

Solid-Phase Peptide Capture and Release for Bulk and Single-Molecule Proteomics

Cecil J. Howard, Brendan M. Floyd, Angela M. Bardo, Jagannath Swaminathan, Edward M. Marcotte,* and Eric V. Anslyn*



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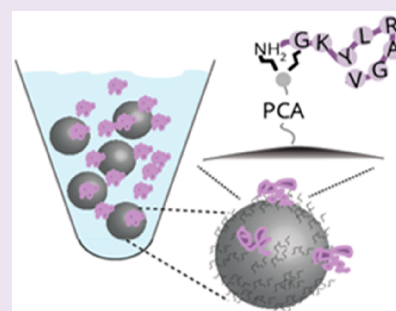


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ABSTRACT: The field of proteomics has expanded recently with more sensitive techniques for the bulk measurement of peptides as well as single-molecule techniques. One limiting factor for some of these methods is the need for multiple chemical derivatizations and highly pure proteins free of contaminants. We demonstrate a solid-phase capture-release strategy suitable for the proteolysis, purification, and subsequent chemical modification of peptides. We use this resin on an HEK293T cell lysate and perform one-pot proteolysis, capture, and derivatization to survey peptide capture biases from over 40 000 unique peptides from a cellular proteome. We also show that this capture can be reversed in a traceless manner, such that it is amenable for single-molecule proteomics techniques. With this technique, we perform a fluorescent labeling and C-terminal derivatization on a peptide and subject it to fluorosequencing, demonstrating that washing the resin is sufficient to remove excess dyes and other reagents prior to single-molecule protein sequencing.



INTRODUCTION

With the increasing sensitivity of proteomic methods, a number of new proteins, protein isoforms,¹ and post-translational modifications³ have been discovered. The increase in sensitivity is due both to improvements of the mass spectrometers and to the ability to generate high quality protein/peptide samples that are often highly derivatized.⁴ In addition, new single-molecule protein sequencing techniques are being developed, including by the use of modified nanopore sequencing pores^{5,6} and fluorosequencing.⁷ These techniques may rely only on conventional bottom-up proteomic preparations using proteases and may also employ selective detection of site-specific fluorescent labeling of amino acids. Simulation studies suggest two or more separate fluorescent labels are required for adequate wide coverage of the proteome, and currently, this would be extremely difficult for standard solution-phase labeling of samples.^{8–10} Moreover, purification techniques are prone to sample losses, and the inclusion of multiple derivatization/purification cycles can lead to low abundance peptides dropping below the detection thresholds.¹¹ This can lead in turn to a bias against rare or low abundance peptides that may be biologically important.¹²

One method that we envisioned could be used to improve sample preparation is to bind the proteins/peptides to a bead or other solid matrix. While such an approach has been done before, immobilization typically relies on either the addition of non-natural amino acids as purification handles,¹³ nonspecific hydrophobic precipitation,¹⁴ charge-based noncovalent bonding, or metal chelation strategies.¹⁵ In many systems, such as in

mammalian cell cultures, amber codon suppression is difficult.¹⁶ There are also limitations in solvent/buffer conditions that are compatible with noncovalent immobilization techniques,¹⁵ which leads to the loss of peptides.

A method that allows for the binding of peptides on resin support in a covalent and reversible manner would enable complex manipulations with higher overall yields. As demonstrated below in the context of fluorosequencing, the method introduced herein allows for the capture, purification, and derivatization of peptides, and in theory, detection of low abundance peptides. Importantly, this procedure allows for derivatization schemes that could otherwise not be performed because of the use of excess reagents and washing steps, analogous to peptide synthesis on resin, where experimental procedures are optimized to impart high yield and speed rather than the need for purification.¹⁷

For such a procedure to be applicable to all peptides generated during a proteolytic cleavage, the ideal capture reagent would only react with the N- or C-terminus of a peptide/protein and not with any of the side chains that may be present. A specific reaction with the N-terminus is an attractive choice, as all peptides generated via protease action

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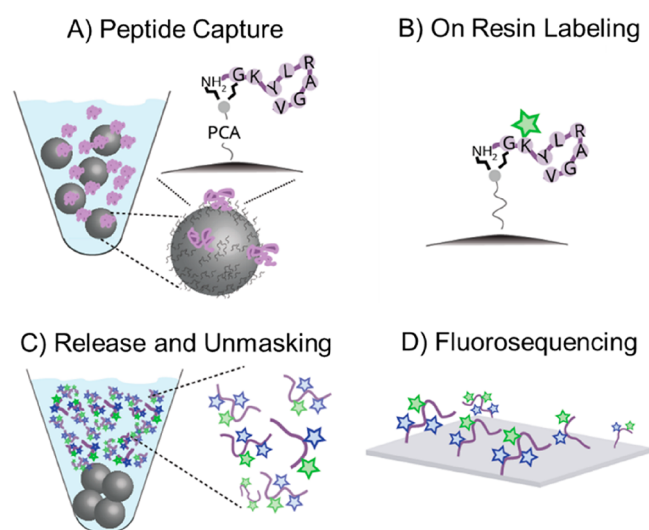
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possess a free amino terminus.¹⁸ The N-terminus is also a good target due to the difference in its conjugate acid's pK_a as well as nucleophilicity when comparing it to the side chain amine on lysine.¹⁹ While there are methods that allow for the selective modification of the N-terminal amine over lysine amines, most of them rely on permanently modifying the N-terminus, such as creating a new amide bond²⁰ or converting the N-terminal amino acid into an aldehyde.²¹ It is also possible to use the C-terminus as a handle for peptide purification. However, due to the similarities between the side chain carboxylic acids of Asp and Glu and the C-terminus, this is a less attractive option, as the pK_a of these three range from 3.3–4.2.²²

Here, we describe the use of imidazolinone formation from the N-terminus of peptides using 2-pyridinyl carboxaldehyde (2PCA) (Scheme 1) as first introduced by MacDonald et al.²

Scheme 1. Overall Scheme of Using an N-Terminal Capture Resin for Fluorosequencing^a



^a(A) N-terminal capture using a pyridinyl carboxaldehyde derivative attached to a resin.² B) Chemical labelling of lysine. C) Release from resin and D) Attachment to surface via C-terminus for sequencing.

This was an attractive option for a next-generation capture resin that would have no cross-reactivity with lysine residues, unlike current styrene-aldehyde resins that rely on unstable imine formation with Lys and the N-terminus.²³ However, to be a tractable method for purification, a chemical trigger to reverse the stable imidazolinone ring in a traceless manner is needed. Thus, as described below, we herein report a method for the covalent, reversible, and efficient peptide capture and release.²⁴

RESULTS AND DISCUSSION

Optimization of Aldehyde Linker. To understand substituent effects on the peptide capture, we screened aromatic and heteroaromatic aldehydes possessing different rings, heteroatoms, and regiochemical placement of the aldehyde. In total, 30 aldehydes were tested and ranked in order of the amount of N-terminally capped product, as an imidazolinone, formed on the model peptide Ser-Gly-Trp in a 6 h reaction at 37 °C in 50 mM sodium phosphate buffer pH 7.5 (Figure 1). Each reaction was analyzed by LC/MS, and amination formation was confirmed by the presence of two distinct peaks in the 218 nm HPLC trace that had masses

corresponding to the imidazolinone capped product. The two peaks are indicative of the two diastereomers formed during the ring closure and were observed in all reactions tested, which was also confirmed in the initial studies describing PCA-based imidazolinone formation.

We found that compounds that contain strongly electron-withdrawing groups (Figure 1A and F) do not lead to imine-intermediates, the step required prior to ring closure. We postulate that this is due to the aldehyde being largely hydrated, which does not reverse to allow imine formation. However, less electron withdrawing character facilitates some product formation but remains at unacceptable yields (Figure 1J, L, N, O, Q, R, W). Imidazolinone formation is also disfavored when the aldehyde is on an electron rich aromatic ring, such as a thiazole/pyrrole (C, D, E, G, H, K), or has a substituent with a large negative Hammett sigma-value (Figure 1M, albeit a Hammett plot was not generated). Aldehydes that are known to promote the formation of the imine complex through intramolecular hydrogen bonding or through a general-acid catalyzed mechanism,²⁵ albeit having a negative Hammett value, can promote product formation (Figure 1V).

Clearly some electronic withdrawing character is necessary to facilitate nucleophilic attack of the N-terminal amine and ring closure with the adjacent amide, but not so much as to favor hydration. Thus, it appears that electron withdrawing heteroatoms adjacent to the aldehydes, exemplified as pyridines, triazoles, imidazoles, and furans (Figure 1Z, AA, BB, CC, and DD) promote the imidazolinone formation.

Traceless Reversal of Imidazolinone Cap. The highly efficient peptide capture suggested that it might prove useful for preparation of single-molecule proteomics samples, in particular by allowing for more effective fluorescent labeling of peptides in preparation for single molecule sequencing using a new proteomics technique known as fluorosequencing. For this approach, peptides with fluorescently labeled amino acids are covalently tethered to a microscope coverslip and imaged at the single-molecule level using total internal reflection fluorescence microscopy (TIRF) following each round of N-terminal amino acid removal by Edman degradation. Edman cycles in which fluorescently labeled amino acids are removed reveal the amino acid sequence positions of the labeled amino acids, thus providing a peptide fingerprint on a molecule-by-molecule basis.

For the FPCA resin to be useful for fluorosequencing, the N-terminus of the peptide must be protected during the initial labeling of amino acids to minimize side reactions when Lys is labeled. Another requirement is that the N-terminus must be free to allow for Edman degradation. Very recently, the Francis lab showed that when peptides attached to a resin bound PCA are treated with hydroxylamine, the peptides slowly are released into solution over 6–10 days²⁶ (Figure 2A). Concurrently, we optimized a similar reaction and found it was amenable to analysis of peptides. To do this, we took inspiration from the field of Native Chemical Ligation²⁷ and its use of thiazolidine, a protected variant of Cys, where a formaldehyde is condensed with the N-terminus and side chain to form a 5-membered ring.

To reveal the Cys, it is common to utilize 300–400 mM methoxyamine at 37–42 °C, which quantitatively deprotects Cys in 3–4 h.²⁸ With this as a starting point, we purified peptides that were N-terminally capped with either 4-nitrobenzaldehyde, PCA, or 3-formylisoquinoline. The status of the N-terminal cap was determined by ¹H NMR

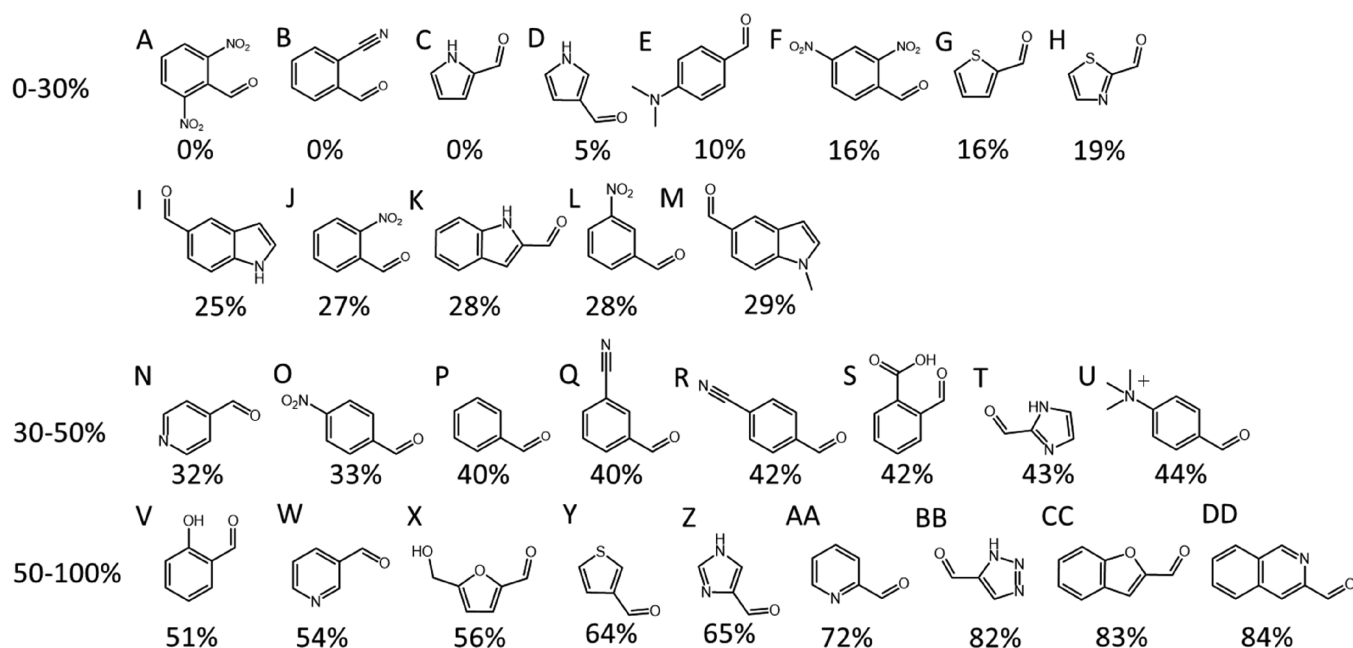


Figure 1. Structures A–DD are the aldehydes screened to find compounds that undergo aminal formation with the N-terminus of a Ser-Gly-Trp peptide. Each shows the structure and the percent aminal formed based on the quantitation of the area under the curve from the HPLC of the reaction. Each reaction was performed in triplicate.

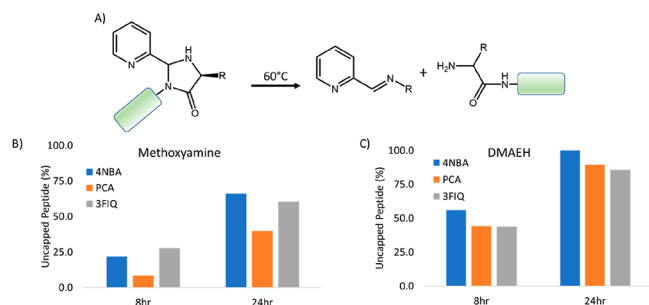


Figure 2. (A) Scheme of imidazolinone removal by a hydrazine. (B) Reversal tests of N-terminally imidazolinone capped SGW peptide with 4-nitrobenzaldehyde (4NBA), 2PCA, or FIQ using either 0.3 M methoxyamine or 0.3 M dimethylaminoethyl hydrazine.

(Supplemental Figures 2–4), and no presence of the imine proton could be detected. Next, the peptides were treated with 300 mM methoxyamine at pH 3 and 60 °C for 24 h (Figure 2B). We observed a range of N-terminal deprotection levels ranging from ~45–70% for the three different aldehydes tested. To optimize this reaction for speed and quantitative conversion, we utilized the more reactive dimethylaminoethyl hydrazine (DMAEH), which is extremely rapid in the formation of hydrazones.²⁹ After a 24 h treatment of 300 mM DMAEH at pH 3 and 60 °C, we observed nearly quantitative deprotection for all three aldehydes (Figure 2C). This deprotection condition was used for subsequent experiments.

Solid-Phase Peptide Capture. Next, we moved on to use the N-terminal specific capture of peptides as a method for the efficient preparation of samples for mass spectrometry-based proteomics. To do this, we used the water swellable PEG amine resin and performed amide bond formation to generate a resin that contained an acid cleavable Rink linker to which we added 6-formyl picolinic acid (FPCA) (Figure 3A). A PCA derivative was used over one of the three derivatives found to

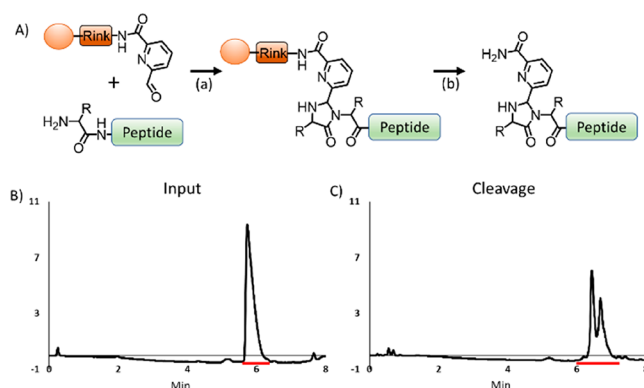


Figure 3. (A) Schematic showing the PEG-Rink-FPCA resin synthesized and the steps for coupling and releasing peptides: (a) peptide in 50 mM sodium phosphate pH 7.5 is added to the resin and incubated at 37 °C for 16 h, (b) the peptide is liberated from the resin using 95% trifluoroacetic acid, 2.5% H₂O, and 2.5% triisopropyl silane for 2.5 h. (B, C) HPLC of angiotensin I input (280 nm trace) (B) and TFA cleavage (C) after capture on PEG-Rink-FPCA resin. Red line indicates area under curve used to quantitate percent of peptide captured.

perform more efficient capture as FPCA is available commercially, and this will ensure this method can be used by the proteomics community more easily.

To evaluate the extent of capture and release by the aldehyde resin, we used angiotensin I as a test peptide. Capture of the peptide was determined by comparing the integrated peaks corresponding to the peptide during RP-HPLC analysis of the initial solution and the peptide resulting from the TFA cleavage. The captured peptide was released from the resin using a TFA cocktail (95% TFA, 2.5% H₂O, 2.5% triisopropylsilane) to free the capped peptide. The resulting pellet was resuspended and analyzed with high-resolution mass spectrometry, and by comparing the input and cleavage sample, we see an overall 70% yield of peptide. The capped

peptide exists as diastereomers, and the two peaks were found in the chromatogram (Figure 3C).

Determining Bias of Resin Capture. While the FPCA modified resin is able to efficiently capture a single pure peptide, we wanted to ensure that there was no bias in regards to what N-terminal amino acids were captured. To test a wide range of peptides, human embryonic kidney (HEK293T) cells were lysed by sonication, and the proteins were denatured by trifluoroethanol and treated with iodoacetamide to alkylate all Cys. Next, this mixture was split to allow for replicate studies and added onto PEGA-PCA resin, and N-methylated trypsin was added to digest the protein mixture. This was incubated for 16 h at 37 °C to perform simultaneous protein digestion and peptide capture. Afterward, the resin was washed extensively, and this step removed the small molecules (iodoacetamide and DTT), salts (phosphate buffer), and unbound trypsin. Finally, the peptides were released using the TFA cocktail, precipitated, and analyzed by high-resolution tandem mass spectrometry to identify the bound peptides.

Mass spectrometry analysis of the released peptides identified 42 171 unique bead-bound peptides common to both replicates. More than half of the peptides observed did not possess a PCA adduct, attributed to nonspecific adsorption to the resin, as is commonly seen in resin-based purification from total cell lysates.³⁰ To determine the bias between PCA-captured vs nonspecifically adsorbed peptides, we sorted peptides by their N-terminal amino acid and compared the relative frequencies of these found with an N-terminal imidazolinone cap. From this analysis, we found only minor bias for N-terminal Ala (~2.5×) and against Met (~3×) (Figure 4). Additionally, we found no peptides with a Pro at

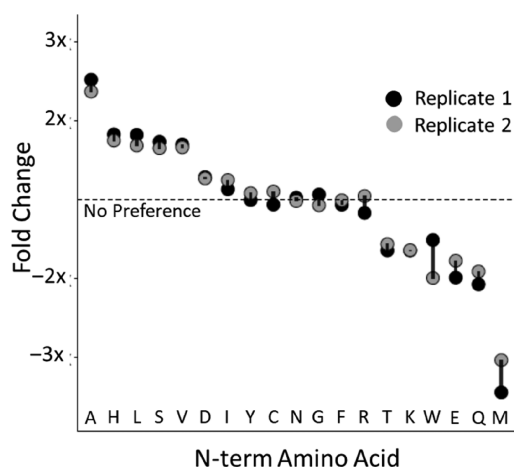


Figure 4. Bias of the PCA capture reagent as a function of differing N-terminal amino acids of captured peptides. Across 42 171 unique peptides identified with high confidence (FDR < 1%) in both of two replicate HEK293T cell lysate proteomics experiments, we measured the fold enrichment in PCA-specific vs nonspecifically bound peptides based on a total of 59 612 tandem mass spectra (peptide-spectral matches) over the two replicates.

the second position due to the fact that Pro contains a tertiary amide and is incapable of undergoing imidazolinone formation. We would like to point out that there are a number of reversible side products that can form (thioacetals forming with Cys residues, for example); however, these are minimized due to the irreversible nature of the imidazolinone product, effectively trapping the peptide as the desired product.

Integration into Fluorosequencing. With a working capture and reversal protocol in place, we turned to applying this technique for fluorosequencing. We considered a scenario in which peptides would be captured by their N-termini on resin, their C-termini modified with an alkynyl linker, one or more of their internal amino acids fluorescently labeled, and then peptides released. This scenario would test a number of important chemical steps and the ability to remove free fluorescent dyes to a level that cannot be detected by single molecule proteomics. To test if such a strategy was feasible with the PCA resin, we captured a short peptide (H₂N-Ala-Lys-Ala-Gly-Ala-Gly-Arg-Tyr-Gly-COOH) onto the resin. Note that this peptide lacks acidic residues to avoid double labeling them with the C-terminus. Peptides from native sources would require C-terminal differentiation or be generated by a protease such as GluC, whose action leaves all acidic residues positioned at peptide C-termini. We labeled the C-terminus with propargyl amine using HCTU/DIEA chemistry in DMF. Next, the Lys was labeled using Atto647N NHS esters in DMF. Finally, the peptide was cleaved from the resin using the TFA cocktail and precipitated, and the pellet was allowed to air-dry.

Fresh azide-silane slides were cleaned, and the fluorescent peptide was attached to their surface by Cu(I)-Click. The slide was washed extensively in ddH₂O, and the PCA was deprotected by submerging the slide in a bath of 500 mM DMAEH for 16 h at 60 °C. Next, the slide was washed in water, assembled into the flow cell of the TIRF microscope, and subjected to fluorosequencing as previously described (Figure 5A). We observed a strong loss of fluorescence after the second Edman cycle in 50% of the molecules, indicating that the Lys-Atto647N was removed at the appropriate Edman cycle as expected (Figure 5B). The remaining peptide losses are accounted by errors produced during the physicochemical processes such as photobleaching, Edman inefficiency, etc. and are similar to the results obtained with Fmoc protected peptides. These errors have been previously described and modeled.⁷ Thus, this capture/label/release strategy offers a novel and elegant solution for proteomics experiments.

CONCLUSION

Here, we have shown the ability to covalently bind peptides from a complex mixture on a solid phase substrate, anchoring them by their N-termini and allowing for their efficient purification and handling for multiple types of proteomics experiments. Further, we show that this method allows for the removal of salts, small molecules, and other unbound reagents used to prepare samples. We expect this methodology should also allow removal of nonpeptide contaminants in the sample such as detergents.

Importantly, binding the peptides to the resin allows for the use of a number of organic solvents, as the PEG resin is compatible with many solvents that peptides generally are not fully soluble in. We utilized this feature to chemically modify the C-termini of peptides bound to the resin using standard peptide synthesis techniques and fluorescently label Lys residues of bound peptides using an NHS ester in DMF and organic bases.

For experimental techniques that require multiple chemical reactions such as fluorosequencing or nanopore sequencing,³¹ we expect the resin will prove valuable for the analysis of proteomic samples with small sample sizes. Working with smaller protein concentrations will be important for these

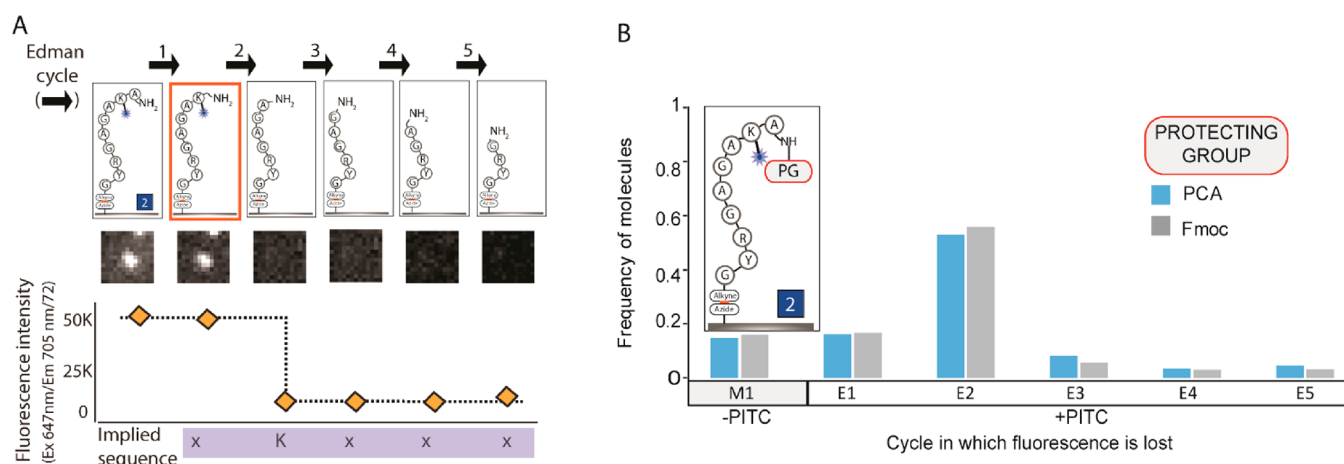


Figure 5. (A) Top: Scheme showing the peptide sequence as it undergoes fluorosequencing. Bottom: Light emitted from one peptide molecule across Edman cycles and its measured fluorescence intensities at each cycle. (B) The frequency of molecules exhibiting a loss of fluorescence after each Edman cycle is plotted for both a PCA or Fmoc protected peptide, showing data from 103 671 and 59 720 molecules, respectively. M1 is a “mock” cycle where the slide is washed with all reagents used in fluorosequencing without the phenyl isothiocyanate (PITC) being present to remove nonspecifically bound peptides or free fluorophores. E1–E5 represent consecutive Edman cycles.

single-molecule techniques and helps advance proteomic techniques to be better able to analyze limiting samples, as for clinical biopsies.

METHODS

Materials. Chemicals were purchased and used as received without any purification. 6-Formyl picolinic acid was purchased from Enamine. Atto647N-NHS was purchased from Attotek. Azide functionalized slides were purchased from PolyAn. All other chemicals were purchased from Sigma-Aldrich.

Peptide Synthesis. All peptides were synthesized using automated microwave-assisted solid-phase peptide synthesis (Liberty Blue Microwave Synthesizer, CEM Corporation). Synthesis was performed using standard Fmoc chemistry using DIC/Oxyma coupling strategies (1:1:1 ratio with amino acids). Coupling steps were performed at 90 °C for 120 s, and deprotection was performed using 20% piperidine in DMF at 90 °C for 60 s. All peptides were cleaved from resin using trifluoroacetic acid (TFA), triisopropylsilane (TIS), and H₂O (95:2.5:2.5) for 2.5 h prior to the cleavage solution being concentrated under nitrogen stream. The peptide is precipitated with ice cold diethyl ether and collected by centrifugation at 12 000g for 10 min. Peptides were purified using a Grace-Vydac C18 column (Buffer A: H₂O + 0.1% formic acid; Buffer B: methanol + 0.1% formic acid) over a 10–60% gradient.

In-Solution N-Terminal Peptide Capture. Peptides were mixed with four molar equivalents of aldehyde in 50 mM phosphate buffer pH 7.5 and then incubated for 8–16 h at 37 °C prior to purification or analysis. All aldehydes used were solubilized in DMF at 100 mM and then diluted to final concentration. Samples were analyzed by LC/MS. Amino formation was determined by quantitation of unreacted peptides at the zero hour and end point of the reaction using HPLC by measuring the area under the curve.

Reversal of Amino Cap. Peptides were first allowed to react with 4-nitrobenzaldehyde, 2-pyridinylcarboxaldehyde, or 3-formylisoquinoline following standard in-solution reaction procedures (4 mM aldehyde and 1 mM peptide). Peptides were then purified using a Grace-Vydac C18 RP-HPLC column, analyzed by LC/MS, and lyophilized to dryness. Complete imidazolinone conversion was checked by ¹H NMR (MR400 MHz, Agilent). For the reversal tests, capped peptides were resuspended in either 0.3 M dimethylaminoethyl hydrazine or 0.3 M methoxyamine. Samples were incubated at 60 °C and then analyzed by HPLC and mass spectrometry at each time point. Percentage released was determined by comparing the integration of the HPLC peak of the capped peptide over time.

Aldehyde Capture Resin Preparation. Amino PEGA resin (Novabiochem) was used and was functionalized with Fmoc-Peg₂-OH, Rink linker, and 6-formylpyridine-2-carboxylic acid using HCTU/DIEA (1:1:1.1 ratio) chemistry coupling for 45 min. Deprotection was done using 20% piperidine in DMF two times for 5 min each. Resin was stored in DMF at 4 °C prior to use.

Resin-Based Peptide Capture. Capture resin was washed in DMF, water, and 50 mM phosphate buffer pH 7.5. Each wash included a 5 min incubation in the solvent. Peptide was then added to the resin in 50 mM phosphate buffer pH 7.5 and incubated at 37 °C for 16–24 h. Next, the resin was washed extensively in incubation buffer, water, and finally DMF. After derivatization, the resin was washed extensively in water, DMF, and finally DCM. The peptide was cleaved from resin in 95% TFA, 2.5% TIS, and 2.5% H₂O. The TFA was concentrated under N₂ stream and ether precipitated prior to mass spectrometry analysis.

Cell Growth Conditions. HEK-293T cells were grown in Dulbecco’s modified Eagle’s Medium with 10% fetal bovine serum at 37 °C and 5% CO₂. Cells were passaged when between 70–80% confluence.

HEK Lysate Digestion and Capture. Cells were grown to 80% confluence, harvested in PBS, and pelleted at 500g for 3 min. Cells were then suspended in hypotonic 50 mM Tris-HCl buffer pH 8 and placed on ice. Protease inhibitor (Mini cOmplete, EDTA free protease inhibitor cocktail, Roche) was added to 1× concentration. Cells were sonicated (Branson 2510) for 1 min at 42 kHz and placed on ice for an additional minute. This was repeated 3 times. The solution was then centrifuged at 17 000g for 10 min at 4 °C, and the supernatant was collected. Protein content was then measured using a Bradford Assay. Two hundred and fifty micrograms of protein was denatured in 2,2,2-trifluoroethanol (TFE) and 5 mM tris(2-carboxyethyl)phosphine (TCEP) at 45 °C for 45 min. Proteins were alkylated in the dark with 5.5 mM iodoacetamide, and then the remaining iodoacetamide was quenched in 100 mM dithiothreitol. MS-grade reductively methylated trypsin (Pierce) was then added to the solution in a ratio of 1:25. This mixture was added to PEGA-FPCA beads and diluted with 100 mM phosphate buffer pH 7.5. The peptide mixture was incubated at 37 °C for 18 h. Next, the resin was washed extensively with H₂O, DMF, and DCM prior to cleaving the resin with a TFA cocktail (95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% H₂O) for 2.5 h. The peptides were precipitated with ice cold ether and allowed to air-dry.

Mass Spectrometry. Peptides were separated on a 75 μm × 25 cm Acclaim PepMap100 C-18 column (Thermo Scientific) using a 3–45% acetonitrile +0.1% formic acid gradient over 120 min and

analyzed online by nanoelectrospray-ionization tandem mass spectrometry on an Orbitrap Fusion (Thermo Scientific). Data-dependent acquisition was activated with parent ion (MS1) scans collected at high resolution (120 000). Ions with charge +1 were selected for collision-induced dissociation fragmentation spectrum acquisition (MS2) in the ion trap using a Top Speed acquisition time of 3 s. Dynamic exclusion was activated with a 60-s exclusion time for ions selected more than once. MS proteomics data were acquired in the UT Austin Proteomics Facility and have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with data set identifiers PXD016291 and 10.6019/PXD016291.

Peptide Identification. Peptides were identified with Proteome Discoverer 2.3 (Thermo Scientific), searching against the Uniprot human reference proteome. To distinguish PCA protected peptides, searches incorporated an optional peptide N-terminal dynamic modification (132.032 Da) corresponding to the PCA modified peptide, thresholding identifications with a false discovery rate of 1%. A total of 133 793 tandem mass spectra were collected and assigned, corresponding to 39 581 (from the bound sample) and 25 049 (from the flowthrough) in replicate 1, and 43 986 (from bound) and 25 177 (from flowthrough) in replicate 2.

On Bead Labeling of Peptides. Peptides were captured on PCA resin as described. After rinsing, the C-terminus was first coupled with 100 mM propargylamine, 100 mM HCTU, and 100 mM DIEA in DMF for 2 h at RT. The resin was extensively washed with DMF, and the Lys residues were labeled with 0.5 mM Atto647N-NHS (Attotec) and 1 mM DIEA overnight at RT. The resin was washed extensively in DMF and DCM and all of the peptides cleaved from the resin with a TFA cocktail (95% TFA, 2.5% H₂O, and 2.5% TIS) for 2.5 h. The supernatant was collected and concentrated with a N₂ stream. Ice cold diethyl ether is added (10 vol) and the peptides collected by centrifugation for 10 min at 17 000g. The peptide was analyzed by high-resolution mass spectrometry to confirm the double labeling.

Single Molecule Peptide Sequencing. Approximately 200 pM of peptides were immobilized on an azide-silane slide (custom slides from PolyAn, Germany) using standard Cu(I)-Click chemistry. Briefly, a 2 mL solution comprising peptide (200 pM), CuSO₄/tris-hydroxypropyltriazolylmethylamine (THPTA) mix (1 mM/0.5 mM), and freshly prepared sodium L-ascorbate (5 mM) was incubated on the azide-silane slide at RT for 2 h. Following the incubation, the slides were rinsed with water, and fluorosequencing was performed as previously described⁷ with minor modifications. To deprotect the N-terminal PCA cap, the slides were bathed in 0.5 M DMAEH at 60 °C for 16 h. Deprotection of the Fmoc group was performed by incubating slides with 20% piperidine solution in DMF for 1 h. The images were processed using custom developed scripts available at <https://github.com/marcottelab/FluorosequencingImageAnalysis/>.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.0c00040>.

¹H NMR, additional proteomics information, chemical structures, and LC-MS information (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Eric V. Anslyn – Department of Chemistry, University of Texas at Austin, Austin, Texas 78712, United States; orcid.org/0000-0002-5137-8797; Email: anslyn@austin.utexas.edu

Edward M. Marcotte – Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas 78712, United States; Email: marcotte@icmb.utexas.edu

Authors

Cecil J. Howard – Department of Chemistry, University of Texas at Austin, Austin, Texas 78712, United States

Brendan M. Floyd – Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas 78712, United States

Angela M. Bardo – Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas 78712, United States

Jagannath Swaminathan – Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas 78712, United States

Complete contact information is available at: <https://pubs.acs.org/10.1021/acscchembio.0c00040>

Notes

The authors declare the following competing financial interest(s): J.S., E.M.M., A.B., and E.V.A. are cofounders and shareholders of Erisyon, Inc. A patent application based on aspects of this work is pending.

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