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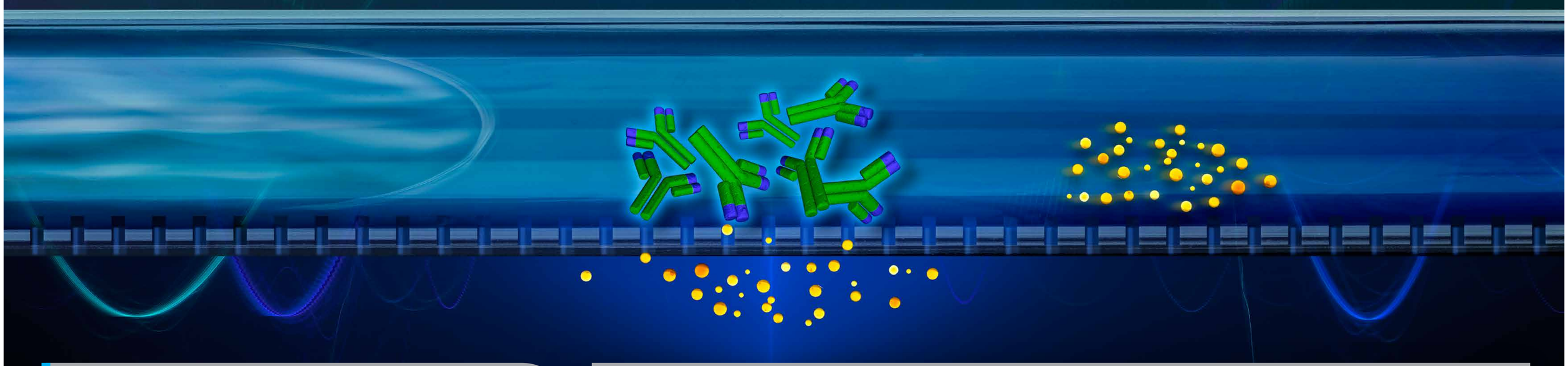


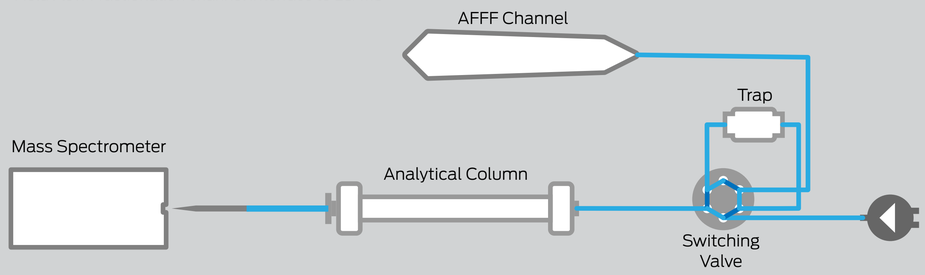
Solid-Phase-Free Immunocomplex Isolation for Mass Spectrometry-Based Immunoassays

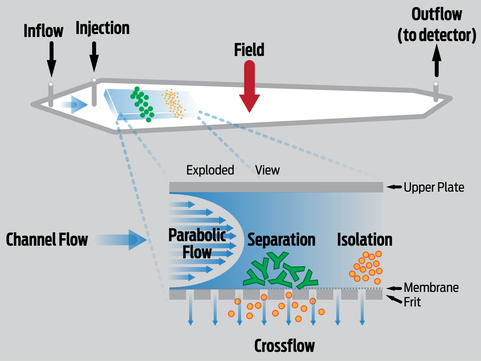
Mats Leeman[1], Benedikt Lang[2], William Scott[3], Matilda Storm[1], Ray Runyon[1], Calvin Wiese[3], Oliver Poetz[2], Thomas Joos[2]

[1]SOLVE Research, Lund, Sweden, [2]NMI, Reutlingen, Germany, [3]Wellspring Clinical Lab, Altamonte Springs, FL USA

6Σ Isolator

Field Flow Fractionation channel interface to LC/MS





ABSTRACT

A novel method integrating solid-phase free immunocomplex isolation for LC/MS based immunoassay approaches was established. Commonly, LC/MS based immunoassays include an immunoprecipitation step using antibodies conjugated to solid surfaces in bead or column format. Here, we present a method where the antibody-peptide complex is not precipitated, but isolated from plasma digest peptides by the hybrid of normal-mode field flow fractionation with ultrafiltration. The use of this hybrid separation method allowed a solid-phase free immunocomplex enrichment from plasma digest, thereby avoiding contamination by high abundant peptides through solid-phase interaction with polymer surfaces and allowing the detection of C-reactive protein.

Isolating "Needles" from "Haystacks"

THE PROBLEM

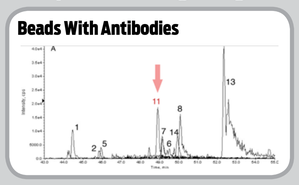
The vast dynamic range of the plasma proteome (spanning more than 10 orders of magnitude) has chronically plagued its analysis and exploration. The massive analytical power of mass spectrometry continues to be challenged when exploring low abundance proteins by this massive dynamic range. State of the art methods such as SISCAPA that report enrichment of low abundance proteins up to 100,000 times merely reduce the complexity for proteins like cardiac troponin from 1 protein for every 10 billion albumins, to 1 protein for every 100,000 albumins.

With such complexity, ionization suppression characteristic of mass spectrometry makes it highly difficult to analyze low abundance proteins of interest. While liquid chromatography is useful, the narrow elution peaks of the low abundance proteins of interest continue to be overwhelmed by the wide elution peaks associated with high abundance proteins in plasma.

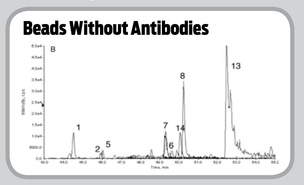
Immunoaffinity isolation of surrogate peptides along with stable isotope internal standards of plasma proteome digests is a useful method for quantifying plasma proteins with LC/MS. Typical isolation methods employ solid surfaces on which to immobilize such immunocomplexes. N. Leigh Anderson, et al, demonstrated non-specific binding of high abundance residues to isolation surfaces as being primarily implicated as the source of high abundance residues in the isolated fractions[1].

Nonspecific Binding of High Abundance Peptides

Beads With Antibodies



Beads Without Antibodies



We have demonstrated in the past that high abundance residues in isolated fractions can be largely avoided by eliminating immobilizing surfaces using ultracentrifugation to isolate the immunocomplex fraction (~155kDa) from the peptide digest fraction (< 5kDa)[2]. While demonstrating proof of concept, ultracentrifugation has not been shown to be readily production capable. We have now demonstrated avoiding high abundance residues in isolated fractions using a hybrid separation method that combines normal-mode field flow fractionnation with ultrafiltration to isolate the immunocomplex fraction from the peptide digest fraction that is readily production capable.

1 N. Leigh Anderson, Angela Jackson, Derek Smith, Darryl Hardie,Christoph Borchers, and Terry W. Pearson. SISCAPA Peptide Enrichment on Magnetic Beads Using an In-line Bead Trap. Molecular & Cellular Proteomics 8.5 995-1004.

2 Sonja Volk, Thomas D. Schreiber, David Eisen, Calvin Wiese, Hannes Planatscher, Christopher J. Pynn, Dieter Stoll, Markus F. Templin, Thomas O. Joos, and Oliver Poetz. Combining Ultracentrifugation and Peptide Termini Group-specific Immunoprecipitation for Multiplex Plasma Protein Analysis. Molecular & Cellular Proteomics 11.7.

METHOD

We employ a hybrid isolation method that combines normal-mode field flow fractionation with ultrafiltration. We employ an asymmetric field flow fractionation channel in normal field flow mode using a 30kDa MCWO ultrafiltration membrane. In this configuration, the immunocomplex fraction is isolated by 1) permeation of the peptides across the membrane and 2) lower migration velocity across the channel.

The conditions for performing the separation are:

Sample: 1 ul human plasma digest

Stable isotopic internal standard: 5 pmol GYSIFSYATK

Antibody: 5 ug anti GYSIFSYATK

Incubation: 60 minutes mixing

Carrier solvent: Phosphate buffered solution + 3mM sodium azide

FFF Channel: Wyatt SC

Membrane: Regenerated cellulose 30kDa MWCO

Spacer: 490 um, narrow

Carrier flow rate: 1.0 ml/minute

Crossflow flow rate: 0.5 ml/minute

Output port flow rate: 0.5 ml/minute

Injection time: 3 minutes/0.2 ml/minute

Focusing time: 7 minutes

Immunocomplex fraction elution start time: 4.5 minutes

Immunocomplex fraction elution end time: 7.5 minutes

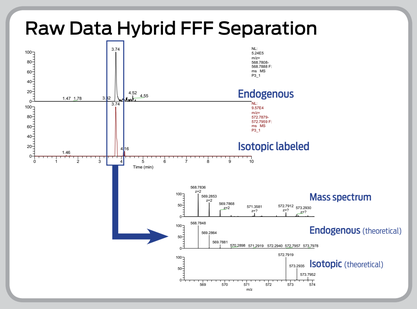
Immunocomplex fraction volume: 1.5 ml

The isolated fraction was concentrated by magnetic bead solid phase extraction. The concentrated fraction was injected into LC/MS for analysis.

RESULTS

The isolated fraction was analyzed by LC/MS for presence of high abundance protein residues. 19 protein residues were identified. None of the identified residues are associated with high abundance plasma proteins. The identified residues were associated with antibody development. The signals associated with the identified proteins were much lower than the signals associated with the surrogate peptides.

Raw Data Hybrid FFF Separation



Protein Search Hybrid FFF Separation

Database search: human and rabbit
Acceptance criteria for proteins: min. 2 peptides identified (MS/MS)

Sample 1

Protein	Cov. [%]	# pep.	# PSMs
Ig kappa chain C region (human)	16.3	3	45
Ig gamma-1 chain C region (human)	12.8	4	37
Cytochrome C (rabbit)	21.9	2	13

Sample 2

Protein	Cov. [%]	# pep.	# PSMs
Ig kappa chain C region (human)	15.9	2	20
Ig lambda-2 chain C regions (human)	27.4	2	11
Ig gamma-3 chain C region (human)	6.9	2	8

Sample 3

Protein	Cov. [%]	# pep.	# PSMs
Ig kappa chain C region (human)	16.3	3	37
Ig lambda-2 chain C regions (human)	27.4	2	33
Ig gamma-1 chain C region (human)	10.3	3	19
Cytochrome C (rabbit)	21.9	2	9

Just peptides from antibody fragments found (need in depth analysis for similarity search of rabbit and human)

No peptides from high abundant proteins present

In general fewer PSMs in comparison to conventional bead-based system

Protein Search TXP-IP/MS (comparison case)

Database search: human and rabbit
Acceptance criteria for proteins: min. 2 peptides identified (MS/MS)

Sample 1

Protein	Cov. [%]	# pep.	# PSMs
Serum Albumin (human)	7.7	7	433
Ig kappa chain C region (human)	22.2	4	65
Alpha-1-acid glycoprotein (human)	11.9	2	24
Cytochrome C (rabbit)*	21.9	2	24
Alpha-2-macroglobulin (human)	2.4	2	23

Sample 2

Protein	Cov. [%]	# pep.	#PSMs
Serum Albumin (human)	7.7	7	259
Ig kappa chain C region (human)	21.8	3	126
Alpha-1-acid glycoprotein (human)	11.9	2	17
Alpha-2-macroglobulin (human)	2.4	2	14
Ig gamma-3 chain c region (human)	3.6	2	5

Sample 3

Protein	Cov. [%]	# pep.	# PSMs
Serum Albumin (human)	7.7	7	249
Ig kappa chain C region (human)	22.2	4	100
Alpha-1-acid glycoprotein (human)	11.9	2	27
Cytochrome C (rabbit)*	21.9	2	22

Peptides from high abundant proteins (albumin, alpha-1-glycoprotein identified)

Unspecific binding of peptides

*Cytochrome C from rabbit might also be from human (91.4% sequence similar)

6Σ ISOLATOR

This device is being designed to separate immunomplexes from high complexity peptide digests with very high resolution. Peptide digests with target-bound immunocomplexes are injected into a field flow fraction channel where the immunocomplexes are separated from the peptide digest by a hybrid of normal-mode field flow fractionation and ultrafiltration.

The isolated immunocomplex fraction is pH shifted to unbind targets and loaded onto the pre-column where targets are captured while antibodies pass through to waste. The loaded pre-column is switched in-line with the analytical column for gradient elution and electrospray ionization into MS/MS.

With human plasma tryptic peptide digests, no high abundance peptides are detectable in the isolated fraction. Recovery of targets is expected to exceed 70%.

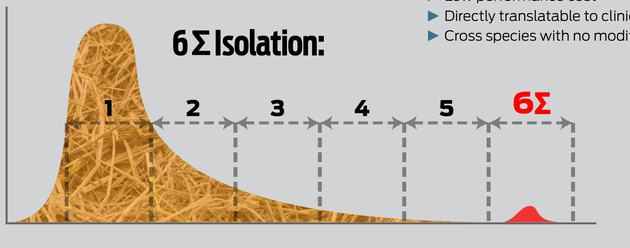
This device is an effective and efficient tool for developing and performing high sensitivity, high selectivity, multiplexed protein assays in complex matrixes.

Advantages to targeted proteomics:

- Minimize ionization suppression
- Increase sensitivity
- Simplify assay development
- Universal assay protocol
- Increase automation of sample preparation

Predictable assay development method:

- For proteins in complex matrix such as blood plasma
- Highly multiplexible
- Clinical precision (cv<10%)
- ng/ml sensitivity
- Production capable
- Low performance cost
- Directly translatable to clinical environment
- Cross species with no modifications



CONCLUSIONS

The demonstrated isolation method is useful for LC/MS low-abundance plasma protein assays. Assay development is streamlined by avoiding complexities associated with incomplete isolation from digest residues. Higher sensitivity should be achievable by avoidance of ionization suppression from high abundance residues. Elution from LC should be cleaner due to avoidance of wide high abundance elution peaks.

There are alternative configurations for integration with LC/MS. We are developing direct integration of the isolator with the LC. Through appropriate integration with LC/MS, high robust, high throughput assays may be achievable.