Ultra-high pressure (>30,000 psi) packing of capillary columns enhances depth of shotgun proteomic analyses

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Abstract

Extreme sample complexity is an inherent challenge in shotgun proteomics that positions quality of chromatographic separations as one of the key determinants of attainable proteome coverage. In search of better separations, macroscopic physical characteristics of capillary columns, *i.e.*, length and properties of stationary phase particles, are typically considered and optimized, while significance of packing bed morphology is frequently underappreciated. Here we describe a technology that enables packing of capillary columns at excess of 30,000 psi and demonstrate that such columns exhibit reduced backpressure and remarkably reproducible chromatographic performance, improved on average by 23%. These enhancements afford up to 35% increase in the depth of commonplace bottom-up proteomic analyses, owning to augmented sensitivity and resolution of peptide separations. Our findings strongly corroborate advantages of ultra-high pressure packing of capillary columns for diverse shotgun proteomic workflows.

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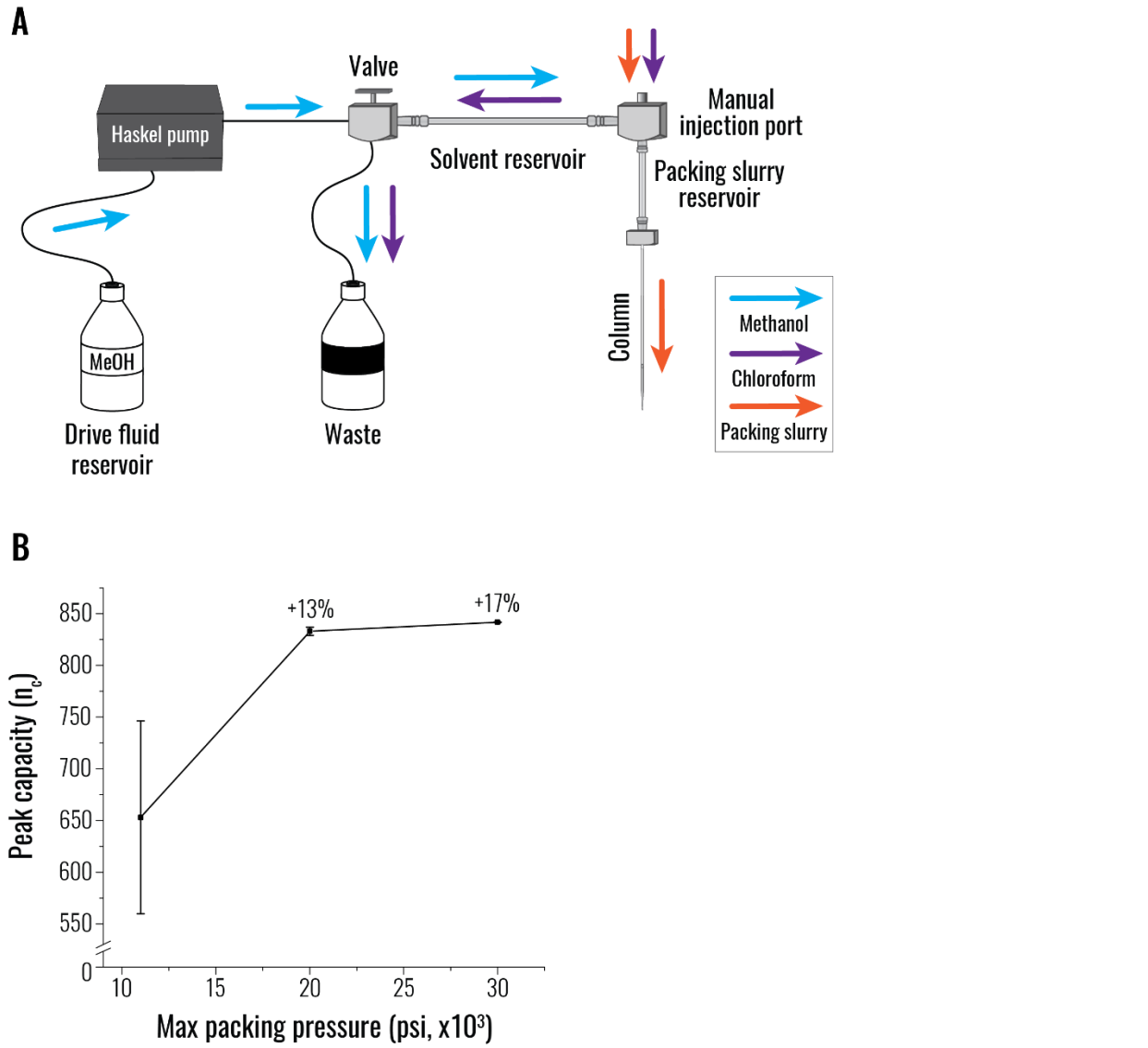
Supplemental Figure S1.

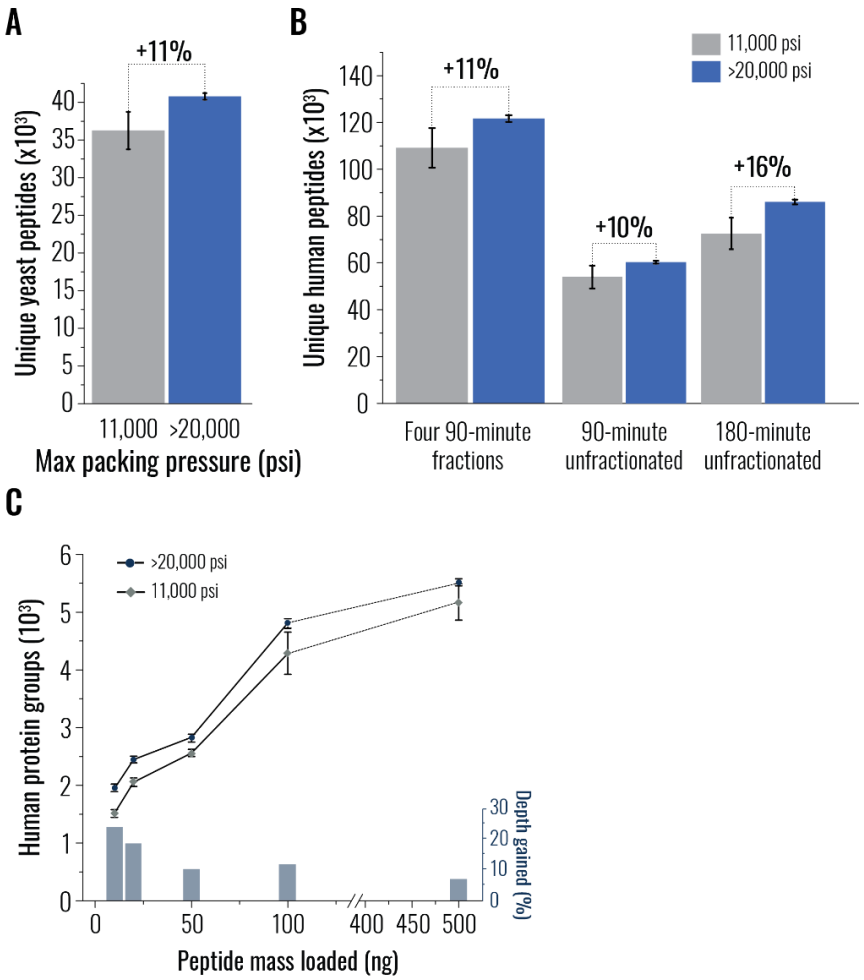


Supplemental Figure S1. Flow path and operation of the ultra-high pressure (uHP) packing station and the impact of uHP packing on column peak capacity.

(A) Schematic illustrating the flow path and operation of the uHP packing station. Flow path of pump driving fluid (methanol) is indicated in blue; flow of chloroform used for backflushing the system is in purple; flow directions of packing slurry (packing material in chloroform) are in orange.

(B) The effect of maximum packing pressure (psi) on column peak capacity (n_c). Peak capacity was estimated for 70-minute separations using full peak widths at the base (full width at 10% height; seconds) of identified peptide precursors, as reported by MaxQuant. Comparisons included three column replicates packed under the indicated maximum pressures; error bars represent standard error of mean.

Supplemental Figure S2.



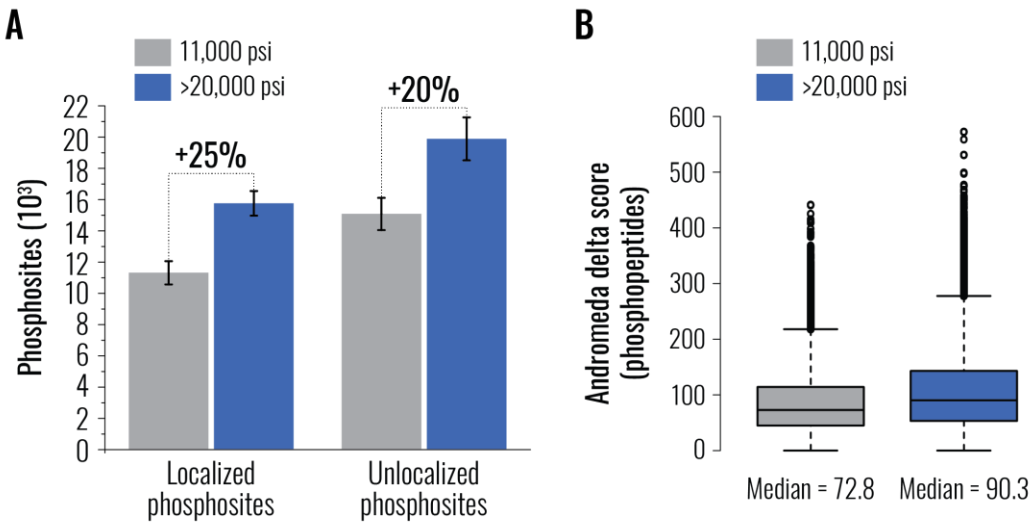
Supplemental Figure S2: Benefits of ultra-high pressure (uHP) column packing for depth of bottom-up proteomics analyses. Error bars represent standard error of mean; “>20,000 psi” and “11,000 psi” groups contained six and three column replicates, respectively, each an average of two injection replicates.

(A) Unique yeast peptides detected using columns packed at low (<11,000 psi in grey) and ultra-high (>20,000 psi in blue) pressures. The whole yeast proteome was digested using trypsin and separated over a 90-minute gradient.

(B) Unique human peptides identified across four fractions using columns packed at the indicated maximum pressure. The whole proteome tryptic digest of K562 cells was fractionated via high pH reverse phase chromatography, and each fraction was analyzed over a 90-minute gradient. Depths achieved in 90- and 180-minute analyses of the same sample without prefractionation are displayed for comparison.

(C) Human protein groups identified using capillary columns packed at low (<11,000 psi) and ultra-high pressure (>20,000 psi). The indicated mass of peptides was analyzed over the 90-minute gradient, and percent increase achieved using uHP packed columns is indicated below each data point.

Supplemental Figure S3.



Supplemental Figure S3: Benefits of ultra-high pressure (uHP) column packing for depth of bottom-up proteomics analyses of phosphorylated peptides.

(A) Phosphosites detected using capillary columns packed at the specified pressure in the enrichment of phosphorylated tryptic peptides from mouse brain lysates. All analyses were performed in injection triplicates over 180-minute gradients. Error bars represent standard error of mean; “>20,000 psi” and “11,000 psi” groups contain two column replicates.

(B) Box plots comparing distributions of Andromeda delta scores calculated for phosphopeptide analyses (**Figure 3B**) using columns packed at the specified maximum pressure. Population medians are indicated below the boxes. Higher delta scores signify greater confidence in the peptide match and phosphosite localization, as compared to the second best match.

Supplemental Experimental Section

Design and operation of the uHP column packing station. The uHP capillary column packing system was manufactured in-house (**Figure 1A & B**). To provide pressures up to 50,000 psi a Haskel air driven liquid pump (model DSHF-300, Burbank, CA) was employed. Methanol was used as the drive fluid, as it had proven to be less harsh on the pump seals than directly pumping organic solvents used to resuspend packing material. All fluid connections from the pump to the slurry chamber were constructed using uHP valves, fittings, and tubing obtained from HiP High Pressure Equipment Co. (Erie, PA), rated for pressures up to 60,000 psi (**Supplementary Figure S1A**). The methanol from the pump was split off to a 0 to 50,000 psi gauge (HiP part number 6PG50), employed to monitor the applied pressure. The methanol not directed towards the pressure gauge flowed to a three-way uHP valve (HiP part number 60-13HF2), used to release pressure and provide an exit for excess solvent used in the back flushing process, as described below. The valve was connected to the slurry chamber by a one-meter length of 316 stainless steel tubing (3.175 mm OD x 0.508 mm ID), sufficient to hold 200 µl of solution. The slurry chamber consisted of a 5 cm length of 316 stainless steel tubing with an inner diameter of 1.016 mm and was connected to the one-meter length of stainless steel tubing by an uHP tee (HiP part number 60-23HF2). The third port of the tee was used to backflush the system and to load the packing slurry. A female taper seal to male uHP fitting (HiP part number 15-21AF1HM2) was modified in-house to complete the coupling of the capillary column (360 µm outer diameter) to the slurry chamber, which then could hold up to 80 µl of packing slurry. The column was additionally restricted through the internal support of a capillary 1/16 to 0.5 mm Vespel ferrule (Restek, France). To prevent leaks, the ferrule had to be replaced after ~10 uses, as prolonged exposure to high pressures caused its substantial deformation and malfunction.

To pack a column, the bottom of the slurry chamber was first blocked with a stopper plug (HiP part number 60-7HM2). Using a syringe and adapter placed on the third port of the tee above the slurry chamber, the system was backflushed with the slurry solvent by opening the three-way valve and flowing fluid from the syringe until it was observed exiting the opened port of the valve. At that point the three-way valve was closed, and the bottom plug from the slurry chamber was removed and replaced by the capillary column and

fitting. 80 µl of packing slurry was added via the same port that was used to backflush the system. The syringe adapter was then removed, and the port was plugged. Next, pressure was gradually applied that caused the pumped methanol to push on the slurry solvent, which in turn pushed on the packing slurry.

The rate of packing was monitored with a Dino-Lite digital microscope series AM4000 attached to a monitor (Dunwell Tech, Inc., Torrance, CA). The position of the column in the front of the microscope was fixed by a custom 3D printed structure, which maintained the columns distance from the microscope while allowing the microscope to be traversed along the length of the column. Vertical movement of the microscope was enabled by a motorized camera slider (Robotshop, Mirabel, Canada). The entire setup was enclosed within a cabinet constructed from T-slot aluminum (80/20 Inc., Columbia City, IN) and polycarbonate panels to protect the user from potential uHP solvent spills. The total price of the materials used to construct the station was approximately $6,300 with the Haskel pump and enclosure parts accounting for 41% and 33% of the costs, respectively.

Column fabrication. A laser puller (Sutter Instruments Co., Novato, CA) was used to generate 75x360 µm inner-outer diameter bare-fused silica capillary columns with electrospray emitter tips. The tips were briefly etched with 100% hydrofluoric acid (Sigma Aldrich, St. Louis, MO) and plugged with ~5 mm of 5 µm 130 Å pore size Bridged Ethylene Hybrid (BEH) C18 particles (Waters, Milford, MA) using an in-house made pressure injection cell (a.k.a. a packing bomb) with maximum gas pressure grading of ~1,500 psi. Then using the same packing unit three columns were filled with 1.7 µm diameter 130 Å pore size BEH particles (Waters, Milford, MA). Two additional sets of three columns were packed with 1.7 µm diameter particles at the uHP column packing station, reaching maximum pressure of ~20,000 psi and ~30,000 psi, respectively. In all cases 1.7 µm packing material was resuspended in chloroform (Sigma Aldrich, St. Louis, MO) at unspecified concentration between 40-160 mg/ml.

Additionally, we packed columns using 360 OD /75 ID 10 um PicoFrit tips (PF360-75-10-N-5; New Objective Inc., Woburn, MA), carrying out the uHP packing procedure as described for in-house made tips. PicoFrit tips withstood pressure of up to 35,000 psi,

and the generated columns exhibited similar performance, as compared to the columns equipped with the in-house made emitters (data not shown).

Cell and mice information. K562 cells were purchased from the American Type Culture Collection (Manassas, Virginia) and cultured according to the ACTT guidelines in IMBM medium with the addition of fetal bovine serum to the final concentration of 10%. Cells were collected at 70-80% confluence. Wild type *Saccharomyces cerevisiae* strain BY4741 (Open Biosystems, Lafayette, CO) was grown in yeast extract peptone dextrose media (1% yeast extract, 2% peptone, 2% dextrose) to the optical density of ~0.6 at 600 nm. Both yeast and human cells were harvested by centrifugation, washed with ice-cold phosphate-buffered saline, pelleted, and frozen for storage at -80ºC. Brains were harvested from C57BL/6J adult female mice after euthanasia and immediately frozen in liquid nitrogen. Mice were housed at the University of Wisconsin-Madison, and all experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the University of Wisconsin-Madison.

Sample preparation. A cell pellet was resuspended in lysis buffer (100 mM Tris, 8 M urea, 10 mM TCEP, 40 mM 2-chloracetamide) and rigorously vortexed. Mouse brain samples were also resuspended in the lysis buffer and homogenized with a probe sonicator (QSonica, Newtown, CT) at 4ºC. Proteins were precipitated by the addition of methanol to the final concentration of 90% and consequent centrifugation at 14,000 g for 10 min. After decanting the supernatant resulting protein pellets were resuspended in the lysis buffer, and Reducing Agent Compatible Protein BCA (Pierce, Rockford, IL) was used to measure protein concentration. Lysate was diluted with 50 mM Tris to a final urea concentration of ~ 1 M before the addition of LysC in 1:50 ratio (enzyme:protein, Wako Chemicals, Richmond, VA). Proteins were incubated overnight at room temperature, followed by additional digestion with trypsin in 1:50 ratio (enzyme:protein, Promega, Madison, WI) for 3 hours, acidified by the addition of 10% TFA, desalted over a StrataX solid phase extraction column (Phenomenex, Torrance, CA), and lyophilized to dryness in a SpeedVac (Thermo Fisher, Waltham, MA). Samples were resuspended in 0.2% formic acid (Pierce, Rockford, IL), and final peptide concentration was determined using Quantitative Colorimetric Peptide Assay (Pierce, Rockford, IL).

Peptide fractionation. For high pH reverse phase fractionation ~200 µg of human peptides were separated across XBridge Peptide BEH C18 Column, 130Å, 3.5 µm, 4.6 mm X 150 mm column (Waters, Milford, MA) at the flow rate of 0.8 ml/min over a 25-minute gradient into 8 fractions using 1260 Infinity II High Pressure Liquid Chromatography system with configured Analytical-Scale Fraction Collector (Agilent, Santa Clara, CA). Mobile phase A consisted of 10 mM ammonium formate (Sigma Aldrich, St. Louis, MO) in uHPLC-MS grade water (Fisher Scientific, Hampton, NH), buffered to pH 10 with ammonium hydroxide (Sigma Aldrich, St. Louis, MO); mobile phase B contained 10 mM ammonium formate at pH 10 in 80% HPLC-MS grade methanol (Fisher Scientific, Hampton, NH).

Phosphorylation and acetylation enrichments. ~1 mg of desalted tryptic peptides obtained from the digest of mouse brain proteins were used to enrich for phosphopeptides with a 50 µl aliquot of MagResyn Ti-IMAC Ti^{4+} (ReSyn Biosciences, Edenvale, South Africa), according to the manufacturer's instruction. Acetylated peptides were enriched from 2 mg of human tryptic peptides using one aliquot of pan-specific Acetylated-Lysine Antibodies (Cell Signaling, Danvers, MA), according to the manufacturer's instruction.

Nano LC-MS/MS. Columns were installed on a Dionex Ultimate 3000 nano HPLC system (Thermo Fisher, Sunnyvale, CA) using a stainless steel ultra uHP union (IDEX, Oak Harbor, WA). Prior to any analysis, every column was equilibrated and compressed by flowing mobile phases A and B at the rate adjusted to achieve backpressure of ~11,000 psi. Columns were heated to 55°C inside an in-house made heater that left ~1 cm of the column tips exposed and thus held at the ambient temperature; the length of the exposed region was kept consistent from experiment to experiment. Peptides were loaded onto a column and separated at a flow rate of 325 nl/min over a 90- and 180-minute gradients, including injecting time, column wash, and re-equilibration. Mobile phase buffer A consisted of 0.2% formic acid in water; mobile phase B consisted of 0.2% formic acid in 70% HPLC-MS grade acetonitrile (Fisher Scientific, Hampton, NH).

Eluting peptides were analyzed on a quadrupole-linear ion trap-Orbitrap hybrid Fusion Lumos® mass spectrometer (Thermo Scientific, San Jose, CA). Orbitrap survey scans were performed at a resolving power of 240,000 at 200 m/z with an AGC target of $1x10^6$ ions and maximum injection time set to 50 ms. The instrument was operated in the Top

Speed mode with 2 s cycles using advanced precursor determination algorithm for monoisotopic precursor selection. Precursors were isolated using a quadrupole isolation window of 0.7 Th. Tandem MS scans were performed in the ion trap on precursors with 2-5 charge states using HCD fragmentation with normalized collision energy of 25 and dynamic exclusion of 20 s with mass tolerance of 15 ppm around the precursor and its isotopic peaks. The ion trap MS/MS ion count target was set to $1x10^4$ and the maximum injection time of 11 ms for turbo scans. Peptide fragments were mass analyzed over the fixed *m/z* range from 200 to 1,200.

Data analysis. Raw data were processed using COMPASS proteomics software suite and MaxQuant quantitative software package, version 1.5.2.8. MS/MS spectra were searched against isoform-containing UniProt *H. sapiens* database (downloaded 2.07.2017), *S. cerevisiae* database (downloaded 6.23.2015), and *M. musculus* database (downloaded 8.05.2015). When analyzing data with COMPASS, carbamidomethylation of cysteine residues was included as fixed modifications and oxidation of methionine – as a variable modification. Average mass tolerances of 125 ppm and 0.4 Da were allowed for MS1 precursor searches and MS2 fragment searches, respectively. Up to 3 missed cleavages on tryptic peptides not following the proline rule were allowed. 1% false discovery rate (FDR) correction was performed on identified peptides and proteins.

MaxQuant analyses were performed using default settings and “Calculate peak properties” advanced option selected. MaxQuant reported full peak widths at the base (full width at 10% height; seconds) and full width at half maximum height (FWHM; seconds) of all elution profiles observed in survey scans. However, only peaks corresponding to peptides systematically observed in all experiments with the same charge and variable modifications were used to calculate median peak widths and intensities reported for each column. Likewise, only peaks corresponding to peptides consistently detected across all experiments were used to calculate peak capacity (n_c), according to the formula:

$$nc = \frac{gradient\ time\ (s)}{FWHM\ (s)}$$

MaxQuant was also used to identify and localize sites of phosphorylation and acetylation. Sites with localization probably greater of equal to 0.75 were considered localized.

All raw proteomics data files were deposited into Chorus repository (Accession #1476).