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.
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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.

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

recommendations. Briefly, 20 µL of human serum was diluted five times with MARS buffer A. Particulates in the diluted serum were moved using 0.22-µm filter before injecting to the MARS column (4.6×50 mm). Flow-through was collected in buffer A at 0.25 mL/min for 4 min. Depleted proteins were concentrated and buffer exchanged to 50 mM ammonium bicarbonate using an Agilent Spin concentrator (5-kDa molecular weight cut-off). The final depleted serum concentration was 2 mg/mL. A tryptic digest of the depleted serum was prepared as follows (see Supplemental material for further characterization of the trypsin digestion protocol): Serum proteins were denatured by addition of methanol to a final concentration of 60% and dithiothreitol (1 M) to a final concentration of 10 mM. The mixture was incubated at 60 °C for 1 h; 1 M iodoacetamide was added to a final concentration of 50 mM, and the sample was incubated at RT for 30 min in the dark. The mixture was diluted with 50 mM ammonium bicarbonate to a final methanol concentration of 20% (final iodoacetamide ∼16.7 mM). Trypsin (Promega, Madison, WI) was added at an initial trypsin-to-protein ratio of 1:100, and a second aliquot of trypsin was added after 2 h of digestion, for a final trypsin-to-protein ratio of 1:50. The total duration of trypsin digestion was 6 h at 37 °C. Lima bean inhibitor of bovine and human trypsin (Worthington, Lakewood, NJ) was added to the final digested mixture at the ratio of 1:1.3 (by weight, trypsin inhibitor:trypsin).

Antigen capture and elution

Magnetic Protein G beads with immobilized antibodies were incubated with tryptic digests of serum at 4 °C overnight. The beads were washed with 10 mM Tris, 1 mM EDTA, 0.5 M NaCl. The bound peptides were eluted by incubating the beads in 8 µL of 5% acetic acid for 3 min at RT. The recovery of TNFα protein was analyzed by SDS–PAGE stained with SilverQuest kit from Invitrogen (Carlsbad, CA) or by Western blot.

Nano-liquid chromatography

An Agilent 1100 nano flow system (Agilent Technologies) equipped with a microwell-plate autosampler and an isocratic capillary pump (for sample loading) was used for liquid chromatography. Solvents used were water/0.1% formic acid (mobile phase A) and acetonitrile/0.1% formic acid (mobile phase B). An IntegraFrit trap column (2 cm $\times$ 100 µm; New Objective, Woburn, MA) was connected to a silica-based C_{18} monolithic column (Chromo-Lith CapRod, Merck, Darmstadt, Germany) via a microcross connector (Upchurch Scientific, Oak Harbor, WA). The trapping column was packed in house at a pressure of 500 psi using Atlantis C_{18} material (5 µm particle, 100 Å pore size, Waters Corp., Milford, MA). Samples were loaded on the trapping column at 10 µL/min and desalted by washing with 2% mobile phase B for 5 min.

The LC gradient for the monolithic column was delivered at 800 nL/min. A linear gradient of mobile phase B was developed from 10 to 40% B for 10 or 30 min depending on sample complexity.

Electrospray ionization linear ion trap mass spectrometer

The nano-LC system was connected to a linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nano electrospray interface operated in the positive ion mode. Typical instrument settings included a spray voltage of 1.5 kV, an ion transfer tube temperature of 200 °C, and a collision gas pressure of 1.3 Torr. Voltages across the capillary and the quadrupole lenses were tuned for optimal signal intensity using the +2 ion of angiotensin I (m/z 649). For each analyte run, a MS survey scan (m/z 400–1600) was followed by two targeted MS–MS scans at the m/z of the monitor peptide and the m/z of the heavy-isotope-labeled version of the peptide. Quantification was performed by integrating the extracted ion chromatogram of the doubly charged parent ion from the MS scan. Ions monitored for quantification of AAC were m/z 531.3 and 534.3 (^{13}C- and ^{15}N-incorporated standard); TNFα ions were m/z 692.2 and 695.2 (^{13}C- and ^{15}N-incorporated standard). MRM experiments were performed by integrating the areas of two transition fragment peaks from the MS–MS spectrum using XCalibur QualBrowser. The inclusion window for each precursor ion was ±1 Da, the inclusion window for the fragment ion was ±2 Da. Transitions monitored for AAC were $531.3 \rightarrow 633.5, \, 819.5$ and $534.3 \rightarrow 639.5, \, 825.5$. Transitions monitored for TNFα were $692.2 \rightarrow 841.5, \, 954.5$ and $695.2 \rightarrow 847.5, \, 960.5$.

Results

Selection of magnetic beads

Magnetic beads have been used in almost all branches of bioscience and biotechnologies for isolation, separation, and purification of various types of proteins and peptides [23]. Many varieties of magnetic beads with a variety of surface chemistries are commercially available. We sought a bead system with high antibody coupling efficiency, good functional orientation of antibody for antigen capture, low background binding of serum peptides and proteins, and production of captured samples appropriate for mass spectrometry (i.e., without chemical contamination). To this end, a panel of seven Dynal magnetic beads (see Supplemental material for more details), including Protein G, Myone streptavidin, M280 streptavidin, M270 streptavidin, tosylactivated, epoxy, and carboxyl beads, was tested. Based on their low background binding, lack of chemical contamination, high coupling efficiency, and proper orientation of cross-linked antibodies, we chose Protein G beads for our peptide antigen capture technology.

Antigen binding, wash, and elution conditions

We sought to optimize conditions for capture and recovery of targeted peptide antigens, minimizing nonspecific binding while maximizing recovery of the target antigens. We used a relatively small protein, tumor necrosis factor alpha (17 kDa), as a test antigen to monitor the capture and elution process by gel electrophoresis (SDS–PAGE). Specifically, 50 ng (2940 fmol) of TNFα was spiked into 1 mL human serum. Anti-TNFα-monoclonal-antibody-coated magnetic beads (20 μL) were added to the serum, and the antigen was captured at 4 °C overnight. The beads were washed four times with 1 mL 0.5 M salt. A series of elution buffers was tested on captured TNFα, and the recovery of protein was determined by Western blot. As can be seen in Fig. 1a, very little antigen is lost in the flow-through or wash collections, demonstrating that the majority of antigen was successfully captured on the beads. Four of the five elution buffers tested yielded considerable recovery of antigen within 3 min. Trifluoroacetic acid (5%) was the only exception; in this case, none of the antigen came off during the elution, although it was released by boiling the beads.

Addition of trypsin inhibitor to antigen captures

For peptide capture experiments, tryptic digests of serum (containing the target peptide antigen) are added to antibody-coupled beads. Theoretically, residual active trypsin in the digested serum could proteolyze and destroy the bead-bound antibodies, thereby reducing the capture efficiency of target peptide antigen. To test this possibility, we compared the recovery of recombinant TNFα spiked into proteolyzed serum in the presence or absence of trypsin inhibitor. As can be seen in Fig. 1b, recovery of TNFα was significantly improved by the addition of trypsin inhibitor. Consistent with the notion that residual trypsin activity is responsible for reducing recovery, an increase in TNFα degradation products in the sample that did not receive trypsin inhibitor can be seen. Given the considerable amount of residual trypsin activity observed in this experiment, we presume that antibodies on the surface of beads are also being proteolyzed. Hence, for all capture experiments using trypsin-digested serum, trypsin inhibitor is added to inhibit the digestion prior to the addition of bead-bound antibody.

Performance characteristics of SISCAPA–MS-coupled measurements

For SISCAPA coupled to LC–MS to be applied to clinical samples for biomarker testing, the technology platform must be capable of precise and accurate measurements. Triple-quadrupole instruments, already entrenched in clinical laboratories for measuring small molecules, have consistently provided measurements with high precision (CVs below 5%), a good linear response range ($>10^3$), and high
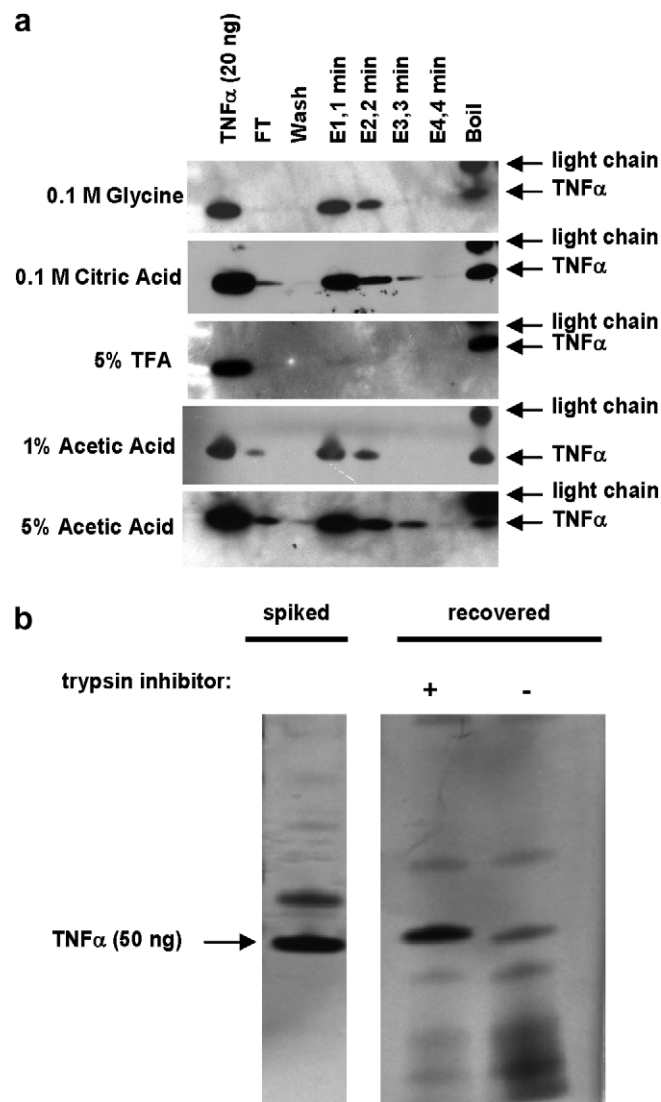

Fig. 1. Optimization of conditions for maximum recovery of captured antigen. (a) Western blot (cropped) showing recovery of 50 ng (2940 fmol) TNFα spiked into 1 mL human serum, captured at 4 °C overnight by 20 μL of anti-TNFα-polyclonal-antibody-coated beads. "FT" refers to flow-through. "Wash" refers to four times washing of beads with 1 mL of 0.5 M NaCl. Multiple elution buffers were tested, as indicated. Four aliquots of elution buffer (labeled E1–E4, 8 μL each) were added to 20 μL of antigen-bound antibody-coated beads at RT in 1-min intervals (total of 4 min). At the end of the elution period, the beads were boiled in SDS buffer to remove residual protein ("Boil"). Note that the secondary antibody used is a goat antimouse antibody, which reveals mouse light-chain fragments released by boiling. (b) SDS–PAGE of antibody enrichment of 50 ng of recombinant TNFα spiked into 400 μL of human serum. The gel image shows recovery of TNFα from a trypsin digest of human serum with (+) and without (−) addition of trypsin inhibitor following the digestion (prior to the antibody-capture step). Addition of trypsin inhibitor results in higher recovery of TNFα protein from trypsin-digested serum.

sensitivity of detection (less than 1 ng/mL). Linear ion trap instruments, far more common in proteomics laboratories involved with biomarker discovery, have only recently been tested in this setting [24] but are nonetheless widely used for

quantification [25–28]. To determine the performance characteristics of our magnetic-bead-based capture system coupled to a linear ion trap, we assessed the linear range, limit of quantification, accuracy, and precision for our two test antigens, AAC and TNFα. We used the extracted ion chromatogram of the target peptide for quantification and verified the identity of the peptide by acquiring a full MS–MS spectrum.

Linear range and limit of quantification of peptides

To ensure the accuracy in quantification using stable-isotope-labeled internal standards, the linear concentration ranges and limits of quantification of the instrument were determined for pure synthetic peptides from AAC and TNFα suspended in 50 mM ammonium bicarbonate. The responses for AAC and TNFα were found to be linear ($R^2 = 0.9984$ and 0.9967, respectively) over 4 and 3 orders of magnitude, respectively (Fig. 2). The limit of quantification (where signal-to-noise = 10) for AAC and TNFα was

approximately 1 fmol ($\sim$1 pg) of peptide injected on-column.

The linear concentration range and limit of quantification for the SISCAPA assays are affected by antigen capture and instrument sensitivity. For example, if capture is inefficient and/or if ion suppression is significant in the eluate, the limit of quantification of a given target captured from serum will differ from the limit of quantification of the pure peptide. Hence, the linear concentration range and limit of quantification were also determined for AAC and TNFα following enrichment from serum on antibody-coated magnetic beads. Human serum was depleted of six abundant proteins and proteolyzed with trypsin. Depletion of serum has been shown to improve reproducibility of quantification [16]. Since AAC can be detected at its endogenous level, the heavy-labeled monitor peptide concentration was varied and the amount of serum was kept constant, thus allowing use of the endogenous peptide as an internal standard. For the TNFα peptide (endogenous level below the limit of detection), synthetic (light) monitor peptide was spiked into serum at increasing concentrations, keeping heavy-labeled peptide concentrations constant (for use as an internal standard). For AAC and TNFα, monitor peptides were subjected to capture by antibody-coated beads and elution in 5% acetic acid. The eluates were analyzed by LC–MS and the peak area response from the extracted ion chromatogram was used to construct a calibration curve (Fig. 3). Overall, the response for captured peptides was linear ($R^2 = 0.9969$ and 0.9946 for AAC and TNFα) over about 2.5 orders of magnitude with limits of quantification around 1 fmol ($\sim$1 pg) and 10 fmol ($\sim$14 pg) of peptide on column for AAC and TNFα, respectively. As expected, the linear concentration range was greater and the limits of quantification were generally lower using pure peptides compared to captured peptides, since nontarget peptides in the eluates cause some degree of ion suppression.

For the SISCAPA methodology to be of utility for testing candidate biomarkers, it must be able to quantify analytes present at $\leqslant$ng/mL, since this is the concentration range of many known diagnostic markers. AAC is present at significantly higher concentrations (endogenous concentration 0.3–0.6 mg/mL [29]). Hence, we chose to demonstrate the capabilities of the SISCAPA–MS assay on TNFα, where the endogenous level (<10 pg/mL) was not detected by the SISCAPA assay (data not shown). TNFα ^{12}C monitor peptide (500 pg; equivalent to $\sim$12 ng protein) was spiked into 1 mL of depleted and digested serum and captured by anti-TNFα magnetic beads, and the entire eluate was injected for LC–MRM–MS analysis (8 μL). The resulting MRM chromatogram had a signal-to-noise >100. Therefore, we conclude that our optimized platform can achieve limits of quantification for proteins on the order of ng/mL, sufficient to detect many biomarkers at physiologic levels (e.g., PSA, CEA, and AFP present at ng/mL).
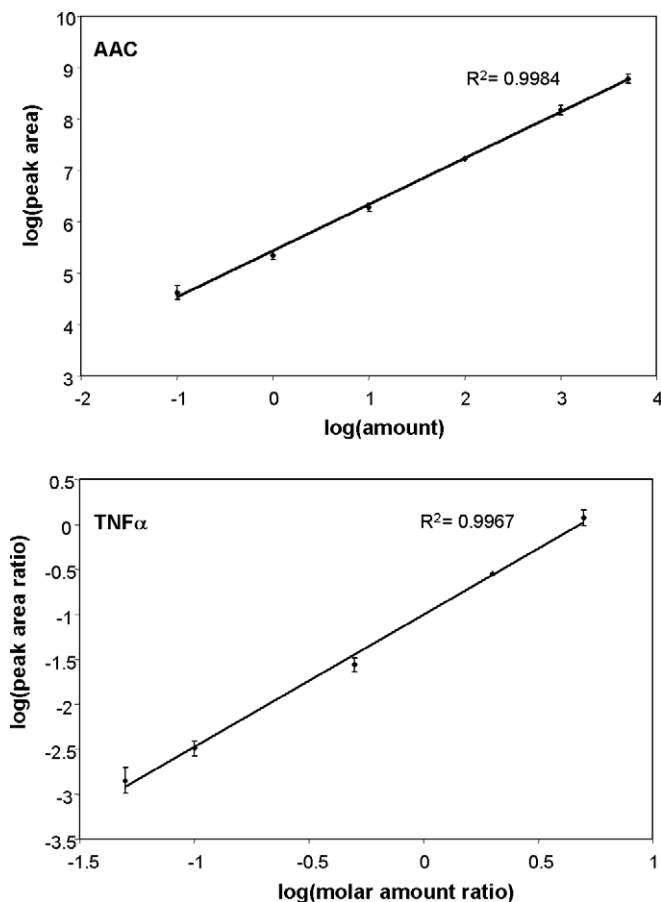

Fig. 2. Calibration curves for quantifying heavy-labeled pure AAC and TNFα peptides. The ion signals for different amounts of pure synthetic heavy peptides were measured using LC–MS and used to determine the linear range of quantification on the linear ion trap instrument. Duplicate analyses were performed for each amount of peptide injected. Error bars show the range for each measurement.
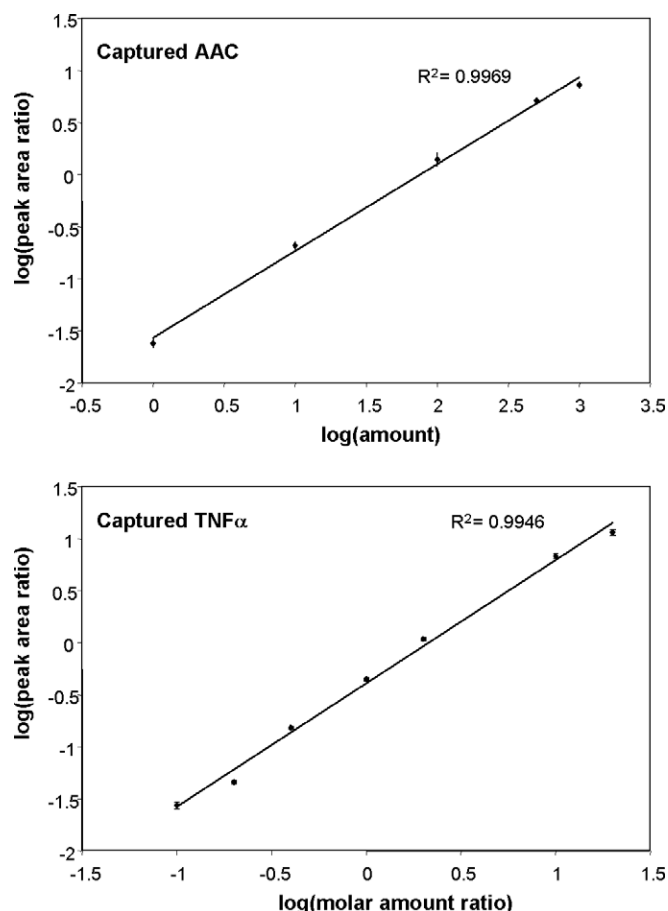
Fig. 3. Calibration curves for quantifying enriched AAC and TNFα peptides. The ion signal ratio for different amounts of peptides spiked into serum and captured on antibody-coated beads were measured using LC–MS and used to determine the linear range of quantification. For AAC, the ratio of heavy peptide:light peptide (endogenous) was determined from the extracted ion chromatograms and plotted versus amount of AAC spiked-in. For TNFα, the ratio of light peptide: heavy peptide was determined from the extracted ion chromatograms and plotted versus the expected molar ratio. Three replicate measurements were performed for each target. Error bars show the standard deviation of the measurements. The amounts of the peptides used for the assay characterization studies (Tables 1 and 2) were selected at the same points along these curves, within the linear range of the instrument.

Reproducibility and accuracy of peptide quantification

To evaluate the reproducibility of LC–MS measurements per se, three independent measurements were made on identical antigen capture samples for each of the study peptides, AAC and TNFα. A single capture was performed for each target, and each of the eluates was subjected to LC–MS analysis three times to determine reproducibility of measurements. The measured concentration, percentage relative error (accuracy), and coefficients of variation (precision) are presented in Table 1. We find that the measurement is highly reproducible; CVs for the target peptides were below 10%. Additionally, the accuracy of the measurements was within 20% of the expected value.

Table 1
Accuracy and precision for SISCAPA–MS analysis of TNFα target peptides spiked into human serum at different concentrations

TNFα (µg/mL)							
	2.32	4.63	9.26	23.1	46.3	231	463
Mean	2.38	3.66	10.1	24.7	53.1	251	390
Accuracy (%RE)	2.6	−20.9	9.2	7.0	14.7	8.4	−15.9
Precision (%CV)	6.3	3.3	2.0	1.1	2.5	4.7	5.4

The mean concentration, accuracy (percentage relative error; %RE), and precision (percentage coefficient of variation; %CV) are presented. Each measurement was performed in triplicate ($n = 3$).

Having confirmed the reproducibility of the measurements, we next evaluated reproducibility of the antibody capture protocol. The heavy-labeled monitor peptide of AAC was captured three independent times from different serum aliquots at three spiked concentrations (keeping serum concentration the same). Eluates were analyzed by LC–MS, and the ratio of heavy peptide to light peptide was determined from the extracted ion chromatograms. The measured concentrations, percentage relative error, and coefficients of variation for the replicate captures are presented in Table 2. The total mean, accuracy, and precision for captures from all 3 days are also shown in Table 2. Overall, the total CVs for the three capture experiments are excellent and range from 8 to 14%. Accuracy for the repeat capture experiments is also good and ranges from −18 to 8% relative error. Based on the known amount of spiked-in isotopically labeled standards, we empirically deter-

Table 2
Quantification statistics for SISCAPA–MS quantification of AAC target peptide spiked into human serum and captured with immobilized anti peptide antibodies

Day	AAC (µg/mL)			
		3.58	17.9	35.8
1	Mean	3.70	17.5	27.4
	Accuracy (%RE)	3.3	−2.4	−23.4
	Precision (%CV)	4.4	4.1	2.6
	n	5	3	3
2	Mean	4.40	20.3	29.4
	Accuracy (%RE)	22.8	13.6	−17.9
	Precision (%CV)	3.7	2.9	5.6
	n	3	3	3
3	Mean	3.03	20.6	34.4
	Accuracy (%RE)	−15.5	15.0	−3.9
	Precision (%CV)	6.8	0.6	—
	n	2	2	1
Total	Mean	3.77	19.3	29.3
	Accuracy (%RE)	5.4	7.9	−18.2
	Precision (%CV)	14.0	8.4	9.2
	n	10	8	7

The mean concentration, accuracy (percentage relative error; %RE), precision (percentage coefficient of variation; %CV), and number of replicates (independent captures from different aliquots of serum) for the measurements (*n*) are presented for quantification performed on 3 days. The total mean, accuracy, and precision were calculated using all measurements from the independent captures.

mined the concentrations of the endogenous AAC peptide to be 0.1 μM. The reported mean concentration of AAC in human plasma is 7 μM [29]. Although our empiric determinations are lower than that reported in the literature, pooling or processing of the reference serum used in our study (which was derived from many individuals) may have altered the concentrations, and errors in amino acid analysis could contribute to inaccuracy. Additionally, incomplete trypsin digestion could contribute to errors in quantification. We have determined our trypsin protocol to be suitable for digestion of serum (see Supplemental material); however, acidic residues adjacent to trypsin cleavage sites within monitor peptides may be predisposed to incomplete digestion. This possibility highlights a great need for internal standards to determine trypsin digestion completion. Nonetheless, based on the results presented in Tables 1 and 2 we conclude that the capture and subsequent LC–MS measurements show sufficient precision and accuracy for screening biomarker candidates [30].

Assessment of enhancement of ion signals of captured target peptides

We next assessed how well our optimized SISCAPA protocols enhance target peptide ion signals over nontarget peptides in serum. We began by studying AAC, capable of being seen by mass spectrometry at its endogenous serum concentration (0.3–0.6 mg/mL [29]).

The AAC ^{12}C monitor peptide from the endogenous serum protein AAC was captured from 20 μL processed (depleted and digested) serum on antibody-coated beads and eluted into 20 μL 5% acetic acid. Both the starting serum digest and the eluates following capture were analyzed by LC–MS. It is important to note that equivalent amounts of peptide were injected for LC–MS analysis for each sample (before and after capture). Therefore, changes in signal intensity for the target analyte are solely due to reduction in ion suppression (by high-abundance serum peptides) and are not due to concentration of the peptide following capture enrichment. Fig. 4a shows the total ion chromatogram for a 30-min gradient LC–MS analysis of the total serum digest. As expected, there is considerable background ion content in the serum run. The endogenous AAC peptide was detected at trace level when analyzed by LC–MRM–MS (Fig. 4b) and its identity verified by a full MS–MS spectrum (Fig. 4c). Fig. 4d shows the total ion chromatogram of the 30-min LC–MS analysis of the anti-AAC antibody-captured sample. Notice that there is a considerable decrease in background ions and that the AAC peptide is the predominant peak in the chromatogram. The LC–MRM–MS chromatographic trace of the anti-AAC antibody-enriched sample is shown in Fig. 4e and the AAC peptide is again verified by a full MS–MS spectrum (Fig. 4f). A large increase in the relative signal-to-noise following antibody capture and elution is apparent from examining these traces. This is due to elimination of background species which contribute to ion suppression of the AAC peptide in serum.

To evaluate the increase in analyte signal relative to other components in the sample, we normalized the analyte signal to the total ion current (TIC) of the starting serum digest and compared this to the normalized analyte signal of the eluates following antibody capture. Based on the ratios of the analyte signal:TIC from the starting serum digest vs the eluates, we estimate that antibody capture resulted in 1453-fold enhancement of ion signal for the AAC peptide. Note that this is a conservative estimate of actual enrichment of the target peptide; the plasma sample in which these measurements were made had already been depleted of six abundant proteins using the depletion column, which is expected to contribute an additional ~5-fold enrichment [31].

We next studied a lower-abundance target, TNFα. The endogenous plasma level of TNFα is <10 pg/mL, which is below the limit of detection of the instrument (when measured directly in plasma). To determine the enhancement of TNFα peptide by antibody capture and LC–MS, we spiked TNFα (light) monitor peptide (along with heavy stable isotope standard) into depleted human serum digest at a concentration of 2.3 μg/mL. The spiked-in peptides were captured by anti-TNFα antibody and analyzed by LC–MS. As illustrated in Figs. 5(a–f), a 573-fold enhancement of the TNFα peptide ion signal was achieved by antibody capture and MRM detection (monitoring two transitions).

Discussion

There is tremendous opportunity to transform medicine through more and improved diagnostic biomarkers. With the application of genomics technologies, the biomarker discovery pipeline now resembles the drug discovery pipeline; there are far more candidates than can possibly be tested in reasonable time with existing resources, and there are no proven ways to prioritize them [32,33]. Hence, although there is widespread acceptance [34] that better and more diagnostics will greatly impact patient care, the number of new diagnostics being approved for clinical use is small and rapidly vanishing [7]. We urgently need technologies that enable measurement of hundreds of biomarker candidates in thousands of clinical samples to allow rapid determination of the potential of candidates, individually or in panels, to impact clinical care.

SISCAPA has several significant advantages over the current gold standard (ELISA) in filling this gap. For instance, the need to generate pairs of antibodies compatible for sandwich assays is largely responsible for the high cost and long lead time for developing ELISAs. SISCAPA minimizes this burdensome step by requiring one antibody, not two as with ELISA. (The mass spectrometer effectively acts as the second antibody, with exquisite specificity.) Furthermore, antibody reagents will be easier to generate for SISCAPA vs ELISA because there are (1) no constraints to target nonoverlapping epitopes, (2) no requirements
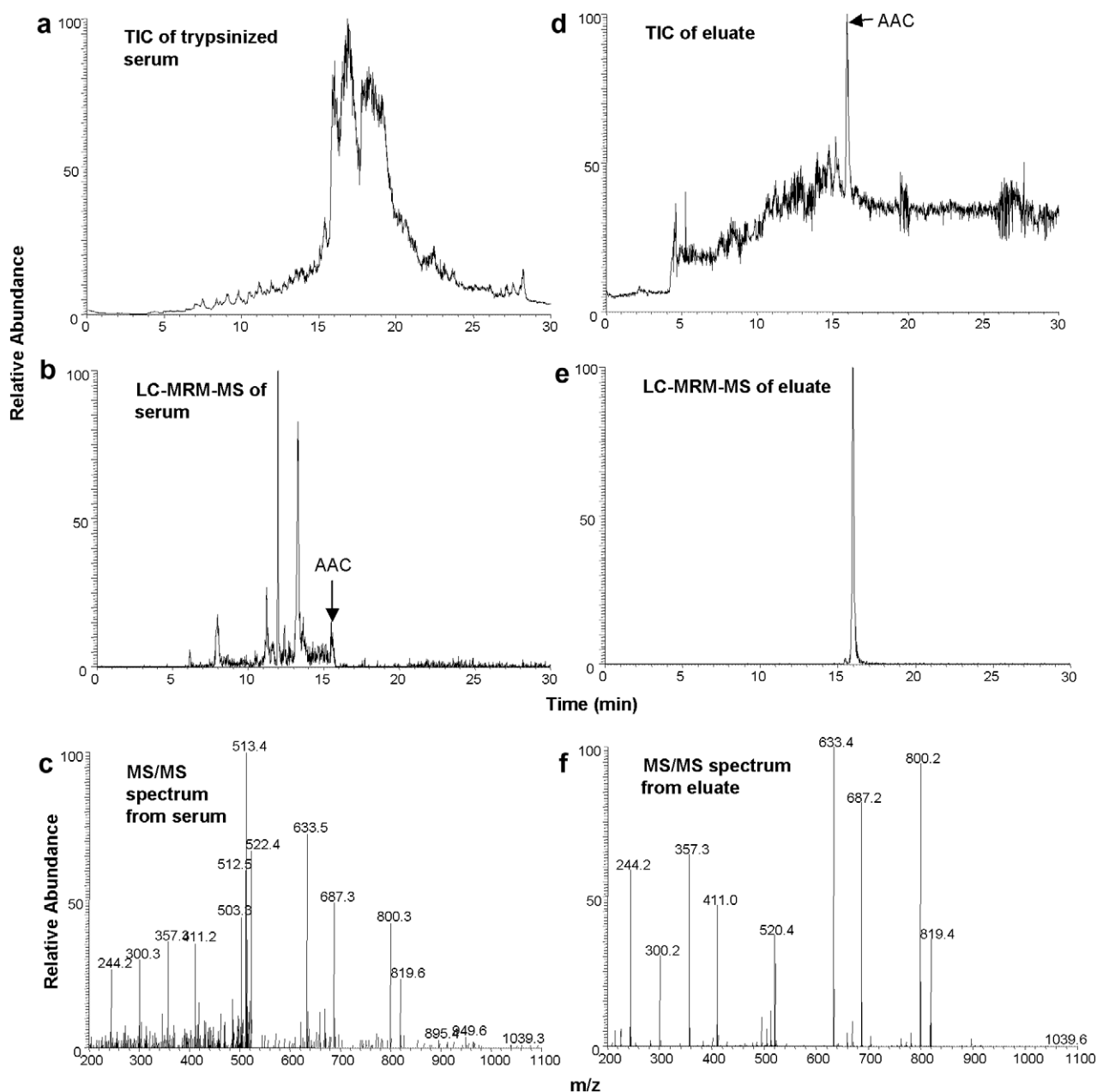
Fig. 4. Enhancement of endogenous AAC ion signals following antibody enrichment assessed by LC–MS. (a) 30-min LC–MS analysis of 20 μL depleted and digested human serum. (b) 30-min LC–MRM–MS analysis of AAC from same serum digest. (c) MS–MS spectrum of AAC from serum digest. (d) 30-min LC–MS analysis of antibody-enriched AAC from 20 μL depleted and digested human serum. (e) 30-min LC–MRM–MS analysis of antibody-captured AAC. (f) MS–MS spectrum of AAC from antibody capture. Equivalent amounts of peptide were injected for LC–MS analysis for each sample (before and after capture).

for the antibodies to recognize the intact protein, and (3) more relaxed requirements for antibody selectivity because of the highly selective nature of the mass spectrometric analysis. For candidates showing initial promise, it may be possible to enhance sensitivity with some investment in further optimizing the assay conditions for specific antibody–antigen pairs. Likewise, for lower-abundance targets where sensitivity must be optimized, carefully screening for highest affinity antipeptide antibodies should provide benefit. (Note the difference in enrichments achieved with the TNFα vs the AAC antipeptide antibodies in our

experiments.) SISCAPA also provides benefits over ELISA in potential throughput. The SISCAPA–MS approach can simultaneously monitor multiple targets within a single sample while the standard ELISA can generally quantify only one target per sample, making SISCAPA desirable with regard to both throughput and sample consumption. Recent data suggest that at least 200 assays could be multiplexed in a single LC–MRM–MS run [16]. Additionally, the magnetic-bead-based platform described in this report is amenable to automation and high throughput, and its flexibility allows for capture from large sample volumes,
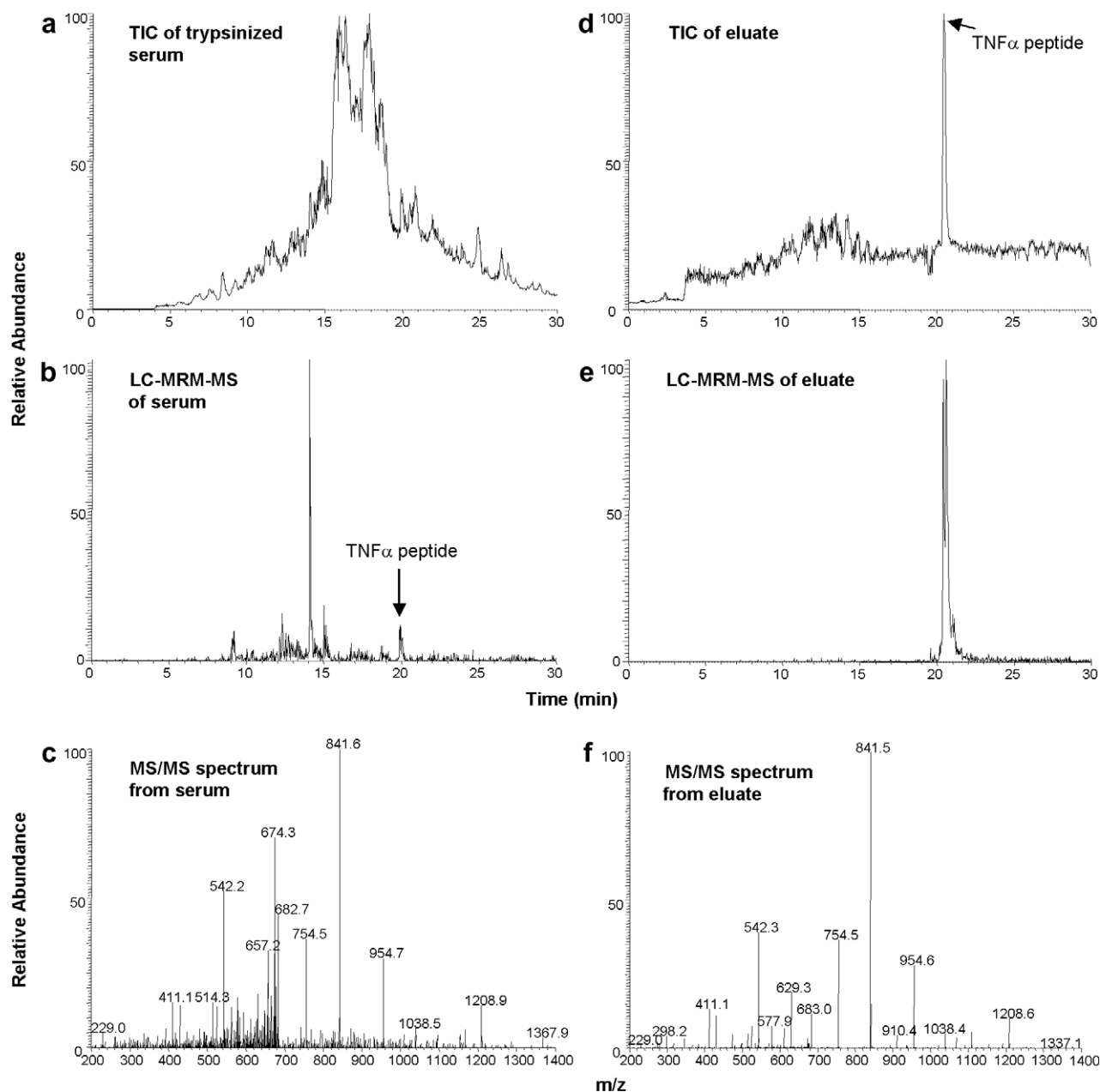
Fig. 5. Enhancement of spiked TNFα ion signals following antibody enrichment assessed by LC–MS. (a) 30-min LC–MS analysis of 20 μL depleted and digested human serum. (b) 30-min LC–MRM–MS analysis of TNFα from same serum digest. (c) MS–MS spectrum of TNFα from serum digest. (d) 30-min LC–MS analysis of antibody-enriched TNFα from 20 μL depleted and digested human serum. (e) 30-min LC–MRM–MS analysis of antibody-captured TNFα. (f) MS–MS spectrum of TNFα from antibody capture. Equivalent amounts of peptide were injected for LC–MS analysis for each sample (before and after capture).

allowing for further enrichment by volume reduction on the order of 10^2–10^3 and extending analysis to beyond the ng/mL range, sufficient for quantifying low-abundance biomarkers. Hence, we believe that SISCAPA holds great promise for facilitating the biomarker discovery pipeline.

In this study we demonstrated the capabilities of the linear ion trap for quantitative assays using an extracted ion chromatogram. This mode of operation has been used for numerous quantitative proteomics studies [25–28]. Although the linear ion trap is unlikely to replace the triple-quadrupole instrument (using SRM–MRM) for

MS-based quantitation in the clinical setting, there are advantages of using the ion trap for early stage testing of candidate biomarkers. First, many proteomic laboratories doing MS-based biomarker discovery are using ion trap instruments and do not have ready access to triple-quadrupole instruments. Second, using the same instrument for discovery and assay development facilitates choosing monitor peptides. For instance, the biomarker discovery dataset can be used to select monitor peptides that were empirically observed to ionize efficiently and produce robust fragmentation. In contrast, if the discovery data were generated on

an ion trap and validation were done on a triple-quadru-pole instrument, the chosen monitor peptides may not be the most optimum with respect to ionization or fragmenta-tion, increasing the risk of assay failure. Finally, in initial validation and screening of biomarker candidates, the capability to rapidly and unambiguously verify the peptide identity (via a full MS–MS spectrum) greatly increases con-fidence in the data.

For this work, we adopted a scan sequence to quantify and conclusively verify the peptide target. The scan sequence consisted of a full-scan MS spectrum followed by two full MS–MS spectra acquired at the mass/charge of the (light) monitor peptide and the heavy-labeled moni-tor peptide (internal standard). The MS spectrum permits quantification of the analytes by using an extracted ion chromatogram and provides information on the back-ground levels of other components in the sample. The full MS–MS spectra allow for unambiguous identification of the peptide. Taking into account the identification of the targeted peptide (via the MS–MS spectrum) in addition to the coelution of the heavy-labeled internal standard pep-tide allows for conclusive verification of peptide identity. Additionally, integrating the peak areas of one or more fragment ions from the MS–MS spectra can be used for SRM–MRM studies. A recent study demonstrated the effectiveness of the linear ion trap operated in such a mode [24].

The capability of multiplexing many peptides into a sin-gle run is an important advantage in using SISCAPA–MS. In this study, the entire scan sequence requires less than 1 s (about 200 ms each for MS scan and MS–MS scans), allowing for sufficient points across an elution peak for accurate quantification, and uniquely performs the desired analysis by providing full MS–MS spectra of the analytes. Dividing a run into multiple time segments will allow for monitoring multiple peptides. The number of possible time segments depends on chromatography and elution times of the peptides. Based on the scan sequence that we have pre-sented, one or two peptides can be monitored per time seg-ment, allowing for a maximum of about five peptides to be monitored in a short run. A recent study [24] using a single-quadrupole ion trap for MRM studies (without acquiring a full MS–MS scan for peptide verification) reports scan times of about 150 ms, suggesting that up to 10 peptides can be monitored in a 10-min run. In contrast, a triple-quadrupole instrument operated in MRM mode at only 18 ms per transition may be capable of monitoring signifi-cantly more peptides (e.g., up to 50–100) in a 10-min run depending on the number of time segments [16]. Ultimate-ly, a triple-quadrupole mass spectrometer is the instrument of choice for such experiments; however, we have demon-strated the utility of an ion trap for quantitative studies, as mentioned above.

Another important aspect of the assay is complete pro-teolytic digestion. Because the assay depends on the enrich-ment and measurement of peptides, incomplete proteolysis can significantly affect detection limits and accuracy. Thus, enzymatic digestion protocols must be strictly optimized, controlled, and monitored. To this end, there is a great need for development of a proteolysis quality control stan-dard. Such a standard could include spiking a heavy-iso-tope-labeled synthetic polypeptide containing internal trypsin cleavage sites into serum to measure the efficiency of digestion of an endogenous or spiked polypeptide.

One can now envision an efficient staged approach to building quantitative assays for testing biomarker candi-dates. For example, initially quantitative LC–SRM–MS, without antibody enrichment, could be attempted to detect peptides derived from the candidate protein in trypsin-di-gested plasma samples that have been depleted of abundant proteins; many moderate- and high-abundance candidates will likely be detectable, down to the ~µg/mL range, with-out the need to generate antibodies [16]. For those candi-dates not detectable without enrichment, the SISCAPA technology can be used. Our data indicate that candidates in the ng/mL range can be quantified on the platform described herein. For very-low-abundance candidates (<<ng/mL) or for candidates refractory to the above approaches, it may still be necessary to generate an ELISA, which may achieve sensitivity in the pg/mL range with good "sandwich antibodies." Emerging immuno-PCR adaptation of ELISAs promises even higher sensitivities [35,36].

In conclusion, we have developed a generalizable auto-matable peptide enrichment and quantification platform for high-throughput testing of biomarker candidates. This platform should provide a desperately needed bridging technology between development-intensive biomarker dis-covery and clinical application. Furthermore, the tech-niques presented here are amenable to protein quantification in any biological investigation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.12.023.

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