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Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers

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Abstract

A major bottleneck for validation of new clinical diagnostics is the development of highly sensitive and specific assays for quantifying proteins. We previously described a method, stable isotope standards with capture by antipeptide antibodies, wherein a specific tryptic peptide is selected as a stoichiometric representative of the protein from which it is cleaved, is enriched from biological samples using immobilized antibodies, and is quantitated using mass spectrometry against a spiked internal standard to yield a measure of protein concentration. In this study, we optimized a magnetic-bead-based platform amenable to high-throughput peptide capture and demonstrated that antibody capture followed by mass spectrometry can achieve ion signal enhancements on the order of 10^3, with precision (CVs <10%) and accuracy (relative error ~20%) sufficient for quantifying biomarkers in the physiologically relevant ng/mL range. These methods are generally applicable to any protein or biological fluid of interest and hold great potential for providing a desperately needed bridging technology between biomarker discovery and clinical application.

Keywords: Biomarkers; Antipeptide antibody; LC/MS/MS; Targeted proteomics; Stable isotope dilution; Peptide quantitation

Protein biomarkers have had tremendous impact on clinical management of human disease, especially cancer. For example, proteins differentially present or mutated in tumor cells have facilitated both the development and appropriate use of targeted therapeutics [1–4], and the prognostication of disease outcomes [5,6]. The application of genomics and proteomics technologies to protein biomarker discovery has enabled hundreds of biomarker candidates to be identified in a single discovery effort. However, as yet the promise of these discovery tools has not been fulfilled, in part due to the downstream bottleneck of clinical validation.

The current gold standard for validating putative biomarkers is the enzyme-linked immunosorbent assay (ELISA)[3]. A well functioning ELISA can be run at high throughput and has extraordinary sensitivity and specificity for quantifying the target analyte. However, ELISA development is costly ($100,000–$2 million per biomarker candidate) and associated with a long development lead time (>1 year) and a high failure rate. The high cost and long lead time are bottlenecks to biomarker testing and make it impractical to develop an ELISA for all putative biomarkers. It is not surprising that the number of new bio-

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[3] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; SRM, selected reaction monitoring; SISCAPA, stable isotope standards with capture by antipeptide antibodies; AAC, α_1-antichymotrypsin; TNFα, tumor necrosis factor α; RT, room temperature; MARS, multiple affinity removal system; TIC, total ion current.

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markers validated in the past 5 years has been remarkably small [7].

Although the ELISA may remain the gold standard for clinical application, relieving this bottleneck to preclinical biomarker testing will require developing more affordable "bridging" methodologies with shorter development lead times. Mass spectrometry (MS) is a well established tool for quantification of metabolites in clinical samples [8,9]. With the development of soft ionization techniques [10,11], it is now possible to extend its application to quantifying peptide components of biomarker candidates in clinical samples. A specific tryptic peptide can be selected as a stoichiometric representative of the protein from which it is cleaved (a monitor peptide) and quantitated against a spiked internal standard (a synthetic-stable-isotope-labeled peptide) to yield a measure of protein concentration [12–14]. As already used in analytical chemistry to quantify drug metabolites and other small molecules [8], MS offers high precision (coefficients of variation (CVs) below 5%), a good linear response range ($>10^3$), and high sensitivity of detection (less than 1 ng/mL).

Although selected reaction monitoring (SRM–MS) has been applied to the quantitation of plasma-protein-derived peptides [15–21], a major limitation is that, due to ion suppression, the majority of biomarker proteins (e.g., PSA, CEA, and AFP present at ng/mL) cannot be detected in plasma in a mass spectrometry experiment without enrichment relative to large quantities of interfering proteins (e.g., albumin 50 mg/mL, globulin 35 mg/mL). To this end, we previously described a technology, stable isotope standards with capture by antipeptide antibodies (SISCAPA) [22], in which antipeptide antibodies immobilized on 100-nL affinity columns were used to enrich specific peptides along with spiked stable-isotope-labeled internal standards of the same sequence. Upon elution from the antipeptide antibody supports, electrospray ionization–MS was used to quantify the peptides (natural and labeled). In a series of pilot experiments, binding and elution from these supports were shown to provide an average 120-fold enrichment of the antigen peptide relative to others, as measured by selected ion monitoring or SRM–MS. These MS experiments generated peptide ion current measurements with cycle-to-cycle CVs near 5%.

In this current study, we extended the previous work by (1) optimizing a magnetic-bead-based platform amenable to high throughput for peptide enrichment, (2) using this optimized platform to demonstrate for the first time that antibody enrichment followed by SRM–MS can achieve ion signal enhancements of $>10^3$, sufficient for quantifying biomarkers in plasma at the ng/mL range, and (3) demonstrating the capabilities of the commonly available linear ion trap mass spectrometer for quantitative testing of biomarker candidates. These highly sensitive and specific methods are generally applicable to any protein and biological fluid of interest.

Materials and methods

Materials

Polyclonal antibodies against α_1-antichymotrypsin (AAC; accn. P01011) and tumor necrosis factor alpha (TNFα; accn. P01375) peptides were described previously [22]. Stable isotope standards of AAC (EIGELYL*P*K, ^{13}C- and ^{15}N-labeled amino acid in italics) and TNFα (DLSLISPLAQA*V*R) were obtained from Cell Signaling Technologies (Beverly, MA). The ^{12}C version of TNFα peptide was synthesized by AnaSpec (San Jose, CA) and quantified by amino acid analysis. The labeled version of peptides resulted in a mass shift of +6 Da. Monoclonal antibody against human TNFα was purchased from R&D systems (Minneapolis, MN). Dynabeads Protein G, Myone, M280, M270 streptavidin, tosylactivated, carboxyl, and epoxy magnetic beads were obtained from Dynal Biotechnology (Lake Success, NY).

Immobilization of antibody on magnetic beads

Antibody (100 μg) was added to 250 μL (7.5 mg) of Protein G beads and incubated at room temperature (RT) for 1 h to allow antibodies to attach to the surface. Approximately 10 μg antibody per 1 mg beads was bound (see Supplemental material for more detail). The beads were then washed with 1 mL 0.2 M triethanolamine, pH 8.2. For cross-linking of antibody on beads, Protein G beads with immobilized polyclonal antibody were resuspended in 1 mL of freshly made cross-linking buffer (20 nM dimethyl primelinidate 2 HCl in 0.2 M triethanolamine, pH 8.2). The mixture was incubated at RT for 30 min and placed on the magnet, and the supernatant was discarded. The beads were resuspended in 1 mL of 50 mM Tris, pH 7.5, and incubated for 15 min to stop the reaction. The amount of antibody bound to Protein G beads was estimated by comparing protein concentrations (measured by Bradford assay) of the supernatants before and after cross-linking. For experiments that determined the effects of trypsin inhibitor, mouse monoclonal antibodies for TNFα protein were biotinylated using the EZ-Link kit from Pierce (Rockford, IL). Biotin (3 μg) was added to 100 μg antibodies and incubated at RT for 2 h. The antibodies were extensively dialyzed using Slide-A-Lyzer mini dialysis units from Pierce with 2× 500-mL buffer changes to remove free biotin. Biotinylated antibody (10 μg) was added to 1 mg of M270 streptavidin beads and incubated at RT for 1 h.

Serum depletion and digestion

Human serum was purchased from Sigma–Aldrich (St. Louis, MO). The human multiple affinity removal system (MARS) and 0.22-μm filters were purchased from Agilent Technologies (Palo Alto, CA) for depletion of albumin, transferrin, IgG, IgA, antitrypsin, and haptoglobin from human serum and used per the manufacturer's