New On-Bead Digestion Protocols Improve LC-MS Workflows Of Albumin Depleted Samples

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Background & Introduction

Porous silica has been a chromatography industry standard as an advantageous matrix suitable for high performance liquid chromatography. With **NuGEL**™, non-specific sites have been virtually eliminated making it an ideal support for protein separations. Through a proprietary polymer coating, silica is crosslinked forming a reactive Poly-Epoxy functionality stable across a wide pH range (pH 2 to 10). From this foundational chemistry, all of the **NuGEL**™ products are derived.

On-Bead Digestion

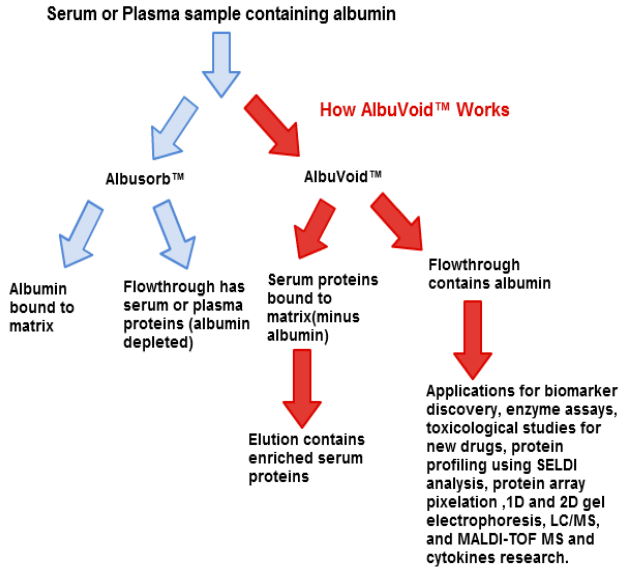
With greater interest in the proteomics community for better workflows and performance for LC-MS analyses, affinity separations have often been coupled to direct proteolytic digestion of the bound protein content. This is commonly called "on-bead digestion" in the literature [1-7]. **NuGel**™ products are rigid with a low water content, ideal for protein concentration and proteolytic digestion. In addition to their use with affinity ligand immobilization for chemical proteomics, we envision designing new strategies for high abundance depletion that can utilize on-bead digestion workflows for highly complex bead-bound proteomes. One such strategy is albumin depletion.

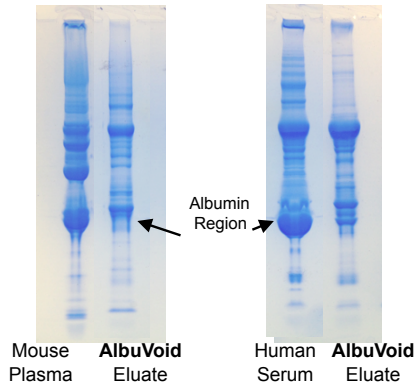
New Strategies for Albumin Depletion

Serum Albumin has the singular highest protein concentration in plasma and sera from all animals. In human serum, albumin represents more than half of the protein mass [8]. To detect low abundance proteins, depletion of one or more high abundance proteins is common and desirable. To address this need, several commercial products incorporating either immobilized monoclonal antibodies or combinatorial peptide libraries have been reported to either deplete high abundance proteins or enrich for low abundance proteins (9).

Derived from the **NuGel**™ silica-based platform, we have devised two strategies for albumin depletion, **AlbuSorb**™ and **AlbuVoid**™ as shown in the diagram.

AlbuSorb™ has been used extensively to selectively bind albumin from a wide range of mammalian sera [10-16]. **AlbuVoid**™ is a relatively new product designed to deplete albumin and enrich for low abundance proteins [17,18]. It derives from the **NuGel**™ silica-based platform. Upon empirically evaluating many chemical mixed-mode ligand/buffer/load binding combinations, a singular surface chemistry was selected that inhibits binding, achieving significant voidance of albumin while adsorbing the vast amount of the remaining protein mass. As it is not immunological, it works across all mammalian species.





These SDS-PAGE profiles show that albumin removal is specific, and suitable for both mouse plasma and human serum with equal efficiency.

In the work presented here, we demonstrate both an improved protein profile after use of AlbuVoid™, and comparable LC-MS/MS spectral output to its standard elution protocol.

METHODS

Samples Received: One **AlbuVoid**™ kit was provided by Biotech Support Group. The CBTC purchased pooled human plasma (EDTA) for testing the kit.

Sample Processing: The protocol outlined in the **AlbuVoid**™ package [18] was followed (in triplicate) to deplete albumin from 200 µls of human plasma. Briefly, 200 µls of sample is conditioned by a binding buffer, and applied to 50 mg of dry powder **AlbuVoid**™ surface, in 500 µl volume micro-spin-filter tubes. After binding and washing, the on-bead digestion protocol followed. On-bead reduction and alkylation were done in the spin tubes provided.

AlbuVoid™ On-Bead Digestion Protocol

After the final wash steps from **AlbuVoid**™ protocol step 7, add 100 µls of 5 mM DTT solution to the beads for complete immersion, mix and incubate at 60°C for ½ hour. After cooling, add 100 µls of 25 mM iodoacetamide to the DTT/bead suspension, mix and incubate in the dark for 1 hour.

Centrifuge at 5000xg (medium setting, not max) for 3 mins, and discard filtrate. Transfer the filter slurry of beads, DTT and iodoacetamide to a clean Eppendorf tube.

On-bead digestion is done by adding 100 µls of a 0.025 ug/uL solution of MS-grade. Trypsin to the beads. Digest overnight at 37°C.

Centrifuge at 5000xg (medium setting, not max) for 3 mins, and retain peptide filtrate.

To further extract remaining peptides, add 100 µls of 10% solution of formic acid to the beads.

Incubate for 15 minutes at 37°C, centrifuge at 5000xg (medium setting, not max) for 3 mins, and add this volume to the first volume.

Reduce to a final volume of 100 µls using a SpeedVac and store at -80 °C until LC-MS/MS.

In-solution digestion was done as follows. The volume was reduced to 100 microliters. Denaturation and reduction were done using 5 microliters of 200 mM DTT for half hour at 60 degrees Celcius. The solution became very cloudy after this. I vortexed the suspension and then added 5 microliters of 400 mM iodoacetamide for alkylation. This proceeded for 1 hour in the dark. The precipitate persisted. I centrifuged the suspension, removed the supernatant and added 0.5 ug (1 uL of a 0.5 ug/uL solution) trypsin to each vial. The digestion proceeded overnight. A small precipitate was observed and this was separated from the supernatant after centrifugation (5 mins at 14 g). I added 10 microliters of 10% formic acid to the digestion solutions. Similarly, 2 microliters were aspirated and mixed with 18 microliters of Solvent A and all 20 microliters were injected. Neat plasma was processed in as similar fashion.

Capillary Liquid Chromatography and Electrospray Ionization Mass Spectrometry:

Our initial aim was not to develop a protocol to identify the maximum number of proteins, but to compare two different digestion protocols under the same parameters. Therefore, the experimental design considered the cost/benefit of LC-MS/MS operation so as to produce spectral output foremost to compare the on-bead digestion protocol to the standard elution protocol. While we expect to optimize separations, performance and operating parameters for maximizing protein/peptide IDs in the future, it was not the primary intent here.

Liquid chromatography was done using an Accela pump and a pre-column flow-splitter (600-PR10-01, Analytical Scientific Instruments, USA). The separation system consisted of a commercially available C18 reversed-phase trap column (CapTrap, Michrom Bioresources) plumbed to the 6-port injection valve, an SCX trap column upstream of the injection valve, and a 100 µm ID pulled tip (sprayer) that was packed with 5µm, 200Å MAGIC C18 beads. (The SCX trap column and analytical column were prepared at the CBTC using a pressure bomb). Three solvents were used for this separation. Solvent A is 97.9% H2O : 2% CH3CN and 0.1% (v/v) formic acid, solvent B is 1.9% H2O : 98% CH3CN and 0.1% (v/v) formic acid, and solvent C is 500 mM NH4OC(O)CH3 in 97.9% H2O : 2% CH3CN and 0.1% (v/v) formic acid. The separation was performed to limit the file size to 2 Gb, a limitation of the software version. Two salt plugs were used, one at 5% and another at 90%. Each salt plug was followed by two separate gradients. For the 90% salt plug, there were also two separate gradients. However, the 5% C was replaced by 90% C. The reversed-phase gradient was the same. On left is the first profile, on right the second, as shown below.

Time (min)	% A	% B	%C	Pump Flow-Rate (uL/min)
0	98	2	0	200
5	98	2	0	200
5.1	93	2	5	100
8.1	93	2	5	100
18	98	2	0	200
318	68	32	0	200
318.1	68	32	0	10

Time (min)	%A	%B	%C	Pump Flow-Rate (uL/min)
0	58	42	0	10
1	58	42	0	200
23	2	98	0	200
43	2	98	75	200
53	98	2	0	200
63	98	2	0	200

Mass Spectrometry

The eluant from the column was coupled to a LTQ XL Orbitrap Discovery mass spectrometer (Thermo, USA). The instrument was operated in DDA (data dependent acquisition) mode with two segments. The first segment comprised a high-resolution full scan mass spectrum is followed by 5 data dependent product ion scans (ion trap), while the second segment comprised a high-resolution full scan mass spectrum followed by 10 data dependent product ion scans. For the 318-minute flow profile, the first segment was 30 minutes long and for the 63-minute flow profile, the first segment was 1 minute long. The first segment selected singly, doubly, and triply charged precursor ions while the second segment selected double and triply charged precursor ions. The dynamic exclusion criteria were as follows:
Repeat = 1
Duration= 30 seconds
Exclusion list size = 500
Exclusion duration = 300 seconds

Informatics

Protein annotation will vary depending upon the search parameters and algorithms used. We therefore opted to look at several different search parameters and compare the on-bead to elution protocols. Database searches included Sequest and X!Tandem which was run through Scaffold (v3.6.2). Search parameters were:
•Trypsin
•Human Uniprot database (most recent)
•fixed modification: carbamidomethyl
•partial modification: deamidated (NQ), oxidation (M)
•3 missed cleavages
•Mass error on precursor (25ppm)
•Mass error on product (2Da)

One set of Sequest searches was used to calculate FDR and Scaffold was used for protein annotations. MaxQuant was used for label-free quantitation.

Results and Discussion
In Scaffold, the cutoff used was 95% confidence proteins, 80% confidence peptides and at least 2 peptides per protein. With these cutoffs, a total 110 proteins were identified out of the three preparations of plasma digest.

Table 1

Test Sample	# Proteins	# Peptide IDs	# Spectra	%IDs
Neat Plasma	63	1335	6407	21%
AlbuVoid On-Bead	84	1925	10132	19%
AlbuVoid Elute	91	1974	8398	24%

Results and Discussion (continued)
This search used 5 dynamic modifications to look for phosphorylated, acetylated, methylated proteins. A reduced number of partial/dynamic modifications is often reported as it increases the probabilities that peptides will have similar nominal mass. Here we report only methionine oxidation as the partial/dynamic modification.

The numbers of peptides at 5% FDR with single modification search (methionine oxidation) (Compare with Table 1, column 3)
Elute - 2113 (100 decoy)
On-Bead - 1869 (92 decoy)
Neat Plasma - 1728 (84 decoy)

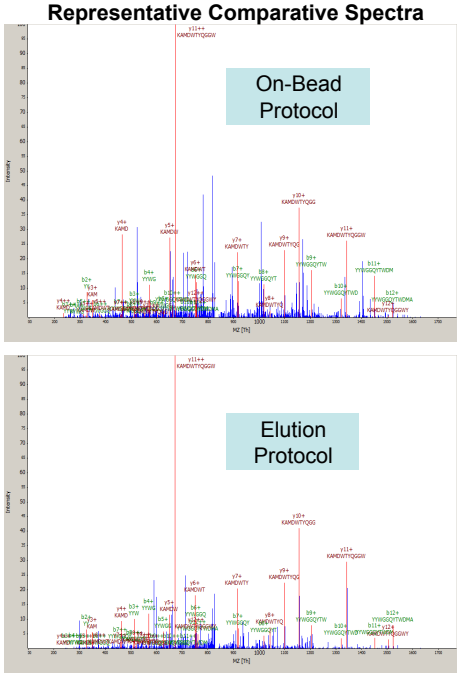
Here are the numbers of protein groups/proteins with 5% FDR for 2 peptide protein hits with single modification search (methionine oxidation). (Compare with Table 1, Column 2)
Elute - 96 groups/313 proteins
On-Bead - 97 groups/292 proteins
Neat Plasma - 72 groups/251 proteins

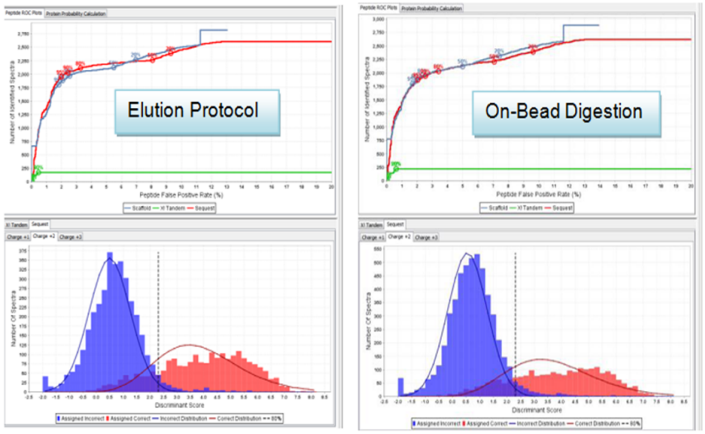
Here are the numbers of protein groups/proteins with 5% FDR for 1 peptide protein hits with only rank 1 peptides and single modification search (methionine oxidation). (Compare with Table 1, Column 2)
Elute - 181 groups/615 proteins
On-Bead - 193 groups/649 proteins
Neat Plasma - 148 groups/516 proteins

A partial list of proteins identified using Scaffold, X!Tandem and Sequest with Spectral Count as the quantitative measurement.

Protein	On-Bead Protocol	Elute Protocol
Ig mu chain C region	42004	36469
Alpha-1-antitrypsin	14641	14831
Beta-2-glycoprotein 1	20399	23697
Alpha-1-antichymotrypsin	14254	15541
IGL@ protein	30848	48412
Vitronectin	24833	16573
cDNA FLJ58826, (similar to Plasma protease C1 inhibitor)	11650	14858
Apolipoprotein B-100	33230	23149
Complement C5	33470	25694
Alpha-1-acid glycoprotein 1	16977	6,839
Inter-alpha (Globulin) inhibitor H4	22859	15122

Representative Comparative Spectra





Quality control for the LC-MS peptides: On left is the standard **AlbuVoid**™ elution protocol, followed by tryptic digestion and LC-MS/MS peptide analysis. On right, using equivalent operating parameters, the on-bead digestion protocol generates comparable peptide spectral output.

Conclusions & Future Directions

By several performance metrics, it appears that the 'on-bead' digestion protocol is comparable to the elution protocol. On-bead protocols provide advantaged speed, simplicity and reduced potential for keratin contamination. We have also demonstrated the efficiency of **AlbuVoid**™ as a simple consumable product for albumin depletion, with 20-30% more protein/peptide identifications achievable relative to untreated plasma. In the future, we will consider optimization to maximize protein identifications. To do this, we plan to use an exclusion list in an attempt to dig deeper into the proteome. Although not a novel approach in itself, it is an appropriate next step in the process.

In addition to the work reported here, the **NuGel**™ **Poly-Amine** support has been reported to be a suitable support for protein interactome analysis by LC-MS [19]. Several other products, based on **NuGel**™ and other metallic oxides are being beta-tested for on-bead digestion. Most notably, in an anecdotal report, on-bead vs. elution comparison of **KinaSorb**™ for kinase enrichment, comparable LC-MS spectral output and protein annotation is achieved. It is foreseen that the range of **NuGel**™-based products, which support enrichment and including hemoglobin depletion [20-24], urine proteins [25], and glycoproteins [26], will be adapted to on-bead digestion.

Future product developments include composite mixtures of **NuGel**™-based chemical ligand architectures, which work to compress protein concentrations across a range of sample types. As new bioinformatics tools become available, along with the optimization of LC-MS operating parameters, we intend to advance protocols for protein enrichment/separations that can improve workflows, and data quality for qualitative and quantitative LC-MS/MS analyses.

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