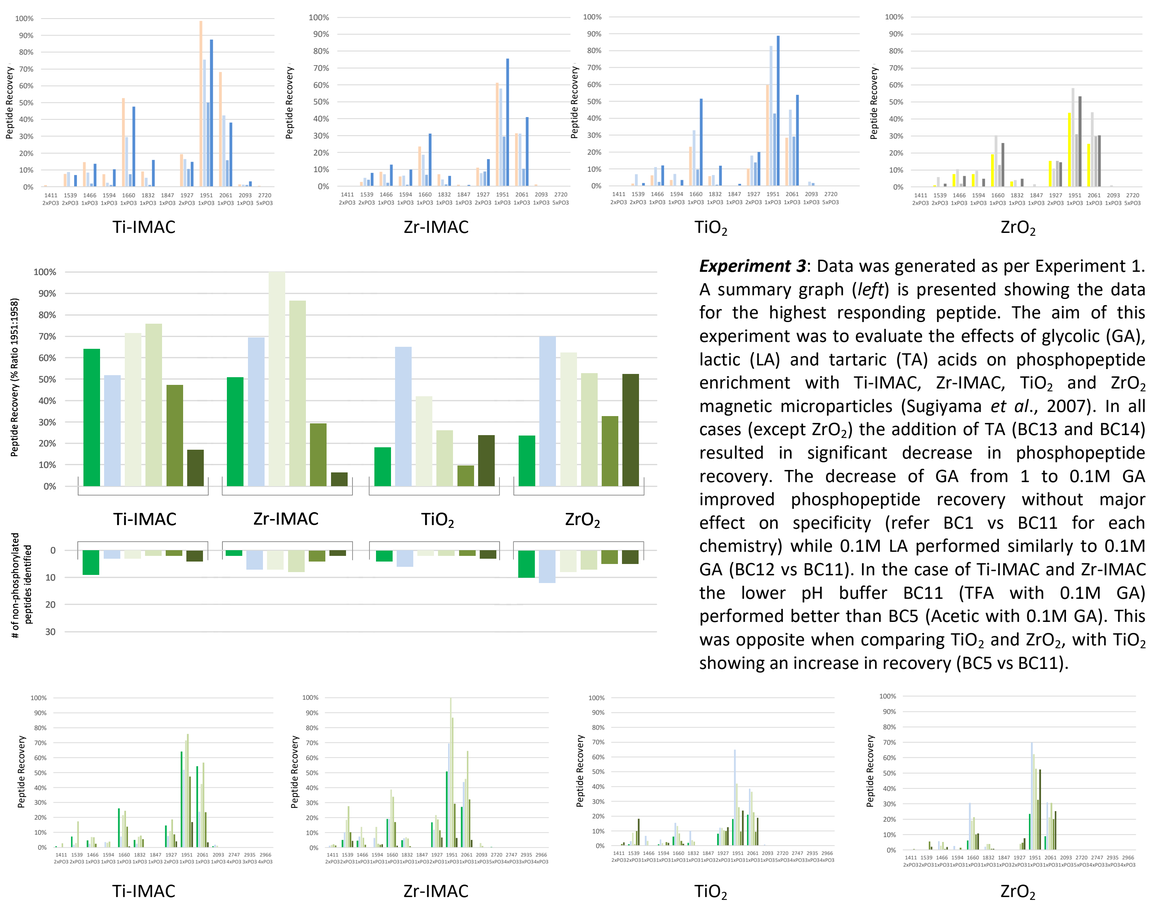
1	80% ACN, 5% TFA, 1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
5	50%ACN/ 0.1% Acetic, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
11	80% ACN, 5% TFA, 0.1M GA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
12	80% ACN, 5% TFA, 0.1M LA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
13	80% ACN, 5% TFA, 0.1M TA	80% ACN, 1% TFA	10% ACN, 0.2% TFA
14	50%ACN/ 0.1% Acetic, 0.1M TA	80% ACN, 1% TFA	10% ACN, 0.2% TFA

RESULTS & DISCUSSION

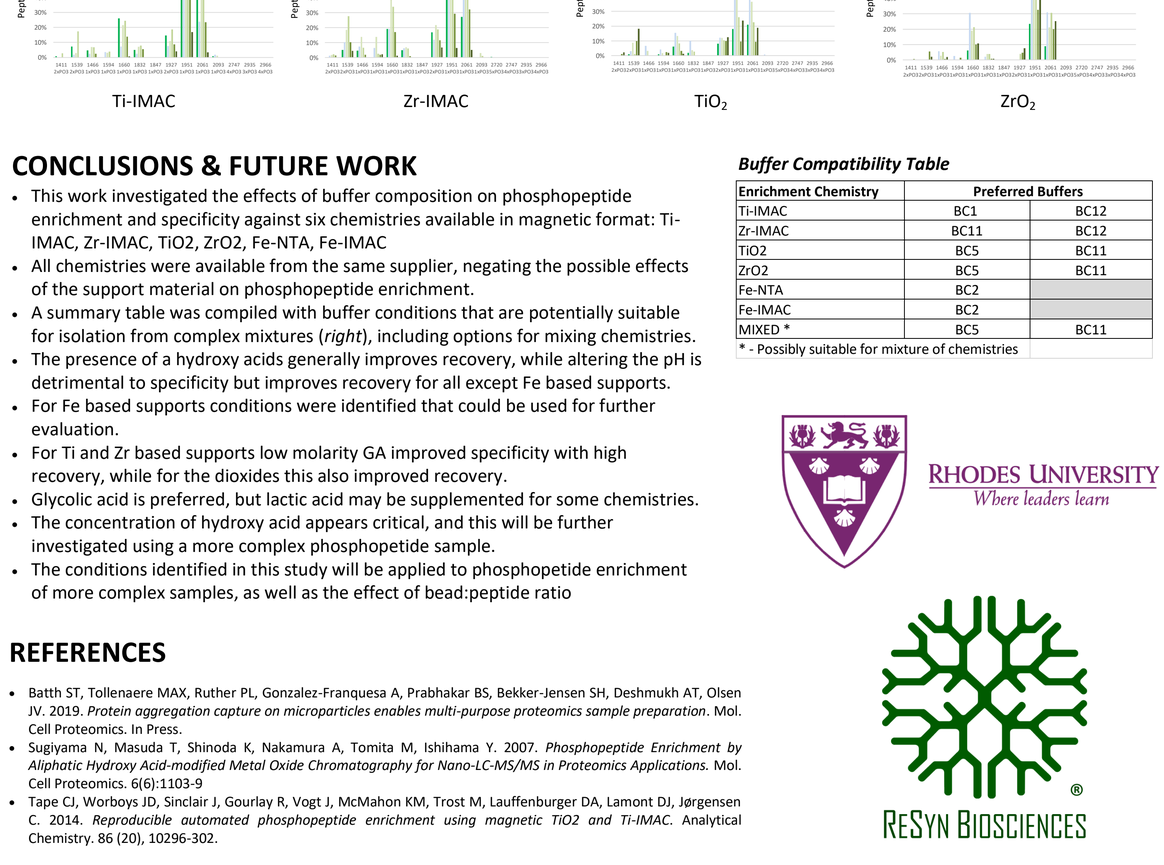
Experiment 1: MALDI-TOF MS1 spectra were generated and the signal for each detected phosphopeptide was normalised against the highest responding heavy internal standard (YKVPQLE[I_C13N15]VPN[pSer]AEER, 1958.4m/z) in order to determine enrichment efficiency under each buffer composition (BC). The data for the highest responding peptide (1951m/z) was plotted to illustrate the general trends (*left*), while the recovery of all phosphopeptides is illustrated for each chemistry (below). Specificity is calculated based on the total number of non-phosphorylated peptides detected. BC 1 is currently the recommended standard buffer system by the bead manufacturer. With all standard phospho-enrichment tools (Ti-IMAC, Zr-IMAC, TiO_2, ZrO_2) this buffer resulted in the highest specificity, but lowest recovery. The use of Acetic acid (Acetic), and the removal of Glycolic Acid (GA, BC2), improved phosphopeptide recovery but reduced specificity. For Fe supports BC2 appears suitable, with a balance between recovery and specificity. BC4 improved the specificity in particular for Zr-IMAC and TiO_2, while retaining high phosphopeptide recovery. Based on these initial results BC4 was selected for further evaluation with Ti-IMAC, Zr-IMAC and TiO_2, while BC3 was selected for further evaluation with ZrO_2 magnetic microparticles.

Experiment 2: Aliphatic hydroxy acids have been noted to affect phospho-enrichment (Sugiyama *et al*., 2007), and were thus investigated for optimization of buffers. MALDI-TOF MS1 spectra were generated as described previously. BC3 and BC4, chosen from Exp 1, were further modified in attempt to improve the specificity while retaining phosphopeptide recovery. In the case of Ti-IMAC the addition of 0.1M GA in the bind buffer (BC5) significantly improved the specificity whilst maintaining recovery. An increase in the ACN concentration (BC7) had a similar effect, i.e. high recovery with improved specificity. For Zr-IMAC the inclusion of GA improved specificity at 0.5M (BC6), but reduced recovery, while BC4 and BC5 (the only difference being 0.1M GA) showed similar recoveries and specificity. Due to possible increased competition in complex samples, these conditions will be investigated further. Unlike Ti-IMAC, for Zr-IMAC high ACN reduced specificity (BC7). For TiO_2 a low concentration of GA (0.1M, BC5) improved recovery with no loss in specificity, while an increased concentration (0.5M, BC6) reduced recovery. The increase in ACN concentration (BC7) improved recovery but reduced specificity. For ZrO_2, the addition of 0.1M GA improved both specificity and recovery (BC8). Similar to TiO_2, increased ACN (BC10) improved recovery but reduced specificity.





Experiment 3: Data was generated as per Experiment 1. A summary graph (*left*) is presented showing the data for the highest responding peptide. The aim of this experiment was to evaluate the effects of glycolic (GA), lactic (LA) and tartaric (TA) acids on phosphopeptide enrichment with Ti-IMAC, Zr-IMAC, TiO_2 and ZrO_2 magnetic microparticles (Sugiyama *et al*., 2007). In all cases (except ZrO_2) the addition of TA (BC13 and BC14) resulted in significant decrease in phosphopeptide recovery. The decrease of GA from 1 to 0.1M GA improved phosphopeptide recovery without major effect on specificity (refer BC1 vs BC11 for each chemistry) while 0.1M LA performed similarly to 0.1M GA (BC12 vs BC11). In the case of Ti-IMAC and Zr-IMAC the lower pH buffer BC11 (TFA with 0.1M GA) performed better than BC5 (Acetic with 0.1M GA). This was opposite when comparing TiO_2 and ZrO_2, with TiO_2 showing an increase in recovery (BC5 vs BC11).



CONCLUSIONS & FUTURE WORK

- This work investigated the effects of buffer composition on phosphopeptide enrichment and specificity against six chemistries available in magnetic format: Ti-IMAC, Zr-IMAC, TiO2, ZrO2, Fe-NTA, Fe-IMAC
- All chemistries were available from the same supplier, negating the possible effects of the support material on phosphopeptide enrichment.
- A summary table was compiled with buffer conditions that are potentially suitable for isolation from complex mixtures (*right*), including options for mixing chemistries.
- The presence of a hydroxy acids generally improves recovery, while altering the pH is detrimental to specificity but improves recovery for all except Fe based supports.
- For Fe based supports conditions were identified that could be used for further evaluation.
- For Ti and Zr based supports low molarity GA improved specificity with high recovery, while for the dioxides this also improved recovery.
- Glycolic acid is preferred, but lactic acid may be supplemented for some chemistries.
- The concentration of hydroxy acid appears critical, and this will be further investigated using a more complex phosphopetide sample.
- The conditions identified in this study will be applied to phosphopetide enrichment of more complex samples, as well as the effect of bead:peptide ratio

Buffer Compatibility Table

Enrichment Chemistry	Preferred Buffers	
Ti-IMAC	BC1	BC12
Zr-IMAC	BC11	BC12
TiO2	BC5	BC11
ZrO2	BC5	BC11
Fe-NTA	BC2	
Fe-IMAC	BC2	
MIXED *	BC5	BC11
* - Possibly suitable for mixture of chemistries		

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