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Evaluation of a Solid-Supported Tagging Strategy for Mass Spectrometric Analysis of Peptides

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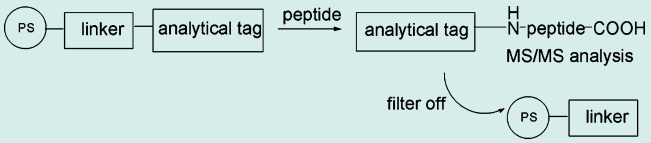
Supporting Information

ABSTRACT: We have explored two divinylbenzene cross-linked polystyrene supports for use in a solid-supported N-terminal peptide tagging strategy. Resin-bound tags designed to be cleaved in a single step at the N-terminus of peptides have been devised and explored as peptide N-terminal tagging reagents (constructs) for subsequent mass spectrometric analysis. While the brominated tagging approach shows promise, the use of these specific solid supports has drawbacks, in terms of tagging reaction scale, for real applications in proteomics.



KEYWORDS: *solid-phase synthesis, bromine, mass spectrometry, peptide labeling, proteome*

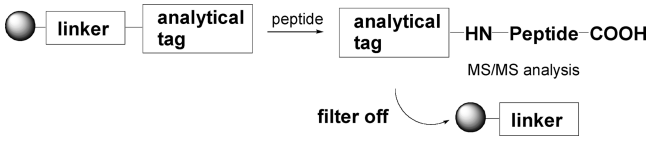
INTRODUCTION

The completion of the human genome and rapid developments in mass spectrometry have led to an explosion of interest in the postgenomic discipline of proteomics, the study of all the proteins within a cell, tissue, or organism. The proteome is dynamic, fluctuating in response to internal and external stimuli. Proteins are complex to analyze and, unlike DNA and RNA, cannot be amplified for analysis; sample complexity and limited quantities of at least some members of the complex proteome are inherent in proteomic studies. In a shotgun proteomic workflow,[1] the protein mixture is digested using a proteolytic enzyme, often trypsin, and separated using 1- or 2-dimensional approaches prior to MS analysis. Clearly, in such an experiment, protein−peptide connectivities are sacrificed, and protein identification relies on the generation of mass and sequence-ion data that allow the peptides to be matched to the database protein sequences from which they originated.

Chemical tagging[2,3] of peptides prior to mass spectrometric analysis can be used to simplify and enrich complex mixtures of peptides[4,5] or to manipulate their mass spectrometric behavior.[6] Chemical tags can be designed to target the N-terminus of peptides[7] or specific amino acid side chains.[4,5] Current commercially available stable isotope-bearing mass tags include ICAT,[8] iTRAQ,[9] and TMT[10] reagents, which are or have been widely used, but all have inherent limitations. ICAT only labels a subset of peptides (those containing cysteine). iTRAQ and TMT achieve relative peptide quantification using low molecular mass tag-derived fragment ions generated in a product ion experiment but suffer from the need to remove unreacted tag and from dynamic range issues.[11,12]

A limited number of solid-phase isotopic tagging approaches have been reported but all, to date, involve "capture and release"[13−17]. An isotopic label is immobilized on a solid support via a cleavable linker, peptides are captured and then loaded resins are cleaved in a separate second step releasing isotope-containing tagged peptides for LC-MS analysis. Although this approach has shown promise it has not been widely adopted by the proteomic community; the reasons for this are currently not clear. Consequently we set out to explore, in depth, two solid supports for use in a solid-phase N-terminal tagging strategy, using resin-bound immobilized tags (analytical constructs) designed to be cleaved not in a separate step but in a single step by the N-terminus of peptides (Scheme 1). This

Scheme 1. Construct N-Terminal Tagging Workflow



results in a simplified workflow for peptide tagging in which unreacted tag and resin-bound byproduct are readily removed by filtration, and which allows facile MS detection via a mass spectral marker allowing tagged peptides to be easily identified from chemical background. A solid-phase approach also has potential for automating the workflow and reusing any excess tag. We have critically evaluated this approach for potential application in real proteomic analyses.

RESULTS AND DISCUSSION

Solid-supported N-terminal peptide tagging with an analytical tagging construct uses the N-terminus of a peptide to cleave, in one step, an immobilized analytical tag from a solid-phase, giving the tagged peptide in solution. It is a labeling strategy

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that targets all peptides via their primary amines, and provides a simplified workflow, as the tag can be used in excess and resin-bound linker (bearing unreacted tag) removed by filtration before analysis (Scheme 1).

The tagging construct consists of three components: the solid-support, the linker and the analytical tag. When selecting the solid-support, we dismissed the usual readily available aqueous compatible supports, such as PEGA[18] and TentaGel[19,20] because of handling difficulties and potentially leachable PEG. Instead, we investigated 2% and 10% divinylbenzene cross-linked polystyrene. The former undergoes limited swelling under aqueous conditions,[21] exposing only surface-bound analytical tags for peptide labeling; the latter macroporous polystyrene has fixed pores, a high internal surface area and readily accessible functionality without the need for resin swelling.[22] Tetrafluorophenyl esters (TFP) were used as the linker because of reasonable hydrolytic stability and efficient cleavage by aminolysis.[23] Macroporous polystyrene was commercially available prederivatized with the TFP linker (1.16 $mmolg^{-1}$); the analogous derivative of 2% cross-linked aminomethyl polystyrene (1 mmol g^{-1}) was prepared.[24]

Three analytical tags were chosen to provide peptides with advantageous mass spectrometric characteristics: 3-carboxypropyl trimethylammonium chloride **1**,[25] 4-bromobenzyl 4-carboxybutyl dimethylammonium bromide **2**, and 4-bromophenyl acetic acid **3** (Figure 1). Tags **2** and **3** incorporate bromine, which acts as a "mass peak splitter" in MS analysis.[26]

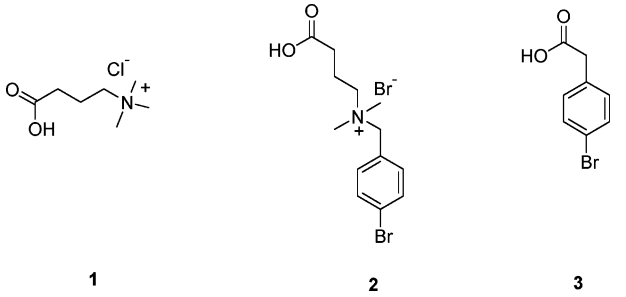

Figure 1.

Each of the three tags were immobilized on TFP-functionalized cross-linked polystyrene using DIC-mediated coupling, to give constructs **4**–**6**; tags **2** and **3** were also immobilized on macroporous polystyrene to give constructs **7** and **8**. Butylamine, Gly-Phe, leucine enkephalin (Leu-Enk) and a tryptic digest of ovalbumin were tagged and analyzed using MS (Table 1).

With simple peptides, expected products were observed for constructs **4** and **6** but not construct **5**. In contrast, the macroporous analogues of **5** and **6** (resins **7** and **8**) both gave labeling of Leu-Enk. No labeled peptides were detected with a tryptic digest of ovalbumin. It was also noted that upon CID fragmentation of peptides labeled with **1**, the first loss was the $-{}^{+}N(CH_3)_3$ group. Such loss of the charge-bearing moiety has been observed by others.[27,28] The general value for shotgun proteomics applications of a tag carrying a positive charge, if it is readily lost upon the central step of CID, is thus questionable. Using construct **7**, an additional bromine free peak was observed in the MALDI mass spectrum of tagged Leu-Enk (m/z 624) corresponding to **9**.

This is consistent with a Hofmann-type elimination reaction occurring either during the tagging reaction or during the MALDI ionization process. MALDI mass spectra were obtained over a range of laser powers. At low power (20%) the most intense peak was tagged Leu-Enk. On increasing the laser power (up to 80%) this peak decreased in intensity and the peak at m/z 624 increased, consistent with elimination occurring in source. This provides further evidence that a fixed positive charge tag has limitations for MS-based workflows.

With the failure to tag the peptides resulting from the tryptic digestion of ovalbumin, the sensitivity of the tagging process was tested by systematically decreasing the amount of Leu-Enk from 60 μg [0.108 μmol in 200 μL of TEAB buffer:1,4-dioxane (50:50)] to 3 μg [0.005 μmol in 200 μL of TEAB buffer:1,4-dioxane (50:50)] while keeping the amount of resin **6** constant (10 mg). The minimum amount of Leu-Enk that could be detected after tagging with **6** was 4 μg. Between 60 and 4 μg, tagged peptide and a low level of untagged peptide were evident; below 4 μg neither tagged product peaks nor untagged peptides could be distinguished from background. This result suggests that this resin is not compatible with this specific peptide (Leu-Enk) but this may not be applicable to other peptides as untagged peptides were observed in the unsuccessful tagging of a tryptic digest of ovalbumin.

In conclusion, the digestion of 80 μg of a 50 kDa single protein may be expected to give rise to perhaps 50 different peptides, and thus approximately 1.6 μg of each peptide. This is below the level that could be detected after tagging. Furthermore, a realistic level of complexity would be at least a subproteome digest which would contain a large number of individual peptides, many of which would be expected to be present at much lower levels.

Our work suggests that there are major limitations in using polystyrene-based resins for application in proteomics workflows. While there are many theoretical benefits to a solid-phase workflow such as potential for automation, minimizing sample handling, there are factors that clearly prevent solid-phase approaches being widely adopted for proteomic reactions. The solid-phase and the microenvironment surrounding the func-

Table 1. Results of MS Analysis of Tagging Reactions using DVB-Crosslinked Resin Constructs[a]

construct	*n*-butylamine[b]	Gly-Phe[b,d]	Leu-Enk[c,d]	ovalbumin[c,d]
4	201 $[M^+]$	350 $[M^+]$	683 $[M^+]$	n.d.
5	355/357 $[M^+]$	n.o.	n.o.	n.d.
6	270/272 $[M+H]^+$	417/419 $[M-H]^-$	750/752 $[M-H]^-$	n.o.
7	n.d.	n.d.	837/839 $[M]^+$	n.d
8	n.d.	n.d.	750/752 $[M-H]^-$	n.d.

[a] m/z values are listed for those reactions giving substantial signal intensities for the expected peaks. [b] Analyzed by ESI-MS. [c] Analyzed by MALDI-MS. [d] Tagging reaction used 10 mg construct in 200 μL TEAB buffer:1,4-dioxane (50:50). n.o. = expected tagged peaks not observed, untagged peptide peaks observed. n.d. = not determined.

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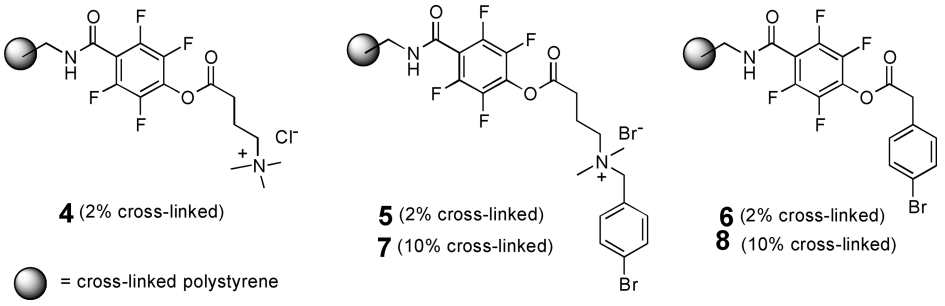

Figure 2. Structures of polystyrene constructs.

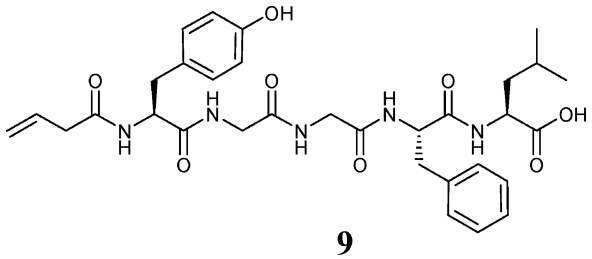

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Figure 3.

tional sites of resins can be complex.[29] The complexities of the factors affecting solid-phase reactions are often underestimated; this issue was addressed by Czarnik[30] who commented that solid-supports are like the solvent in a solution-phase reaction and hence have an influence on reaction kinetics and product distribution. While the resin is often drawn as a simple spherical bead, what is happening within the resin and the interactions with the reagents are difficult to predict. Several papers discuss the issues surrounding choice of support, reaction conditions, and show the variables in this type of chemistry.[29,31,32]

In proteomic scale experiments, samples typically contain a wide dynamic range of protein levels, and hence of their tryptic peptides. This can make recovery of peptides from the solid-phase difficult, especially for peptides with low abundance.[33] It is possible that these factors may underlie the failure of the proteomic community to develop and adopt solid-phase tagging strategies for their applications, in spite of the positive reports in the literature.[13−17,20] There appears to be no consensus in the literature regarding choice of solid-phase, reaction conditions and scale. This suggests that with many variables already affecting peptide tagging reactions for protein identification and quantitation, the use of solid-phase tagging strategies adds a new, unwanted, level of complexity in proteome analysis. However, in spite of the limitations we have identified in the solid-phase approach described, the brominated tags themselves nonetheless show promise and are the subject of ongoing investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental details and selected mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.R., J.T.-O, and S.R.H conceived and designed experiments. S.R.H., F.L.C., E.P., and P.A.H. performed experiments. A.R. and J.T.-O. cowrote manuscript.

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■ ABBREVIATIONS

CID, collision induced dissociation; DCM, dichloromethane; DIC, diisopropylcarbodiimide; PEG, polyethylene glycol; TEAB, trimethylammonium bicarbonate; TFP, tetrafluorophenol

■ REFERENCES

(1) Washburn, M. P.; Wolters, D.; Yates, J. R. III Large-Scale Analysis of the Yeast Proteome by Multidimensional Protein Identification Technology. *Nat. Biotechnol.* **2001**, *19*, 242−247.

(2) Leitner, A.; Lindner, W. Chemistry Meets Proteomics: The Use of Chemical Tagging Reactions for MS-based Proteomics. *Proteomics* **2006**, *6*, 5418−5434.

(3) Zhou, H.; Boyle, R.; Aerbersold, R. Quantitative Protein Analysis by Solid-Phase Isotope Tagging and Mass Spectrometry. *Methods Mol. Biol.* **2004**, *261*, 511−518.

(4) Gevaert, K.; Ghesquière, B.; Staes, A.; Martens, L.; Van Damme, J.; Thomas, G. R.; Vandekerckhove, J. Reversible Labeling of Cysteine-Containing Peptides Allows Their Specific Chromatographic Isolation for Non-Gel Proteome Studies. *Proteomics* **2004**, *4*, 897−908.

(5) Conrads, T. P.; Alving, K.; Veenstra, T. D.; Belov, M. E.; Anderson, G. A.; Anderson, D. J.; Lipton, M. S.; Paša-Tolić, L.; Udseth, H. R.; Chrisler, W. B.; Thrall, B. D.; Smith, R. D. Quantitative Analysis of Bacterial and Mammalian Proteomes Using a Combination of Cysteine Affinity Tags and ^{15}N-Metabolic Labeling. *Anal. Chem.* **2001**, *73*, 2132−2139.

(6) Hennrich, M. L.; Boersema, P. J.; van den Toorn, H.; Mischerikow, N.; Heck, A. J. R.; Mohammed, S. Effect of Chemical Modifications on Peptide Fragmentation Behavior upon Electron Transfer Induced Dissociation. *Anal. Chem.* **2009**, *81*, 7814−7822.

(7) Mason, D. E.; Liebler, D. C. Quantitative Analysis of Modified Proteins by LC-MS/MS of Peptides Labeled with Phenyl Isocyanate. *J. Proteome Res.* **2003**, *2*, 265−272.

(8) Gygi, S. P.; Rist, B.; Gerber, S. A.; Tureck, F.; Gelb, M. H.; Aebersold, R. Quantitative Analysis of Complex Protein Mixtures Using Isotope-Coded Affinity Tags. *Nat. Biotechnol.* **1999**, *17*, 994−999.

(9) Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai,.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J. Multiplexed Protein Quantitation in *Saccharomyces*

cerevisiae Using Amine-Reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* **2004**, *3*, 1154−1169.
(10) Thompson, A.; Schafer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Hamon, C. Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Anal. Chem.* **2003**, *75*, 1895−1094.
(11) Ow, S. Y.; Salim, M.; Noirel, J.; Evans, C.; Rehman, I.; Wright, P. C. iTRAQ Underestimation in Simple and Complex Mixtures: "The Good, the Bad and the Ugly". *J. Proteome. Res.* **2009**, *8*, 5347−5355.
(12) Shirran, S. L.; Bottting, C. H. A Comparison of the Accuracy of iTRAQ Quantification by nLC-ESI MSMS and nLC-MALDI MSMS Methods. *J. Proteomics* **2010**, *73*, 1391−1403.
(13) Qiu, Y.; Sousa, E. A.; Hewick, R. M.; Wang, J. H. Acid-Labile Isotope-Coded Extractants: A Class of Reagents for Quantitative Mass Spectrometric Analysis of Complex Protein Mixtures. *Anal. Chem.* **2002**, *74*, 4969−4979.
(14) Zhou, H.; Ranish, J. A.; Watts, J. D.; Aebersold, R. Quantitative Proteome Analysis by Solid-Phase Isotope Tagging and Mass Spectrometry. *Nat. Biotechnol.* **2002**, *20*, 512−515.
(15) Shi, Y.; Xiang, R.; Crawford, J. K.; Colangelo, C. M.; Horváth, C.; Wilkins, J. A. A Simple Solid Phase Mass Tagging Approach for Quantitative Proteomics. *J. Proteome. Res.* **2004**, *3*, 104−111.
(16) Chowdhury, S. M.; Munske, G. R.; Siems, W. M.; Bruce, J. E. A New Maleimide-Bound Acid-Cleavable Solid-Support Reagent for Profiling Phosphorylation. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 899−909.
(17) Shi, Y.; Xiang, R.; Horváth, C.; Wilkins, J. A. Design and Synthesis of a Solid-Phase Fluorescent Mass Tag. *J. Sep. Sci.* **2005**, *28*, 1812−1817.
(18) Meldal, M. Pega: A Flow Stable Polyethylene Glycol Dimethyl Acrylamide Copolymer for Solid Phase Synthesis. *Tetrahedron Lett.* **1992**, *33*, 3077−3080.
(19) Quarrell, R.; Claridge, T. G. W.; Weaver, G. W.; Lowe, G. Structure and Properties of TentaGel Resin Beads: Implications for Combinatorial Library Chemistry. *Mol. Divers.* **1996**, *1*, 223−232.
(20) Napoli, A.; Athanassopoulos, C. M.; Moschidis, P.; Aiello, D.; Di Donna, L.; Mazzotti, F.; Sindona, G. Solid Phase Isobaric Mass Tag Reagent for Simultaneous Protein Identification and Assay. *Anal. Chem.* **2010**, *82*, 5552−5560.
(21) Mendonca, A. J.; Xiao, X.-Y. Optimization of Solid Supports for Combinatorial Chemical Synthesis. *Med. Res. Rev.* **1999**, *19*, 451−462.
(22) Hori, M.; Gravert, D. J.; Wentworth, P. Jr.; Janda, K. D. Investigating Highly Crosslinked Macroporous Resins for Solid-Phase Synthesis. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2363−2368.
(23) Lee, J. W.; Louie, Y. Q.; Walsh, D. P.; Chang, Y. T. Nitrophenol Resins for Facile Amide and Sulfonamide Library Synthesis. *J. Comb. Chem.* **2003**, *5*, 330−335.
(24) Salvino, J. M.; Vasant Kumar, N.; Orton, E.; Airey, J.; Kiesow, T.; Crawford, K.; Mathew, R.; Krolikowski, P.; Drew, M.; Engers, D.; Krolikowski, D.; Herpin, T.; Gardyan, M.; McGeehan, G.; Labaudiniere, R. Polymer-Supported Tetrafluorophenol: A New Activated Resin for Chemical Library Synthesis. *J. Comb. Chem.* **2000**, *2*, 691−697.
(25) Mirzaei, H.; Regnier, F. Enhancing Electrospray Ionization Efficiency of Peptides by Derivatization. *Anal. Chem.* **2006**, *78*, 4175−4183.
(26) Portal, C.; Launay, D.; Merritt, A.; Bradley, M. High Throughput Physical Organic Chemistry: Analytical Constructs for Monomer Reactivity Profiling. *J. Comb. Chem.* **2005**, *7*, 554−560.
(27) He, Y.; Reilly, J. P. Does a Charge Tag Really Provide a Fixed Charge? *Angew. Chem., Int. Ed.* **2008**, *47*, 2463−2465.
(28) Che, F.-Y.; Fricker, L. D. Quantitative Peptidomics of Mouse Pituitary: Comparison of Different Stable Isotopic Tags. *J. Mass Spectrom.* **2005**, *40*, 238−249.
(29) Hodge, P. Polymer-Supported Organic Reactions: What Takes Place in the Beads? *Chem. Soc. Rev.* **1997**, *26*, 417−424.
(30) Czarnik, A. W. Solid-Phase Synthesis Supports are Like Solvents. *Biotechnol. Bioeng. (Comb. Chem.)* **1998**, *61*, 77−79.
(31) Vaino, A. R.; Janda, K. D. Solid-Phase Organic Synthesis: A Critical Understanding of the Resin. *J. Comb. Chem.* **2000**, *2*, 579−596.
(32) Helliwell, P. A.; Bailey, V. A.; Chevet, C.; Clarke, D. B.; Lloyd, A.; Macarthur, R.; Routledge, A. A Mass Spectrometric Investigation into Microenvironmental Effects in Solid-Supported Radical Chemistry. *React. Funct. Polym.* **2010**, *70*, 110−115.
(33) Iliuk, A.; Galan, J.; Tao, W. A. Playing Tag with Quantitative Proteomics. *Anal. Bioanal. Chem.* **2009**, *393*, 503−513.