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Highly efficient and selective enrichment of peptide subsets combining fluororous chemistry with reversed-phase chromatography

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Abstract

The selective capture of target peptides poses a great challenge to modern chemists and biologists, especially when enriching them from proteome samples possessing extremes in concentration dynamic range and sequence diversity. While approaches based on traditional techniques such as biotin-avidin pairing offer versatile tools to design strategies for selective enrichment, problems are still encountered due to sample loss or poor selectivity of enrichment. Here we show that the recently introduced fluororous chemistry approach has attractive properties as an alternative method for selective enrichment. Through appending a perfluorine group to the target peptide, it is possible to dramatically increase the peptide's hydrophobicity and thus enable facile separation of labeled from non-labeled peptides. Use of reversed-phase chromatography allowed for improved peptide recovery in comparison with results obtained using the formerly reported fluororous bonded phase methods. Furthermore, this approach also allowed for on-line separation and identification of both labeled and unlabeled peptides in a single experiment. The net result is an increase in the confidence of protein identification by tandem mass spectrometry (MS²) as all peptides and subsequent information are retained. Successful off-line and on-line enrichment of cysteine-containing peptides was obtained, and high quality MS² spectra were obtained by tandem mass spectrometry due to the stability of the tag, allowing for facile identification via standard database searching. We believe that this strategy holds great promise for selective enrichment and identification of low abundance target proteins or peptides.

Proteomics researchers often face a complex system comprised of hundreds or even thousands of proteins with a huge dynamic range of concentrations and many forms of related species, such as those derived from alternative splicing and post-translational modifications. To simplify these samples and enhance the detection and quantification of low abundance peptides and proteins, selective isolation and/or enrichment of special subsets of peptides and proteins has become an essential component of targeted proteomics research. For this purpose a number of approaches have been reported. Common strategies include enrichment and quantification of peptides based on antigen-antibody interactions,¹ selective binding of oligohistidine-bearing peptides using Ni-NTA affinity chromatography,² enrichment of phosphopeptides using immobilized metal affinity capturing (IMAC) or titanium dioxide chromatography,^{3,4} enrichment of cysteine-containing peptides with thiopropyl sepharose 6B,⁵ enrichment of diverse glycosylated peptides/

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SUPPORTING INFORMATION Additional supporting information may be found in the online version of this article.

proteins through their highly specific recognition by lectins,⁶ and highly efficient enrichment based on the strong association afforded between biotin and avidin molecules.^{7,8}

Among all available methods, biotin-avidin pairing is probably the interaction most widely exploited in biomedical research due to certain unique merits of these reagents. The biotin-avidin interaction has an extremely high affinity (with a dissociation constant approaching 10^{-15}). Additionally, both the biotin and the avidin species may be readily engineered into different forms with different properties or fused to other molecular species such as capture resins or enzymes used in detection. Thus the rapid and strong interaction between biotin and avidin can be employed as a versatile way to selectively capture or visualize target molecular species. Typical applications of the biotin-avidin pairing in proteomics research involves the conjugation of biotin groups to peptides or proteins via chemical reaction followed by capture of the biotinylated peptides or proteins by immobilized avidin. Some examples include the classical ICATTM reagent for quantitative proteome research,⁸ the biotinylated reagents used for labeling and detection of various forms of protein post-translational modifications: i.e. phosphoproteins,⁹ proteins subjected to oxidative-stress modifications,^{10–14} and protein degradation,¹⁵ etc. However, this strong binding between biotin and avidin may result in complications due to the extremely difficult dissociation of the binding pair and the possibility that the interaction may, in fact, be partly irreversible. To decrease the binding avidity, monomeric avidin has been generated to replace the strongly binding tetramer avidin.¹⁶ Groups that are cleavable, either chemically or enzymatically, have also been incorporated into avidin-biotin chemistry.^{15,17} However, all of these novel solutions may still face certain problems, such as low recovery efficiency, extra expense, and the co-enrichment of proteins that non-specifically bind to avidin or those that are endogenously biotinylated.

Recently, a new strategy based on fluorous chemistry has been gaining interest in chemical biology.^{18–20} Fluorous chemistry was initially introduced and flourished for its use in biphasic catalysis.²¹ More recently, it has been implemented in biochemical research with applications ranging from carbohydrate microarrays to proteomics.^{22–25} The fluorous chemistry approach is based on the following strategy: perfluoroalkyl moieties are first appended to a target compound through covalent addition. The labeled molecular species will then bind strongly to a fluorous-bonded solid phase due to the specific non-covalent interactions that occur between fluorine atoms, while the unlabeled compounds remain unbound. In this way the fluorous-labeled molecular species can be selectively captured.^{26,27} This approach potentially offers more specificity than the use of hydrophobic alkyl tags developed for selective capture of cysteine peptides via reversed-phase pairing²⁸ and may provide a suitable alternative to the commonly used biotin-avidin pairing. Unfortunately, recovery of enriched labeled peptides from the fluorous solid-phase media has been rather low: the recovery rate generally obtained for perfluorinated peptides from a fluorous-bonded solid-phase extraction (FSPE) column was observed only to be in the range of 50–55%.²⁴ A higher rate of recovery is warranted, especially for the application of enrichment of low abundance peptides such as those generated from redox-sensitive proteins.^{10–14} In addition to the low recovery rate problem, another limitation of peptide enrichment approaches from a proteomics standpoint is that if only a small subset of peptides are observed post-purification, data analysis can be more challenging. Low peptide coverage makes protein identification using database searches difficult and, depending on the number of accessible labeled amino acid in a protein (such as cysteine), often results in 'one-hit wonders'.²⁹ An ideal method should achieve the identification of labeled and unlabeled peptides simultaneously while realizing the enrichment of labeled peptides.

Here we demonstrate a significant improvement that we have achieved for the recovery of perfluorinated peptides using reversed-phase (RP) chromatographic approaches instead of

FSPE chromatography. We demonstrate using this Fluorous Labeling Assisted Capture (FLAC) approach using both C18 solid-phase extraction (SPE) micropipette tips and C18 RP chromatography that conjugation of perfluorinated groups increases the hydrophobicity of the labeled peptides and thus enables the selective enrichment of perfluorinated peptides by simple adjustment of the concentration of the organic solvent in the C18 elution phase. Peptides appended with different numbers of perfluorinated groups can also be separated from one another and from unlabeled peptides. This methodology can be readily implemented in standard proteomics work-flows involving RP capillary chromatography combined on-line with electrospray ionization tandem mass spectrometry (ESI-MS/MS) analyses. By this means we illustrate here the on-line selective enrichment of tagged peptide subsets with simultaneous identification of tagged and non-tagged peptides via nano-ESI-MS/MS. We also demonstrate that efficient and informative MS/MS fragmentation of perfluorinated peptides may be observed without loss of potentially labile modifications.

EXPERIMENTAL

Reagents

Acetonitrile (ACN) and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA), and trifluoroacetic acid (TFA) and ammonium bicarbonate (NH_4HCO_3) were purchased from J. T. Baker (Phillipsburg, NJ, USA). The standard peptide Cys-Kemptide (CLRRASLG, $[\text{M}+\text{H}]^+$ m/z 875.49) was purchased from American Peptide Co. (Sunnyvale, CA, USA). Iodoacetamide (IAA), tetrahydrofuran (THF) and protein standards were obtained from Sigma (St. Louis, MO, USA). *N*-[(3-Perfluorooctyl)propyl]iodoacetamide (FIAM) and fluoruous NuTipsTM were obtained from Fluorous Technologies Inc. (Pittsburgh, PA, USA). C18 ZipTipsTM were obtained from Millipore (Billerica, MA, USA). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Applied Biosystems (Foster City, CA, USA). The matrix alpha-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Bruker Daltonics (Billerica, MA, USA).

Tryptic digestion of protein standards

Bovine serum albumin (BSA) or *holo*-myoglobin (100 μmg) was dissolved and reduced in 50 μL 8M urea, 30 mM NH_4HCO_3 and 1mM TCEP at 37°C for 1 h, then 40 μL ACN (final concentration 10%) and 310 μL 100mM NH_4HCO_3 were added to the solution, followed by the addition of 2 μg of mass spectrometry grade trypsin (Promega, Madison, WI, USA). The digestion was performed overnight at 37°C. Afterwards, the tryptic peptide mixture was acidified with 5% formic acid to terminate the digestion. Desalting of the peptides was performed using an LC-18 SPE column (Supelco, Bellefonte, PA, USA), which was first activated by 3mL ACN and equilibrated by 3mL 0.1% TFA/water solution. After the peptide mixture had been slowly loaded, the column was washed with 2mL of a solution of 0.1% TFA in water. Finally, the peptides were eluted with 1.2mL 50% ACN/0.1% TFA solution, aliquotted, then lyophilized and stored at -20°C before further operations.

Labeling of peptides by FIAM reagent

For the FIAM labeling experiment, 40 nmol of a standard peptide or 0.37 nmol of a BSA tryptic digest were first dissolved in 50 μL 50 mM NH_4HCO_3 /4 M urea with 0.5 mM TCEP and incubated at 37°C for 30 min. Then 200 nmol FIAM dissolved in 50 μL THF was mixed with the peptide solution. The labeling reaction mixture was kept for 30 min at 37°C, and then the FIAM reagent was inactivated by addition of 10 mM DTT. The products were stored at -50°C before further experiments.

Fluorous solid-phase extraction (FSPE)

The FSPE of fluorous-labeled peptides was carried out using three different protocols. Protocol A followed the recommended method of Fluorous Technologies;³⁰ protocols B and C followed the methods optimized by Brittain *et al.*²⁴ Table 1 delineates the detailed procedures of each protocol. For each protocol, 25 pmol of labeled Cys-Kemptide was mixed with 25 pmol of a myoglobin digest (without cysteine-containing peptides) and these were used as the starting materials. Purified labeled peptides were lyophilized and dissolved in 25 μ L 60% ACN/0.1% TFA for mass spectrometry analysis.

Stepwise elution of peptides using ZipTip™ SPE columns

For enrichment of fluorous-labeled peptides using Zip-Tips™, the peptide mixture was dissolved in either 20% or 35% ACN in 0.1% TFA. The ZipTip™ procedure was conducted according to the manufacturer's protocol. Stepwise elution was realized by using steps of increasing concentrations of ACN in 0.1% TFA, as described herein.

MALDI-TOF-MS and peptide identification

The matrix CHCA was freshly reconstituted from re-crystallized stock to 20 μ g/ μ L in 50% ACN/0.1% TFA. A volume of 1 μ L of peptide solution was mixed on-target with an equal volume of matrix solution. MALDI-TOF mass spectra were obtained on a Bruker Daltonics Reflex IV™ mass spectrometer operated in positive ion reflectron mode. The intensity of the beam from the nitrogen laser (337 nm, 3 ns pulse width) was adjusted to approximately 6–10% above threshold. External calibration was performed in each case using Bruker Peptide Standards II. For each spectrum, 150 laser shots were summed. The MALDI mass spectra were analyzed with FlexAnalysis 3.0 software (Bruker Daltonics).

LC/ESI-MS/MS analysis

LC/ESI-MS/MS analysis was performed using a nanoACQUITY UPLC system (Waters Corp., Framingham, MA, USA) coupled to a Q-TOF-API-US quadrupole orthogonal TOF mass spectrometer (Waters Corp., Milford, MA, USA) equipped with a Z-spray™ source (Waters Corp.). Solution A (1.5% ACN/1% HCOOH) and solution B (98.5% ACN/1% HCOOH) were used for the UPLC system. Peptides were diluted with solution A and injected with an autosampler, pre-concentrated and desalted using an on-line peptide trap column (5 μ m, Symmetry™ C₁₈, 180 μ m $\times$ 20 μ m, Waters Corp.). Separation was performed using a high-pressure capillary column (1.7 μ m particle size, BEH 130, C18, 150 μ m $\times$ 100 mm, Waters Corp.) at a flow rate of 0.5 μ L/min using the following gradient: 2 min, 3% solution B; 55 min, 55% solution B; 60 min 70% solution B; 62 min 100% B, hold for 8 min. The column was then re-equilibrated with solution A for 20 min before another injection. Mass spectra were acquired in the m/z 400–1700 range under the following conditions: positive polarity, 110°C ion source temperature, 2.1 kV capillary voltage, and 32 V cone voltage. For MS/MS, data-dependent acquisition was programmed as follows: the two most abundant peaks with intensities above 10 counts were selected for MS/MS scans; collision energy was programmed as proportional to the m/z and the charge state of the precursor ion and ranged from 16 to 40 V; two MS/MS spectra were acquired for each precursor ion over the range m/z 100–1800. A 30-s exclusion time window was programmed for previously fragmented parent masses. Data were interpreted with MassLynx software (version 4.1, Waters Corp.).

For direct infusion analyses by ESI-Q-TOF MS, the peptide mixtures were dissolved in 60% ACN/2% HCOOH, and sample were introduced at flow rate of 0.5 μ L/min.

Database searching

MS/MS peak lists were generated by processing LC/MS/MS raw data with ProteinLynx Global Server 2.2.5 using the following parameters: for noise reduction, background subtract type: normal; background threshold: 15%; background polynomial: 5; for deisotoping and centroiding: perform deisotoping: yes; deisotoping type: slow; iterations: 30; threshold: 3%. The fragment ion lists were searched against Swissprot database (version 51.6, 257967 sequences, 93949396 residues) using local MASCOT (Matrix Science) (version 2.2.0) with parameters as follows: species; other mammalian; enzyme, trypsin; variable modifications: fluorous labeling (C), oxidation (M); mass values, monoisotopic; MS and MS/MS tolerance, 0.5 Da, 13C = 1; max missed cleavage, 2; instrument type, ESI-QUAD-TOF.

RESULTS AND DISCUSSION

Design of the strategy

Substantial effort has been expended in many laboratories towards accomplishing targeted labeling and enrichment of peptide/protein subsets from complex biological samples. Considering the various difficulties which exist, as described above, our intent was to develop a procedure that is simple, highly efficient and robust. Fluorous chemistry applications have been shown to be effective in carbohydrate, proteomic and metabolomic research, via conjugation of the perfluorinated moieties to the target molecules, followed by the specific association between the labeled molecules and a fluorine-bonded phase. Since conjugation of perfluorinated moieties to a peptide increases the peptide's hydrophobicity, we speculated that it might be possible to selectively capture the perfluorinated peptides using reversed-phase columns by adjusting the concentration of elution solvent. In this way, it should be possible to achieve on-line separation of both the labeled and unlabeled peptides, thus allowing for complete sample analysis in a single run by LC/MS/MS. This should then afford higher confidence in identification of target proteins or peptides and minimize so-called 'one-hit wonders' which plague many of the labeling approaches now widely employed in proteomic research.

Enrichment of a perfluorinated peptide by FSPE

A synthetic peptide, Cys-Kemptide, containing a chemically reactive cysteine, was chosen for method development. The peptide was tagged with FIAM reagent following a nucleophilic substitution reaction. Representative MALDI-TOF-MS spectra obtained before and after labeling are shown in Fig. 1. The results demonstrated that, upon appending the perfluorinated group, the peak corresponding to the $[M+H]^+$ ion of Cys-Kemptide at m/z 875.60 (Fig. 1(A)) shifted to m/z 1392.47 (Fig. 1(B)), corresponding to a mass increase of 517.03 Da. Following the reaction, no peaks were observed at the position corresponding to the unmodified peptide, which indicates that a labeling efficiency approaching 100% was achieved in 30 min at 37°C. Low abundance peaks observed at masses higher than the labeled peptide corresponded to salt adducts, while lower mass peaks were also present in the original peptide profile and could be attributed to impurities of Cys-Kemptide synthesis. The labeling conditions were transferrable to other standard peptides and to the peptide mixtures reported here. Complementary and more quantitative results were obtained by ESI-Q-TOF-MS with the addition of internal standard peptide. As shown in Supplemental Figs. 1(A) and 1(B) (see Supporting Information), we calculated the recovery efficiency to be more than 95%.

We first evaluated the efficiency of FSPE following three recommended methods proposed by Brittain *et al.*²⁴ or Fluorous Technologies Inc. using fluorine NuTip SPE tips. The detailed conditions used are shown in Table 1. Equal amounts of the peptide mixture

consisting of a myoglobin tryptic digest and perfluorinated Cys-Kemptide were used as the starting materials for each method. As shown in Figs. 2(A) and 2(B), appreciable amounts of the perfluorinated peptide could be recovered with both methods A and B. However, with method C, no MS signal corresponding to the perfluorinated peptide could be observed, which suggest that the 50:50 ACN/H₂O washing solution was too strong to retain the perfluorinated peptide on the column (Fig. 2(C)). To estimate the approximate percent recovery of perfluorinated peptide using FSPE, the flow-through fraction from method A was lyophilized and desalted in 35% ACN/0.1% TFA with a ZipTip™ (as described later), and analyzed by MALDI-TOF-MS. The remnant perfluorinated peptide was recovered as shown in Fig. 2(D). Comparison of the intensity of the peaks in Figs. 2(A) and 2(D) indicated a recovery rate of about 50–60% using the NuTip SPE column, which is in agreement with the value reported in the literature.²⁴ Similar results were obtained by recovering remnant labeled peptides from FSPE using method B. As performing quantitation with MALDI-TOF-MS may be used for rough estimates of relative quantitation, we verified the results by performing a direct infusion analysis of the samples after FSPE procedures, with the addition of 1 pmol/μL of unmodified Cys-Kemptide as an internal standard. Example data are shown in Supplemental Figs. 2(A)–2(C) in the Supporting Information. The recovery for FSPE method A was about 29%, while method B yielded about 42%.

A few hydrophobic peptides were shown to elute with higher percentage of organic solvent (m/z 1378, 1885) indicating that partitioning occurred; thus LC/MS analysis should be expected to yield results with improved spatial resolution, as illustrated later. This also means when dealing with more complex digests the presence of numerous highly hydrophobic peptide sequences, species modified with prenyl or palmitoyl groups etc., may compete with the fluorinated-labeled peptides and decrease the enrichment effect. We surmise that the normal peptide desalting approach used after protein cleavage may help to remove the highly hydrophobic species.²⁴

Enrichment of perfluorinated peptides by fluorinated labeling affinity capture using RP extraction

Within a biological system or protein sequence, the bulk of biological tryptic peptides have a moderate hydrophobicity and generally elute below 40% organic solvent when partitioned via reversed-phase chromatography in the presence of proper ion-pairing reagents. In contrast, perfluorinated entities are extremely hydrophobic, and a compound containing 60–70% fluorine by weight may only readily dissolve in fluorinated or partially perfluorinated reaction solvents.²⁷ We reasoned that a peptide bearing such a fluorinated group might possess properties representing a compromise between solubility in the LC mobile phase due to the polarity of the amino acid sequence and enhanced binding to C18 material due to the hydrophobicity of the fluorinated tag, and these properties would allow for enhanced partitioning compared with unlabelled peptides. We have called this new procedure Fluorinated Labeling Assisted Capture (FLAC) to differentiate it from Fluorinated Solid-Phase Extraction (FSPE).

We explored this hypothesis using mixtures of peptides obtained from a standard protein digest of myoglobin which was spiked with the fluorinated-labeled Cys-Kemptide. After loading the peptide mixtures onto ZipTip™ SPE tips, peptides were eluted from the tips using a stepwise elution of 20%, 35% (or 40%), 60% and 80% levels of ACN in 0.1% TFA. MALDI-TOF mass spectra are presented in Fig. 3 for each of the elution conditions in comparison with the original mixture. Several peptides from the myoglobin digest were observed to be efficiently eluted in 20% ACN (Fig. 3(B)) and the majority of peptides promptly eluted with 35% ACN (Fig. 3(C)). The fluorinated-labeled peptide was not observed until the content of ACN was increased to 40% (Fig. 3(D)) and most of the peptides could

be recovered by increasing the ACN to 60% (Fig. 3(E)) with no further MS signal for the peptides observed at 80% ACN (Fig. 3(F)). As demonstrated, the stepwise elution offered a rough separation between non-labeled and labeled peptides according to the differences in their hydrophobicity. Since the peptide recovery efficiency for the reversed-phase tip column can reach 75% or higher,³¹ we expect equal efficiency can be reached for the FLAC method.

We further explored the FLAC technique using a complex mixture of normal and fluorinated-labeled peptides. We chose bovine serum albumin (BSA) as a target protein since it contains 35 cysteine residues and produces a relatively complex peptide mixture following reduction and tryptic digestion. BSA was subjected to digestion, and resulting peptides were reduced and labeled with the FIAM reagent. The MALDI-TOF mass spectrum of the BSA peptides recovered after fluorinated labeling and ZipTip™ cleanup with a single 80% ACN elution showed that the majority of ion signals arose from non-cysteine-containing peptides (Fig. 4(A)). However, using a dual elution of first 35% ACN to selectively elute normal peptides, followed by 80% ACN, we observed an array of peptides predominantly bearing perfluorinated cysteines (Fig. 4(B)), which otherwise were absent in the spectra recorded before FLAC enrichment.

Since the number of fluorinated groups on a peptide may also introduce variation in the peptide hydrophobicity, we applied a stepwise gradient elution to the ZipTip™-captured perfluorinated peptides. The resulting spectra showed that with 35% followed by 50% ACN elution, most of the peptides with a singly labeled cysteine were eluted, yielding a profile similar to the one observed using 80% ACN (Fig. 4(C)). After performing a second stepwise elution (35%, 50% then 80% ACN), we observed peptides that predominantly contained more than one cysteine (Fig. 4(D)). This suggested that the ionization of these peptides was suppressed by the presence of the more easily ionized species bearing lower numbers of fluorinated moieties. Along with the fluorinated-labeled peptides seen with 80% ACN elution, minimal additional peaks were observed which we speculate may arise either from the fluorinated reagent reactions with other species or from non-specific adduction. Our results show that this stepwise elution method can selectively differentiate peptides based on the number of fluorinated labels that they bear. This approach may afford greater control in purification schema of cysteine-containing peptides. Peptide identities and labeled cysteine elution profiles corresponding to the profiles shown in Fig. 4 are provided in detail in Table 2.

On-line separation and fragmentation of FLAC-purified perfluorinated peptides

Although reversed-phase SPE tips proved to be efficient for off-line enrichment of perfluorinated peptides, we postulated that higher resolution separation and simultaneous identification might be obtained using on-line reversed-phase LC/MS/MS. In order to explore this possibility, we performed comparative nano-ESI-MS/MS analyses of both IAA-labeled and fluorinated-labeled BSA peptides. The resulting base peak chromatograms are shown in Fig. 5(A) for IAA labeling and Fig. 5(B) for fluorinated labeling. Corresponding peptide identities and labeled cysteine elution profiles are provided in detail in Table 2. The fluorinated-labeled peptides eluted significantly later in the ACN gradient than did the IAA-labeled peptides. For example, a retention time shift was observed for the fluorinated-labeled species of both the T483–489 and T139–155 peptides with respect to their IAA-labeled species, as indicated in Fig. 5. For the fluorinated-labeled sample, no cysteine-containing peptides were observed early in the gradient, whereas all the fluorinated-labeled peptides appeared later, in a distinct retention time window between ca. 38 min to 55 min.

We observed that peptides with more than one fluorinated label tended to be retained longer on the column in comparison with singly labeled peptides; results which agreed with our stepwise elution results from SPE. Furthermore, the increase in the retention times (RTs) for

each cysteine-containing peptide after fluorous labeling showed an approximate ($R^2 = 0.6374$) linear negative correlation with comparable RTs obtained before fluorous labeling (Supplemental Fig. 3(A), see Supporting Information) suggesting that the shift in RT is less pronounced as the length of the peptide increases. This weak correlation could be taken advantage of to predict the approximate change in RT after fluorous labeling. A more thorough examination of the change in RTs was explored using changes in hydrophobicity which may be calculated relative to the amino acid sequence and RT of the peptide.³² Using the SSRCalculator software,³³ based on the RT information from the BSA peptides bearing carbamidomethylation, a linear function ($R^2 = 0.9745$) was obtained for the LC/MS/MS experiment where $HP = -11.986 + 1.5288 * (RT)$ (Supplemental Fig. 3(B), see Supporting Information). We observed that after fluorous labeling the HP increase ranged from an average of 195% for singly labeled peptides to 694% for doubly labeled and 345% for triply labeled (Table 2). Examination of the peptide sequences suggests that several variables will affect the change in the HPs after fluorous labeling (Table 2: notes): (a) for a very polar peptide with low RT before fluorous labeling the effect of labeling on RT will be more pronounced in comparison with other peptides indicating a stronger contribution due to labeling; (b) for peptides containing multiple cysteines the increase in RT will be alleviated if labeling occurs on adjacent cysteines; (c) the RTs of longer peptides will experience a smaller change post-labeling due to the increased peptide effect on polarity; (d) labeling of non-adjacent cysteines will increase the RT shift; (e) peptides with polar residues adjacent to cysteine will shift more after labeling suggesting that that label will mask the effect of the polar amino acid on the RT. In order to further explore these phenomena we examined the relationship between the estimated solution charge state and the number of fluorous labels on the peptide retention time. This distribution is graphically represented in Fig. 6 and presented in detail in Table 2. Peptides were observed to be easily segregated based on their cysteine content and their retention times. We observed that for peptides with equal numbers of cysteines, those with a higher solution charge state observed by ESI-MS eluted earlier, in agreement with an increase in the peptide's apparent polarity. Overall this non-simplistic change in RT and HP is a result of contributions from the number of free cysteine residues in a peptide which may accept a label, the location of the cysteine(s) within the amino acid sequence and the original amino acid sequence of the peptide.

Integration of MALDI-TOF-MS and LC/MS/MS results of 1 pmol sample loads showed that 23 out of the 25 cysteine peptides from BSA were readily identified by MS and their sequences included 32 out of the 35 cysteines (Table 2). The remaining two cysteine-containing peptides (T106–117, ETYGDMAECCEK, and T337–340, DVCK) containing cysteines at positions 114, 115 and 339 were not seen. These results are in agreement with results obtained with a similar approach that employed the ICAT technique.³⁴ We attributed this to the intrinsic biochemical properties of the peptides in question including various forms of oxidation on cysteine,^{10–13} a potential chymotryptic cleavage site and the short 4 amino acid length of the latter peptide. Improved results were obtained by increasing loading of the FBSA digest to 10 and 20 pmol. This resulted in 85% coverage of BSA sequence in which all the cysteine peptides were observed via a standard database search using Mascot (Supplementary Results 4, see Supporting Information).

As a last measure of the utility of the FLAC approach, we evaluated the quality of the MS/MS spectra recorded for the labeled peptides. We were concerned that incorporation of the large perfluorinated group into the peptide during the FLAC procedure might have a detrimental effect on the collision-induced dissociation (CID)-type fragmentation of labeled peptides. However, the qualities of the MS/MS spectra were excellent. Typical examples are shown in Fig. 7 for two labeled peptides from the BSA digest, one of which contains a labeled cysteine in the middle of the peptide sequence (Fig. 7(A)) and the other possesses a labeled cysteine near the end of the sequence (Fig. 7(B)). Additional examples are shown in

Supplemental Fig. 5 (see Supporting Information) for MS/MS spectra of peptides with two or three fluorinated cysteine residues. In all cases, the quality of the MS/MS spectra were very good, with multiple *b* and *y* ion series and apparently no detrimental effect due to the incorporation of the label. These results are in good agreement with those observed by Brittain *et al.*²⁴ and Frahm *et al.*²⁸ Database search of the LC/MS/MS data using Mascot (Supplemental Results 4, see Supporting Information) yielded facile identification with good ion scores indicating that this labeling strategy may be used for high-throughput proteomics analyses. In total, these results constitute the first illustration of combining on-line selective enrichment of peptide subsets with simultaneous identification of both non-labeled and labeled peptides by tandem mass spectrometry. This approach provides increased confidence in the identification of labeled peptides, since the additional non-labeled peptide information can be used to overcome the low confidence assignment problem caused by 'one-hit wonders' that plague some methodologies, such as ICAT-based experiments.²⁹

CONCLUSIONS

Selective enrichment of target peptides is critical when characterizing proteomic samples possessing extremes in dynamic range and sequence diversity. Through a combination of fluorous labeling chemistry and reversed-phase separation we have demonstrated highly efficient and selective enrichment of perfluorinated-labeled peptides from model proteins. Based on differences in the hydrophobic interactions between the C18 bonded phase in RP chromatography and peptides with or without fluorous labeling, the FLAC approach offers a simple and robust means for enrichment with minimal sample handling and minimal potential sample loss. The conjugation of perfluorine groups increases peptide hydrophobicity to such an extent that perfluorinated peptides are retained during stepwise elution with increasing percentages of organic solvent and effectively partition in a separation space different from that of non-labeled normal peptides. Specifically, our results show that perfluorinated peptides bind efficiently to reversed-phase ZipTip™ tips or RP columns and elute at solvent strengths greater than 35% ACN, while unlabeled peptides tend to elute below this concentration. We also found that it is possible to use RP chromatography to separate peptides according to differences in their numbers of fluorous moieties. This feature is important since peptides with two or more reactive sites may be separated from those with only one reactive site, similar to the enrichment strategy that has been demonstrated for tryptic peptides that bear various numbers of ubiquitin modifications.²⁴ Although the model protein we used (BSA) does not reflect the heterogeneity of a complex biological sample, for which additional protein- or peptide-level prefractionation methods may be necessary, we have demonstrated the capability for on-line identification of labeled and unlabeled peptides in a single reversed-phase nano-LC/ESI-MS/MS experiment. This feature can guarantee higher confidence in protein identifications via a database search by achieving more peptide identifications in a single experiment. This FLAC approach has advantages over the previously reported fluorous proteomics approach based on FSPE, in that it provides higher on-column recovery rates and may be performed in a simpler on-line mode. In comparison, early developed techniques like COFRADIC have succeeded in visualizing and identifying special subset of peptides.^{35,36} However, with the COFRADIC approach substantial fractionation is required between multiple steps of chemical modification.

Overall, the FLAC technique provides a powerful new method as an alternative to traditional avidin-biotin affinity proteomics. Nevertheless, there still are areas for development to make the FLAC methodology more practical and widely applicable. While the high concentrations of organic solvent necessary to maintain the solubility of fluorous reagent are compatible with peptide chemistries, there may be incompatibilities with the application of this approach to low-solubility proteins which may precipitate under these

conditions. Therefore, optimization of labeling conditions may be required for comprehensive protein-level labeling. One potential way to perform the labeling reactions at the protein level would be the utilization of high efficiency bio-orthogonal reactions.^{37,38} Another point of concern is that while we have shown that the fluororous group does not interfere with efficient CID fragmentation in MS/MS, conjugation with the high-mass fluororous group brings about a large increase in peptide mass. This suggests that the mass range required for analysis of a complex mixture of labeled peptides with one or more fluororous groups may surpass the capability of a typical LC/MS/MS system. In that regard, it may be expected that development of a cleavable linker, similar to the development of cleavable ICAT reagents, could alleviate this problem and allow for improved methodologies. Finally, a major advancement in the FLAC methodology would be incorporating the capacity for quantification. Although label-free quantification based on spectral counting or ion chromatogram intensities is feasible with the current reagents, the incorporation of stable isotopes into the fluororous tags, either during synthesis of the reagents or through enzyme-catalyzed reactions, would allow for preferred quantitative approaches.³⁹ Indeed, during the preparation of this manuscript, Pal *et al.*⁴⁰ and Qian *et al.*⁴¹ presented preliminary reports on derivatives of some of these concepts, thus reinforcing the utility of fluororous-based approaches for mass spectrometry based proteomics research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative MALDI-TOF mass spectra of the cysteine-containing peptide Cys-Kemptide (CLRRASLG): (A) before and (B) after labeling with the fluoros reagent. The peak corresponding to the unlabeled $[M+H]^+$ peptide ion at m/z 875.60 was observed to shift by the mass of the fluoros group (517.043 Da) to m/z 1392.47. Peaks in the mass region below the peptide ion peak correspond to impurities in the synthetic peptide which were still observed post-labeling. Peaks in the higher mass region can be attributed to salt adducts. To confirm these results, ESI-Q-TOF-MS analyses were performed with the addition of an internal standard, as indicated in Supplemental Fig. 1 (see Supporting Information); the labeling efficiency reached higher than 95%.

Figure 2.

Representative MALDI-TOF mass spectra showing a comparison among three methods for enrichment of fluorinated-labeled peptides from a peptide mixture using fluorinated NuTipsTM. (A), (B), (C) correspond to methods A, B, C, respectively, in Table 1. Roughly equivalent recovery was shown between A and B, while limited recovery was observed for method C. (D) The flow-through fraction from method A was subjected to C18 ZipTipTM column enrichment. The results indicated that a significant portion of the fluorinated-labeled peptide did not bind to the NuTipTM and could be recovered on the C18 material. Based on the peak intensities in (A) and (D), we estimate that a 50% recovery efficiency for NuTipTM can be accomplished. To confirm these results, ESI-Q-TOF-MS analyses performed with the addition of an internal standard indicated an approximate recovery of 42%, as indicated in Supplemental Fig. 2 (see Supporting Information).

Figure 3.

Representative MALDI-TOF mass spectra showing the selective enrichment of a fluorously-labeled peptide from a peptide mixture using a C18 ZipTip™. A tryptic peptide mixture of myoglobin was mixed with fluorously-labeled Cys-Kemptide with a molar ratio of 1:1 and subjected to following analyses. (A) Reference spectrum from 1 pmol of the peptide mixture. (B) The peptide mixture was dissolved in 20% ACN/0.1% TFA and a ZipTip™ column was used to extract the peptide. The unbound fraction was detected by MALDI-TOF-MS. Peptide concentration, 1 pmol/ μ L. (C) or (D) First-step elution of the fraction in 35% ACN/0.1% TFA or in 40% ACN/0.1% TFA. (E) Second-step elution of the fraction in 60% ACN/0.1% TFA after the 35% ACN/0.1% TFA wash. (F) Third-step elution of the fraction in 80% ACN/0.1% TFA after the 60% ACN/0.1% TFA wash. The gray bar denotes the fluorously-labeled Cys-Kemptide $[M+H]^+$ ion at m/z 1392.47.

Figure 4.

Representative MALDI-TOF mass spectra showing selective enrichment of fluorinated-labeled peptides from tryptic digests of bovine serum albumin. (A) The labeled BSA peptide mixture was desalted with 0.1% TFA and eluted with 80% ACN/0.1% TFA. (B) Peptide mixture eluted from a ZipTip™ with 80% ACN/0.1% TFA after selective removal of non-fluorinated peptides via washing with 35% ACN/0.1% TFA. In experiments (C) and (D), fluorinated peptides were bound to the ZipTip™ column as in (B), but eluted in a stepwise mode with (C) 50% ACN/0.1% TFA and (D) 80% ACN/0.1% TFA, respectively. Peaks correspond to *, non-cysteine-containing peptides; +, peptides containing one fluorinated cysteine; ^, peptides containing two fluorinated cysteines; @, peptides containing three fluorinated cysteines; #, peaks which were observed with a 42-Da increase from the fluorinated peptides, which may have resulted from acetylation of the peptide *N*-terminus during the reaction procedure. Peak assignments can be found in Table 2.

Figure 5.

Base peak chromatograms showing separation and identification of BSA peptides by nano-LC/MS/MS. (A) IAA-labeled peptides or (B) fluoruous-labeled peptides were separated under the same conditions. The elution profiles from 38–50 min are magnified by 3 $\times$ to aid the comparison. The increased delay in retention time for two representative peptides, T139–155 and T483–489, are indicated; each of these contains one cysteine. The MS/MS spectra of the fluorinated peptides are shown in Fig. 7. Peak assignments can be found in Table 2.

Figure 6.

Influence of charge state and label incorporation on peptide retention times. RT was affected by solution charge state and the hydrophobicity change that resulted from labeling of fluorine groups. The dashed line shows the change of the gradient, as scaled on the right y-axis. Peptides observed with higher charge states in ESI-MS tended to elute earlier than those with fewer charges, and peptides with incorporation of more than one fluorine group conjugated to the peptide eluted later. Peak assignments can be found in Table 2.

Figure 7.

Examples of tandem mass spectra of fluorinated peptides after CID. (A) Product ion spectrum of $[M+2H]^{2+}$ precursor at m/z 827.19; (B) product ion spectrum of $[M+2H]^{2+}$ precursor at m/z 679.64. Both spectra contain distinct and abundant b and y ion series. Internal fragment ions+are also observed, as labeled in (B). As can be seen from the two tandem mass spectra, the fluorinated groups were very stable. The position of the cysteine, either in the center or close to one end of the peptide, did not affect the production of ion series. The collision energy was automatically set by the MassLynx software using the charge-state-recognition module.

Table 1

Evaluation of three methods for capture of fluoruous-labeled peptides using fluoruous NuTips™

Method	A	B	C
Equilibrate	methanol 3×20 µL 60mM NH ₄ HCO ₂ in 50:50 methanol/H ₂ O (5×20 µL)	methanol 3×20µL 10mM NH ₄ HCO ₂ in 60:40 methanol/H ₂ O (5×20 µL)	methanol 3×20µL 10mM NH ₄ HCO ₂ in 60:40 methanol/H ₂ O (5×20µL)
Bind	60mM NH ₄ HCO ₂ in 50:50 methanol/H ₂ O	10mM NH ₄ HCO ₂ in 60:40 methanol/H ₂ O	10mM NH ₄ HCO ₂ in 60:40 methanol/H ₂ O (5×20µL)
Wash	60mM NH ₄ HCO ₂ in 50:50m ethanol/H ₂ O (5×20 µL)	10mM NH ₄ HCO ₂ in 60:40 methanol/H ₂ O (5×20 µL)	50:50 ACN/H ₂ O
Elute	methanol 40µL	methanol 40 µL	methanol 40 µL

For each sample binding or eluting step, the fluoruous NuTip column was equilibrated 20 times in corresponding solution. For each method, a 20-pmol sample of the starting peptide was used. The final elution fraction was lyophilized and the residue was taken up in 20 µL 50% ACN/0.1% TFA prior to MALDI-TOF-MS measurement. MALDI-TOF mass spectra are shown in Fig. 2.

Table 2

MALDI-TOF-MS and nano-LC/MS/MS assignments of fluorous-labeled tryptic peptides from BSA digests

# Cysteine	From-To	Sequence	# Missed cleavages	[M+H] ⁺ m/z	MALDI 50% ACN	MALDI 80% ACN	LC/ESI-MS/MS	RTf	RTI	HP increase (%)	Notes [#]
1	45–65	GLVLIAFSQYLQQCPFDEHVK	0	2952.28				51.7	36.2	54.5	c
	45–75	GLVLIAFSQYLQQCPFDEHVKLVNELTEFAK	1	4096.89				51.5			
	89–100	SLHTLFGDELCK	0	1879.71				46.2	26.3	108.3	
	118–138	QEPERNECFLSHKDDSPDLPK	2	3001.18				38.8	19.4	144.0	c
	123–138	NECFLSHKDDSPDLPK	1	2361.88				41.7	20.2	148.7	
	139–155	LKPPDPNTLCDEFKADEK	1	2479.98				41.2	22.5	129.4	c
	139–160	LKPPDPNTLCDEFKADEKK	2	2608.08				39.4	21.6	126.6	c
	139–164	LKPPDPNTLCDEFKADEKKFWGK	3	3126.34							
	198–204	GACLLPK	0	1218.43				48.0	20.3	231.4	c
	223–228	CASIQK	0	1166.37				44.4			
	223–235	CASIQKFGERALK	2	1967.82				41.0			
	286–297	YICDNQDTISSK	0	1903.65				44.8	16.9	325.6	e
	310–318	SHCIAVEVK	0	1532.52				41.5	14.5	412.8	e
	387–401	DDPHACYSTVFDKLK	1	2255.84				42.5			
	402–420	HLVDEPQNLIKQNCDAQFEK	1	2815.15							
	413–420	QNCDAQFEK	0	1528.45				43.5	13.6	525.4	e
	413–433	QNCDAQFEKLGEYGFQNALIVR	1	2989.23							
	469–482	MPCTEDYLSLILNR	0	2184.85				50.4	34	63.3	a
	483–489	LCVLHEK	0	1358.49				44.0	17.2	266.1	c
	483–495	LCVLHEKTPVSEK	1	1999.83				40.8			
	483–498	LCVLHEKTPVSEKVTK	2	2328.04				38.9			
	508–523	RPCFSALTPDETYVPK	0	2340.93				43.7	24.4	106.5	
	524–544	AFDEKLFTFHADICTLPDTEK	1	2958.2				44.0			
	529–544	LFTFHADICTLPDTEK	0	2367.93				45.8	28	85.8	a
	588–597	EACFAVEGPK	0	1567.53					20.4		
2	76–88	TCVADESHAGCEK	0	2383.61				52.7	10.9	1795.1	a, d
	298–309	LKECCDKPLLEK	1	2452.81				52.3	16.9	330.2	b
	300–309	ECCDKPLLEK	0	2211.63				56.5	16.9	408.2	b

# Cysteine	From-To	Sequence	# Missed cleavages	[M+H] ⁺ m/z	MALDI 50% ACN	MALDI 80% ACN	LC/ESI-MS/MS	RTF	RTI	HP increase (%)	Notes*
3	372–386	LAKEYEATLEECCAK	1	2734.85				55.6			
	375–386	EYEATLEECCAK	0	2422.64				59.9	18.8	383.1	b
	460–468	CCTKPESER	0	2086.52				55.8			
	499–507	CCTESLVNR	0	2058.52				61.1	16.9	551.6	b
	184–204	YNGVFQECQAEDKGACLLPK	1	3867.15				64.5			
	267–285	ECCHGDLLLECADDRDLAK	1	3627.98				64.5	21.4	293.1	b
	581–597	CCAADDKEACFAVEGPK	1	3307.83				64.8	19.7	397.6	b

The fluoruous-labeled peptides were enriched through stepwise elution with increasing concentrations of ACN and subjected to MALDI-TOF-MS analysis (Figs. 4(C), 4(D)), or loaded directly for nano-capillary UPLC separation and ESI-MS/MS analysis (Fig. 5, lower BPC). Of the 35 cysteines in BSA, 32 were observed. The non-observed three cysteines came from two peptides, including one peptide containing only 4 amino acids.

RTF: retention time fluorinated peptides. RTI: retention time carbamidomethylated peptides.

Notes (for full explanation, see text): (a) polar peptide with low RT before fluoruous labeling; (b) peptide with adjacent cysteines; (c) short amino acid length peptide; (d) non-adjacent cysteines; (e) polar peptide with polar residues adjacent to cysteine.

*The hydrophobicity (HP) for each fluorinated peptide was calculated: HP=−11.986+1.5288 (RT), while the HP for each carbamidomethylated peptide was predicted using SSRCalculator³³ software.³⁰