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Quantitation of binding, recovery and desalting efficiency of peptides and proteins in solid phase extraction micropipette tips

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Abstract

Micropipette-tip solid phase extraction (SPE) systems are common in proteomic analyses for desalting and concentrating samples for mass spectrometry, removing interferences, and increasing sensitivity. These systems are inexpensive, disposable, and highly efficient. Here, we show micropipette-tip solid phase extraction is a direct sample preparation method for ^{14}C-accelerator mass spectrometry (AMS), removing salts or reagent from labeled macromolecules. We compared loading, recovery and desalting efficiency in commercially available SPE microtips using ^{14}C-labeled peptides and proteins, AMS, and alpha spectrometry ion energy loss quantitation. The polypropylene in the tips was nearly ^{14}C-free and simultaneously provided low-background carrier for AMS. The silica material did not interfere with the analysis. Alpha spectrometry provided an absolute measurement of desalting efficiency.

Keywords: Solid phase extraction; Quantitation; Recovery; Peptides; Proteins; Accelerator mass spectrometry

1. Introduction

Micropipette-tip solid phase extraction (SPE) is commonplace in proteomic and other analytical applications: desalting, concentrating, or fractionating peptides and proteins prior to matrix-assisted laser desorption or electrospray mass spectrometry [1–3]. Some efforts have been made to quantify sample recovery using ^{125}I-labeled peptides with γ-counting [4] or ^{18}O-labeled peptides for measurement of ^{16}O/^{18}O ratios using mass spectrometry [5]. High-energy β^- from ^{131}I and ^{32}P have also been used to measure residual binding to polypropylene tubes [6]. We used ^{14}C-labeling and accelerator mass spectrometry (AMS) to measure sample binding and recovery in SPE micropipette-tips. Among the advantages of AMS are the inherent sensitivity (low attomole ^{14}C) [7] and absolute quantitation independent of the chemical nature of the analyte [8–10].

AMS is a sensitive mass spectrometric method for measuring rare isotopes, especially radioisotopes such as ^{3}H and ^{14}C [11] currently expanding into analytical and pharmaceutical industries. AMS measures isotope ratios independent of half-life or decay products, since it directly counts the rare isotope (e.g. ^{14}C). We used AMS and ^{14}C-labeled peptides and protein to demonstrate the utility of AMS for absolute quantitation of binding and recovery in solid phase extraction micropipette-tips and similar devices. Low-energy β^- from isotopes such as ^{3}H and ^{14}C do not penetrate the polypropylene of the tips and could not be used with decay counting methods to measure the isotope in the tips in situ. This technique, like high-energy β^- and γ-counting, can accurately measure remaining sample (^{14}C) in the tip, but uses million-fold lower amounts of radioisotope, increasing safety.

We also measured the desalting efficiency directly from normal saline (0.9% NaCl) using ion-energy loss quantitation of the total sample mass (MELQ) [12] compared to the amount of labeled protein determined by AMS.

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2. Materials and methods

2.1. Samples

Unlabeled BSA and ^{14}C-methylated BSA (^{14}C-BSA) were acquired from Calbiochem (San Diego, CA, USA) and Sigma (St. Louis, MO, USA), respectively. This ^{14}C-BSA is labeled through reductive alkylation of primary amines with ^{14}C-formaldehyde and sodium cyanoborohydride according to the protocol of Dottavio-Martin and Ravel [13]. Through serial dilutions in unlabeled BSA, 167 fmol (11 ng), 400 pCi (15 Bq) ^{14}C-BSA was added to make a 1 ml, 1 mg/ml 400 pCi BSA stock solution. This was further diluted to make 0.1 mg/ml BSA sample solutions in normal saline (0.9% NaCl, Merck KGaA, Darmstadt, Germany), 0.1% TFA and, for C_4 tips, also 2.0 M Guanidine–HCl (Sigma). A 100 μl aliquot (100 μg) of the stock solution was digested by trypsin (Promega, Madison, WI, USA, Sequencing Grade) following a protocol previously used to digest BSA [14] using 2.5 μg trypsin in 500 μl 100 mM NH_4HCO_3 (Sigma) for 24 h. The BSA digest was diluted to make 1 ml 0.1 μg/μl digest in 0.9% NaCl. All solid-phase extractions were made from 40 μl, 0.1 μg/μl samples.

To verify that the ^{14}C-methylated peptides are representative tryptic peptides, the BSA digest was run on an LC Packings UltiMate™ Capillary/Nano LC System (Dionex, Sunnyvale, CA, USA) using a 15 cm, 75 μm i.d. PepMap™ C18 column (Dionex) and pre-blended H_2O:ACN 10:90 and 90:10 with 0.1% TFA (v/v/v) mobile phases (Sigma) and a gradient from 10 to 66% ACN in 50 min after 10 min isocratic elution with 10% ACN, both at 300 nl/min. Fractions were collected in 9 mm × 6 mm tin capsules (Elemental Microanalysis Limited, Devon, UK) in a standard microtiter plate every 25 s using an Probot Micro Fraction Collector (Dionex), which simultaneously added 1.0 μl (±4%) tributyrin carrier compound to each fraction using the integrated syringe pump.

2.2. Solid phase extraction

One and two microliter aliquots of the 0.1 μg/μl protein and peptide sample solutions were taken to measure the specific activity of the protein. Fresh solutions for the desalting procedure were prepared according to the manufacturers' instructions using HPLC grade water (Sigma), acetonitrile (Sigma) and TFA (Fluka, Buchs, Switzerland). A BioHit ePet electronic 0.1–10 μl pipettor (BioHit Oyj, Helsinki, Finland) was used for all aspiration and dispension of sample and solvents. According to the manufacturer's specifications [15], this pipettor has an inaccuracy of 0.9% (0.5% imprecision) at 10 μl, 2.5% (1.5% imprecision) at 1 μl and 12% (10% imprecision) at 0.2 μl. Interpolating between these values, the inaccuracy is about 2% (1.1% imprecision) at 2 μl and 5% (3.5% imprecision) at 0.5 μl. The pipettor was used at the lowest speed setting, aspirating and dispensing 10 μl in 1.2 s. The pipettor, pipette tips and all solutions and samples were at room temperature (20 °C).

Sample binding, recovery, and desalting efficiency were compared using bovine serum albumin (BSA) and a BSA tryptic digest in Millipore (Bedford, MA, USA) ZipTip® tips, both standard ($ZipTip_{C18}$) and microbed ($ZipTip_{U\text{-}C18}$) C_{18} and C_4 ($ZipTip_{C4}$) tips, Varian (Lake Forest, CA, USA) OMIX® C18 and microbed (OMIX® C18MB) C_{18} tips and Eppendorf (Hamburg, Germany) PerfectPure C-18 C_{18} tips.

Sample eluate and washes were rapidly collected in custom made quartz tubes containing MeOH (J.T. Baker, Phillipsburg, NJ, USA) and dried in a Jouan RC 10.10 centrifugal evaporator (Jouan, Winchester, VA, USA) at ambient temperature. To give sufficient carbon for AMS analysis, 1 μl tributyrin was added as carrier carbon to each tube, the contents combusted to CO_2 and subsequently reduced to graphite for AMS [8–10,16]. The ends of the pipette tips containing the chromatographic material were cut using a PEEK capillary cutter (UpChurch, Oak Harbor, WA, USA) with a holder made from a larger pipette tip to make identical cuts 1 mm above the packed end of the tip. The tip ends were dried, weighed and placed in quartz tubes. Control tips wetted and equilibrated according to the manufacturers' protocols and cut using the same tool were used as controls and background measurements. The absolute carbon content in the combustible fraction of each type of tip was measured separately in an Exeter Analytical (North Chelmsford, MA, USA) Model CE440 elemental analyzer. The experiments were carried out in random order to reduce systematic errors.

In this non-exhaustive comparison of commercially available SPE micropipette-tips, individual polypropylene SPE micropipette-tips were combusted to CO_2, reduced to graphite [8–10] and the $^{14}C/^{13}C$ ratio measured by AMS [16]. The absolute amount of ^{14}C-labeled peptide remaining in the tip was derived from the mass of carbon in the sample, i.e. the weight of the tip and its carbon content. Eluted sample was measured in the same way, except a well-defined amount of carbon carrier was added in the form of 1 μl tributyrin [8–10].

To measure desalting efficiency, samples were eluted onto 80 nm SiN windows and total mass (salt and protein) measured by MELQ [12]. The windows were placed in quartz tubes, supplemented with 1 μl tributyrin, and quantified by AMS measurement of the ^{14}C-labeled peptides or protein.

3. Results and discussion

The $^{14}C/^{13}C$ ratios of the wash, eluate and pipette tips are normalized to identically prepared standards of known isotope concentration [4]. The resulting isotope ratio can be expressed in fraction Modern, Curie (Ci) or moles ^{14}C per gram carbon. Contemporary carbon is about 1 Modern or 97.8 amol ^{14}C per milligram carbon. All nine controls were near instrumental background, 0.02–0.03 Modern, due to the fossil origin of the carbon in the polypropylene (equivalent to a few nanograms of labeled protein which was subtracted from the measured ^{14}C). The specific activity of ^{14}C in the peptide or protein sample (6.4 amol ^{14}C per ng BSA) translates the

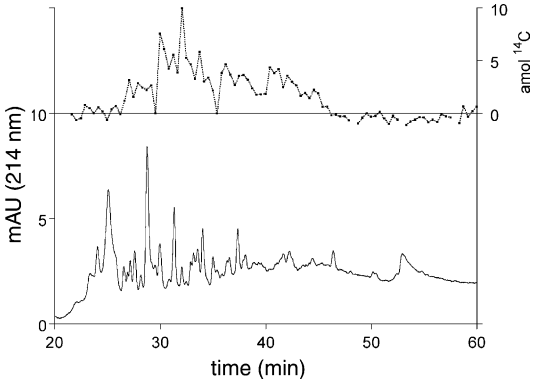

Fig. 1. The BSA tryptic digest was analyzed by reversed-phase liquid chromatography and AMS to verify digestion of ^{14}C-labeled peptides. The ^{14}C-methylated peptides (dashed, AMS data) have different and slightly longer elution times than the unlabeled (solid, UV absorbance) due to the hydrophobic methyl group and/or missed cleavages due to the methylation of lysines. Less than 0.05% of the total absorbance is due to labeled peptides and the ^{14}C from unlabeled peptides is negligible, <0.05% of carrier (background) ^{14}C in each fraction.

measured isotope ratios into an amount of recovered or bound peptide or protein.

The carbon contents of the combustible fractions of the tip ends were found to be 86.8 ± 1.7% (ZipTip$_{C18}$), 85.9 ± 1.7% (OMIX® C18) and 85.5 ± 1.7% (PerfectPure C-18), the remainder being hydrogen (individual measurements with experimental uncertainty). These are consistent with polypropylene, $[C_3H_6]_n$, the specified plastic material of the tips with 85.6% carbon content. The tip polypropylene provided sufficient carbon without addition of carrier.

The reversed-phase separation of the BSA digest shows that the labeled peptides are representative for tryptic peptides in their elution in reversed phase (Fig. 1). The peptides are not uniformly labeled, but a wide range of peptides does contain the isotopic signature. The majority of these elute in less than 50% ACN, or before 55 min in the chromatogram.

3.1. Sample binding and wash

The manufacturer's protocol suggests using between 3 and 10 sample loading cycles for ZipTip® tips. ZipTip$_{C4}$ tips are not saturated at a 0.1 μg/μl BSA concentration after 3 loading cycles (Fig. 2). The recovery of bound BSA eluting in 2 μl 50% ACN was ∼210 of ∼280 ng (77 ± 9%) BSA bound after 3 cycles and ∼440 of ∼640 ng (66 ± 16%) bound after 10 cycles. Most of this, or ∼370 ng BSA, eluted in the first 1 μl. The sample loss in the 3 × 10 μl 0.1% TFA wash corresponded to ∼11% of the sample in the tip (bound and free) after the 10th binding cycle. The sample loaded onto the tips, or the maximum amount of peptide or protein that would be bound assuming 100% retention and perfect mixing of the sample between each aspiration is 2.31 μg after 3 cycles and 3.77 μg after 10 cycles (out of the 4 μg peptide or protein in the 40 μl samples).

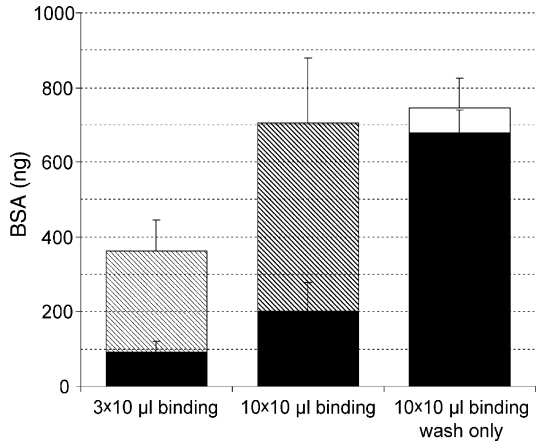

Fig. 2. Recovery of BSA in MilliPore ZipTip$_{C4}$ tips using the manufacturer's protocol and 3 or 10 loading cycles; remaining BSA in tip after wash (solid) and eluted in 2 μl (hatched). The top error bars are standard deviations of the total bound protein after wash (n = 4). The right bar shows sample lost in the 3 × 10 μl 0.1% TFA wash (white) and sample remaining in tip (solid, n = 4).

3.2. Sample recovery

The relative recovery, the eluted fraction of loaded BSA digest, was comparable in the ZipTip$_{C18}$ (75 ± 3%) and OMIX® C18 tips (74 ± 1% discarding one outlier) and significantly higher in the PerfectPure C-18 tips (92 ± 1%) (Fig. 3). However, the ZipTip$_{C18}$ bound and recovered more sample than the other two types of C18 tips from identical samples using the same loading and elution conditions. The binding and recovery of BSA was less than that of the peptides in ZipTip$_{C18}$, ZipTip$_{C4}$ and OMIX® C18 tips, as well as the smaller bed-volume ZipTip$_{U\text{-}C18}$ and OMIX® C18 MB (Fig. 4).

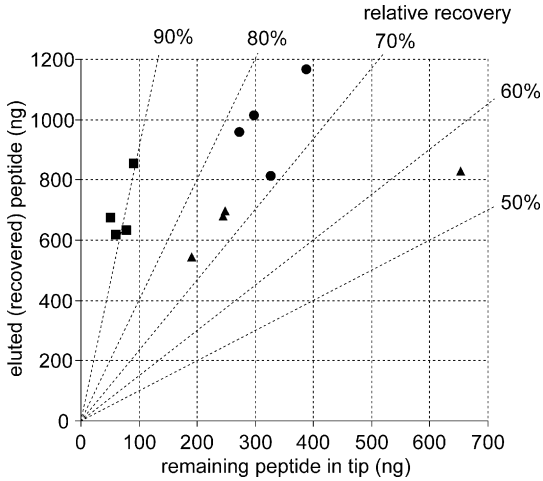

Fig. 3. Recovery of a BSA tryptic digest in commercially available micropipette SPE tips. Eppendorf PerfectPure C-18 (■), MilliPore ZipTip$_{C18}$ (●), and Varian OMIX® C18 (▲). The AMS measurement uncertainties are within the size of the markers.

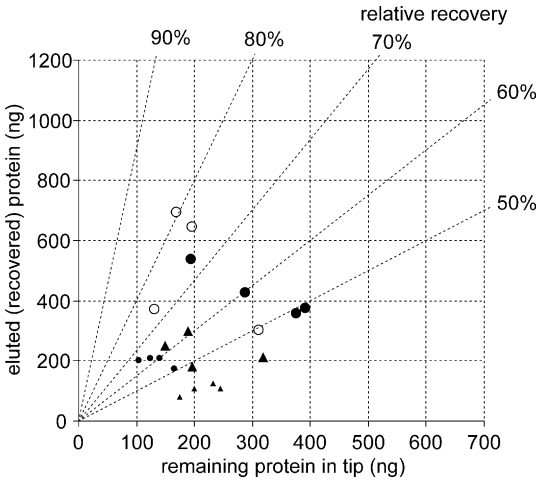

Fig. 4. Recovery of BSA in MilliPore ZipTip$_{C4}$ (◯), MilliPore ZipTip$_{C18}$ (●), and Varian OMIX® C18 tips (▲) in 2 μl 50% ACN. Small symbols represent recovery in 0.5 μl 50% ACN 0.1% TFA using ZipTip$_{U\text{-}C18}$ and OMIX® C18MB, respectively. The AMS measurement uncertainties are within the size of the markers.

3.3. Desalting efficiency

Others have focused on the improvements in mass spectra to characterize desalting efficiency [1,17], but we quantified the desalting efficiency directly using alpha spectrometry to determine the total eluted mass [12], measurement of eluted protein using AMS, and knowledge of the initial NaCl concentration (0.9%). Fig. 5 shows the reduction in salt concentration for the three types of tips compared. The desalting efficiency was generally high, 98–99% for most tips, with occasional ZipTip$_{C4}$ and OMIX® C18 MB tips found to perform less well. The number of analyzed tips is insufficient to determine the frequency of poor-performing tips, although the ZipTip$_{U\text{-}C18}$ tips does seem to have a higher and more consistent desalting efficiency than the OMIX® C18 MB tips. Large air bubbles were frequently observed when using ZipTip® tips, especially during multiple sample loading cycles of BSA. The PerfectPure tips released small air bubbles into the sample, even after pre-wetting and equilibration. No air bubbles were observed in the OMIX® tips.

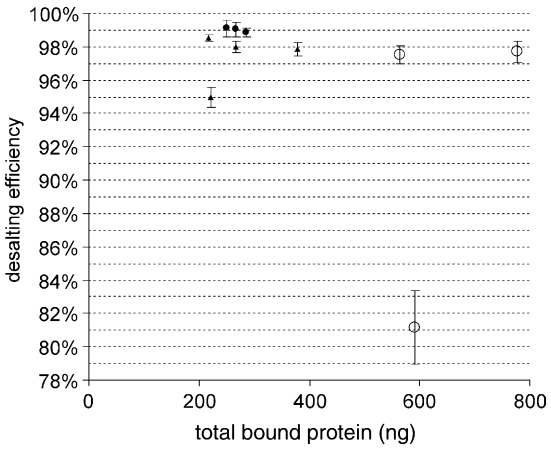

Fig. 5. Desalting efficiency in MilliPore ZipTip$_{C4}$ tips (◯), MilliPore ZipTip$_{U\text{-}C18}$ (●), and OMIX® C18MB (▲) measured by alpha spectrometry. Since alpha spectrometry is non-destructive, the eluted protein can subsequently be quantified by AMS and subtracted from the total measured mass. The error bars show estimated error propagated from MELQ (±10%) and AMS (±2%).

3.4. Utility for AMS sample preparation

The measured desalting efficiency, if extrapolated to a small, polar (small capacity factor) species used to transfer a label (e.g. ^{14}C) to a macromolecule of interest, may be sufficient to determine specific reactivity or binding to the macromolecule within ∼10% using MELQ and AMS, as long as the concentration of the reagent is less than 10 times higher than the concentration of the macromolecule. If a larger excess of reagent is used, two or more purification steps may be required. This presents a possible tool for high throughput quantitation of molecular (drug, toxin, etc.) binding to macromolecules without initial immobilization of the macromolecule to SPE, beads or other solid media. The molecular binding would take place homogeneously in a low concentration, non-denaturing solution using safely low amounts of isotopic labels.

4. Conclusions

The results illustrate on one hand that low-level ^{14}C-labeling and AMS is a sensitive method to quantify binding and recovery in SPE and on the other hand how disposable micropipette-tip SPE systems can be used for AMS sample preparation, particularly for labeling studies. The choice of micropipette SPE tip is obviously dependent on many factors not taken into account here, and the results should be interpreted accordingly. In these studies, the manufacturers' standard protocols were used throughout and optimization of these is likely to improve recovery from the tips in a particular analytical situation.

In addition to the throughput, accuracy, precision, and sensitivity inherent to AMS, the specific advantage of using AMS in this context is that the solid phase and eluted sample are combusted and measured in an identical manner, independent on the chemical nature of the analyte. It should be emphasized that this technique allows million-fold lower levels of radioactivity than using ^{125}I or other γ- or high-energy β^- emitters as in earlier studies, reducing concerns regarding storage, handling and disposal of radioactive materials. The method should be adaptable to follow sample recovery in most microfluidic systems. Micropipette-tip solid phase extraction is a direct sample preparation method for AMS, potentially removing ^{14}C-labeling reagent from labeled biomolecules such as proteins. With successful purification and concentration in a small volume, micropipette tip SPE is an ideal complement for sample definition by desalting prior to mass quantitation using ion-energy loss, yielding

the specific binding or reactivity of a small, isotope-labeled compound to a macromolecule.

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