Abstract

The central role of tyrosine phosphorylation (pTyr) in the initiation of cellular signal transduction and cell signaling has consistently inspired chemist to identify and design sorbents with pTyr recognition properties. To date, the use of pTyr-antibodies is indisputable in targeted pTyr proteomics. Other affinity materials like the super-binder Src Homology 2 (sSH2) domains have recently emerged and proved to have performance comparable with antibodies. The synthesis of a Molecularly Imprinted Polymer (MIP) for the phosphotyrosyl moiety (pTyr-MIP) was first published in 2008, while its premises for targeted phosphoproteomic were initially investigated in 2011 in a simple LC-MS experimental setting using a model peptide. However, a more comprehensive analysis of the applicability of pTyr-MIP's in the enrichment of endogenous pTyr-peptides from a biological sample has been missing, leaving behind a mystery about their characteristics, and potential use in large-scale phosphoproteomics.

In our quest for an independent, unbiased and systematic evaluation of pTyr-MIP, we performed initial experiments and developed the pTyr-MIP protocol using a semi-complex mixture and MALDI MS as an analytical tool. The results from those initial experiments paved the way towards experiments using murine liver and later, pervanadate-stimulated human cervical carcinoma epithelial (HeLa line) cells for the highest representation of endogenous pTyr-peptides.

Equipped with the power of nanoLC-separation and MS/MS analysis (Velos-Orbitrap system), we found that pTyr-MIP had a higher affinity for pTyr compared to phosphoserine (pSer) and phosphothreonine (pThr). However, due to the significant co-enrichment of other peptides, pTyr-MIP based SPE (solid phase extraction) did not meet the criteria for a targeted pTyr- enrichment method on its own. As such, by combining pTyr-MIP with the robust TiO2 (titanium dioxide)-affinity chromatography we developed two protocols applying TiO2 either prior to or post pTyr-MIP enrichment: TiO2–pTyr-MIP and pTyr-MIP–TiO2.

The main finding is that pTyr-MIP concentrate the vast majority of the pTyr-peptides –an effect that is manifested with their intensities boost and achievable only when pTyr-MIP is followed by the robust TiO2. The pTyr-MIP-concentration effect was studied by comparing those pTyr-peptides that were identified both by pTyr-MIP–TiO2 and TiO2. We found that 70% of the pTyr-peptides enriched by pTyr-MIP–TiO2 had higher intensities than their TiO2 pTyr-counterparts. When we extended the analysis to pSer- and pThr-peptides, we found that 50% of pSer-peptides, concurrently found by both TiO2 and pTyr-MIP–TiO2, had higher intensities when enriched with the pTyr-MIP–TiO2 protocol than TiO2. This result suggests that pTyr-MIP have a higher affinity for phosphotyrosyl than phosphoseryl moiety. To our surprise, the pThr-peptides analysis showed a similar enrichment

outcome to pTyr-peptides; 80% of pThr-peptides deriving from pTyr-MIP–TiO2 had higher intensities than their pThrpeptides counterparts identified with TiO2 –a finding that deserves future research. However, pTyr-MIP–TiO2 numerical ratio of pSer: pThr: pTyr was not different from TiO2.

The two combined methods enriched a comparable number of phosphopeptides from 600 µg peptides per method (three technical replicates per protocol, 200 µg per replicate): 1693 and 1842 respectively—the reference, TiO2 enrichment yield 1328 phosphopeptides. The increased phosphopeptide coverage from the combined methods is a common effect when orthogonal methods, for example, pTyr-MIP and TiO2, are used. The set of unique pTyr-MIP–TiO2 phosphopeptides show more similarities with phosphopeptides (a modest number) derived by pTyr-MIP-SPE than with the set of TiO2 unique phosphopeptides. They are frequently short, mono-phosphorylated and with the phosphosite located in the proximity of the peptide N-terminal.

The developed combined method pTyr-MIP–TiO2 offers another alternative for global phosphoproteomic sample preparation with a tweak for pTyr-peptides, increasing the coverage and depth not only of pTyr-but also pSer- and pThr-peptides.