

High Sensitivity Detection of Plasma Proteins by Multiple Reaction Monitoring of *N*-Glycosites*[§]

Jianru Stahl-Zeng[‡], Vinzenz Lange^{§||}, Reto Ossola[¶], Katrin Eckhardt^{||}, Wilhelm Krek^{||}, Ruedi Aebersold^{¶**‡‡}, and Bruno Domon^{¶§§}

The detection and quantification of plasma (serum) proteins at or below the ng/ml concentration range are of critical importance for the discovery and evaluation of new protein biomarkers. This has been achieved either by the development of high sensitivity ELISA or other immunoassays for specific proteins or by the extensive fractionation of the plasma proteome followed by the mass spectrometric analysis of the resulting fractions. The first approach is limited by the high cost and time investment for assay development and the requirement of a validated target. The second, although reasonably comprehensive and unbiased, is limited by sample throughput. Here we describe a method for the detection of plasma proteins at concentrations in the ng/ml or sub-ng/ml range and their accurate quantification over 5 orders of magnitude. The method is based on the selective isolation of *N*-glycosites from the plasma proteome and the detection and quantification of targeted peptides in a quadrupole linear ion trap instrument operated in the multiple reaction monitoring (MRM) mode. The unprecedented sensitivity of the mass spectrometric analysis of minimally fractionated plasma samples is the result of the significantly reduced sample complexity of the isolated *N*-glycosites compared with whole plasma proteome digests and the selectivity of the MRM process. Precise quantification was achieved via stable isotope dilution by adding ¹³C- and/or ¹⁵N-labeled reference analytes. We also demonstrate the possibility of significantly expanding the number of MRM measurements during one single LC-MS run without compromising sensitivity by including elution time constraints for the targeted transitions, thus allowing quantification of large sets of peptides in a single analysis. *Molecular & Cellular Proteomics* 6:1809–1817, 2007.

The discovery and validation of protein biomarkers for the early detection of disease, for the assessment of a therapy's

From [‡]Applied Biosystems, 64293 Darmstadt, Germany, [¶]Institute of Molecular Systems Biology and ^{||}Competence Center for Systems Physiology and Metabolic Diseases, Institute of Cell Biology/ETH Zurich, CH-8093 Zurich, Switzerland, ^{**}Faculty of Sciences, University of Zurich, CH-8006 Zurich, Switzerland, and ^{‡‡}Institute for Systems Biology, Seattle, Washington 98103

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efficacy and side effects, and for drug development is one of the most active areas of proteomics research. Blood plasma represents a promising source of such markers as it has the advantage of being easily accessible. Plasma is circulating and exchanges analytes with cells and tissues within the entire body. In particular, proteins from dying cells and tumors that are shed, secreted, or otherwise released into the blood stream are expected to provide a "fingerprint" of the actual health status of the patient in general and of specific organs in particular. However, the large volume of fluid accounts for a substantial dilution of the analytes secreted or shed by an organ or a tissue. Low concentrations together with rapid clearance of some of the proteins create major challenges to a proteomics-based biomarker discovery approach that remain unresolved despite recent advances of proteomics technology. More specifically, the detection and quantification of proteins in plasma at concentrations in the ng/ml or sub-ng/ml range, at which meaningful biomarkers can realistically be expected (1), has remained difficult.

Two types of approaches have been taken to detect plasma proteins at high sensitivity. In the first, ELISA or other immunoassays are developed to detect and quantify a specific target protein(s) in plasma samples. This approach has the advantage that the assay, once developed, can be applied to high numbers of samples with a high degree of robustness and at low cost. Such assays are sensitive with limits of detection in the sub-ng/ml range being routinely achieved. Unfortunately the development of a reliable and sensitive immunoassay is slow and expensive. It has been estimated that the cost for the development of such an assay is in the order of one million United States dollars. Therefore, immunoassays are most powerful for the routine testing of validated biomarkers in large numbers of samples or population screening but less suited for the discovery and validation of new markers.

The second type of approach is based on profiling plasma proteins by (quantitative) mass spectrometry. Typically sets of plasma samples classified as healthy control or disease-affected are analyzed, and the results are compared for the detection of proteins with consistently increased or decreased abundance in the respective groups. Although this approach is conceptually simple, its successful practical im-

plementation has been exceedingly difficult mainly for two reasons. The first is the complexity of the plasma proteome, which is thought to contain tens of thousands of different proteins. If this proteome is digested with trypsin as it is required for most mass spectrometric methods, hundreds of thousands of peptides are generated. Such complexity clearly exceeds the peak capacity of any state-of-the-art LC-MS system, and low abundance components are masked by the (bio)chemical background. This can be overcome, at least to some extent, by extensive fractionation of the sample at the protein and/or peptide level and the sequential analysis of each fraction, a procedure that is costly and slow. The second reason is the limited dynamic range of mass spectrometers used for most proteomics studies (e.g. quadrupole/time-of-flight or ion trap instruments), which is typically 3 orders of magnitude. This is considerably less than the range of protein abundances in plasma, which has been estimated to exceed 10 orders of magnitude (2). The nominal dynamic range of mass spectrometers can be further constrained by ion suppression effects and, if the analyses involve systematic (shot-gun) tandem mass spectrometry of selected peptides, by undersampling (3). As a consequence of these unresolved limitations, the many and diverse plasma proteome profiling approaches such as two-dimensional gel electrophoresis, SELDI-MS, or two-dimensional LC-MS/MS have largely failed to detect proteins below the level of the most abundant plasma components. Recently the use of the most advanced mass spectrometers with high mass accuracy and high sensitivity in conjunction with extensive sample prefractionation has extended the sensitivity limit of detection of plasma protein profiles to the $\mu\text{g}/\text{ml}$ or ng/ml concentration range (4–7). However, the comparison of a single pair of samples using these methods consumed weeks of mass spectrometer and data processing time. Therefore, although such profiling methods may reach impressive sensitivity levels, they are impractical for the routine quantitative analyses of larger numbers of clinical samples. Directed MS/MS strategies in which specific precursor ions are targeted for CID increase the sequencing efficiency (8) and potentially overcome some of the limitations of random precursor selection in data-dependent approaches. They do not only allow sequencing of lower intensity signals, but they can also focus specifically on the peptides of interest (e.g. peptides that exhibit differential expression) and can exclude peptides that have been observed previously. Nonetheless such an approach does not provide precise quantification and still suffers from limited dynamic range and sensitivity. Recently removal of the six most abundant proteins in plasma and analysis of the tryptic digest by multiple reaction monitoring (MRM)¹ has been proposed to increase the dynamic range for quantifying major plasma proteins (9).

¹ The abbreviations used are: MRM, multiple reaction monitoring; S/N, signal-to-noise ratio; LOD, limit of detection.

As an alternative to chromatographic or electrophoretic fractionation of the plasma proteome we recently introduced the selective isolation of the deglycosylated forms of those peptides that are *N*-glycosylated in intact proteins (*N*-glycosites) by a solid-phase method and their subsequent mass spectrometric analysis (10). The method is based on the observation that most plasma proteins as well as proteins secreted or shed from cell surfaces are glycosylated (11) and that the selective analysis of *N*-glycosites leads to increased sensitivity due to the dramatic reduction of the sample complexity (12). In addition, it was shown that proteins originating from a range of tissues can be detected in plasma by this method (13).

In the present study we applied a selective, highly sensitive quantitative mass spectrometry method for the targeted analysis of specific *N*-glycosites in samples isolated from plasma. To reach the sensitivity level required for analyzing relevant biomarkers multiple reaction monitoring performed on a triple quadrupole type instrument was applied. The high degree of selectivity of MRM results from two sequential levels of mass selection, first the selection of the precursor ion and second the detection of specific fragment ions derived from the targeted peptide. The narrow window of the first mass filter transmits a small ion population and thus minimizes the overall chemical background while increasing the overall signal-to-noise ratio. The second mass filter selects fragment ions (generated in the collision cell) that are typical for the targeted precursor ion and thus further reduces chemical noise and signals unrelated to the analyte of interest. Furthermore the non-scanning nature of this technique results in high data sampling/integration times. The typical dwell times of 20–50 ms used in such experiments compare very favorably to classical scanning experiments (e.g. 400–1400 m/z in 1 s). The combination of these factors accounts for a significant increase in sensitivity by several orders of magnitude compared with the limit of detection achieved in a typical LC-MS experiment or even a product ion scan (14).

In this study, we demonstrate that the application of MRM to *N*-glycosites isolated from plasma resulted in a limit of detection of peptides in the low ng/ml to sub- ng/ml range concentrations (15). In addition, we also showed, using stable isotope-labeled reference peptides, that the targeted analytes could be accurately quantified over a dynamic range of near 5 orders of magnitude. Furthermore the number of transitions to be monitored during an entire LC-MS run could be expanded by implementing a modification of the data acquisition and instrument control software that allowed the use of the elution times of the targeted analytes as a constraint. This expands the capacity of the method to several hundred transitions per LC-MS/MS experiment, thus allowing multiplexed quantitative biomarker analyses with high sensitivity and selectivity.

EXPERIMENTAL PROCEDURES

N-Glycosite Samples—Samples were prepared using the *N*-glycosite isolation protocol described previously (10) starting with 50 μl of

TABLE I
Reference peptides used in this study

Letters in bold refer to isotopically labeled amino acids. M*, Met oxide; C#, carbamidomethyl-Cys.

	Protein	Accession no.	Peptide sequence
1	Sex hormone-binding globulin precursor	IPI00023019	LDVDQALDR
2	Complement C2 precursor	IPI00303963	TM*FPDLTDVR
3	Megakaryocyte-stimulating factor	IPI00024825	DGTLVAFR
4	Low density lipoprotein receptor-related protein 1 precursor	IPI00020557	FDSTEYQVWTR
5	TMEM27	IPI00010191	EATEISHVLLC#DVTQR

serum. The resulting mixture of N-glycosite peptides was dissolved in 40 μ l of solvent (5% aqueous acetonitrile) prior to the LC-MS analysis. Typically 1–2 μ l of the solution was used for a single LC-MS run. Briefly human serum samples (50 μ l, containing approximately 4 mg of total protein), human plasma, or serum pools (Sigma-Aldrich) were treated with sodium periodate to oxidize the diol groups of the glycan residues attached to the protein backbone. The coupling to a solid support occurred by reaction of the aldehyde groups thus formed with functionalized hydrazide beads. The immobilized glycoproteins were extensively washed to remove non-covalently bound molecules, and disulfide groups were reduced and alkylated with iodoacetamide on the solid support. The sample was subsequently treated with trypsin, and non-glycosylated peptides were removed by washing. Finally N-glycosites were released by treatment with peptide-N-glycosidase F, an enzyme that cleaves the covalent bond at the carbohydrate attachment site, thus releasing deglycosylated peptides from the solid support while leaving the glycan moiety ligated to the solid phase. In the process of the enzyme action the asparagine residue of the NX(S/T) consensus sequence is converted into an aspartic acid. The resulting peptide mixture was then analyzed by LC-MS using a reversed phase column.

Reference Peptides—Peptides used as internal standards were chemically synthesized (Cell Signaling Technology Inc., Danvers, MA and Sigma-Aldrich). They were isotopically labeled by incorporating $^{15}\text{N}/^{13}\text{C}$ in one amino acid residue, typically Val, Phe, Arg, or Leu, near the C terminus. The peptide sequences and the site of isotope labeling are indicated in Table I. Concentrations of the synthetic peptide stock solutions were established by *independent* amino acid analysis.

Generation and Calibration of Reference Protein TMEM27—Glycosylated human TMEM27 protein (UniProt accession number Q9HBJ8) was generated by transiently transfecting HeLa cells with a TMEM27 full-length construct. The concentration of TMEM27 in the cell extract was determined by multiple reaction monitoring using the isotopically labeled peptide (reduced and alkylated) as reference.

Aliquots of the HeLa cell extract containing TMEM27 were spiked into human serum (Sigma) to reach a final concentration of TMEM27 ranging from 5 to 750 ng/ml. After performing the glyco-capture procedure, an aliquot of serum was analyzed by multiple reaction monitoring. Based on the heavy labeled peptide EATEISHVLLCDVTQR spiked into the serum the concentration of TMEM27 in the serum was determined.

Peptide Separation—HPLC separations were performed either on a Dionex/LC Packings or Tempo™ Nano HPLC system (Applied Biosystems/MDS Sciex, Foster City, CA). A C₁₈ capillary column with 75- μ m inner diameter was used at a flow rate of 200–300 nl/min. A gradient from 2 to 62% acetonitrile (containing 0.1% formic acid) over a 60-min period was used.

Mass Spectrometry—The LC-MS and MS/MS analyses to characterize the N-glycosite samples were performed on hybrid linear ion trap FT-ICR instrument (Thermo, Bremen, Germany). Data were processed and visualized using in-house software (Pep3D) (18). In parallel, peptide identification was performed on the linear ion trap instrument in data-dependent mode (selection of the three most

abundant peaks). The identification was performed using Bioworks (version 3.2.) using the International Protein Index (IPI) human protein database.

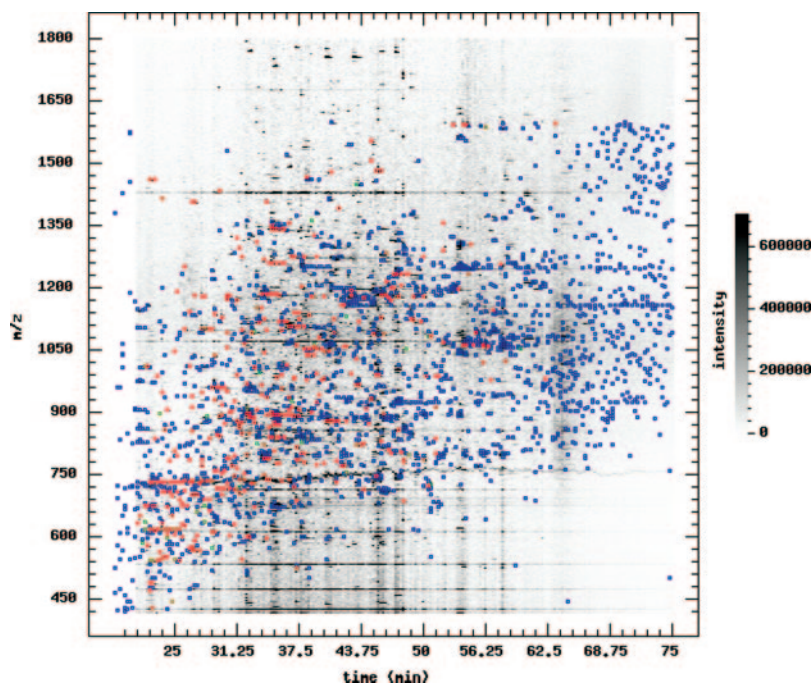
All the MRM experiments described were carried out on a hybrid quadrupole/linear ion trap mass spectrometer (4000 QTrap, Applied Biosystems/MDS Sciex, Concord, Canada) using a nanoelectrospray source. The spectrometer is capable of operating the final quadrupole as either a conventional transmission radiofrequency/direct current resolving quadrupole mass filter or as an axial ejection linear ion trap mass spectrometer. The instrument was operated in triple quadrupole mode with Q1 set on the specific precursor *m/z* value (Q1 is not scanning), and Q3 was set to the specific *m/z* value corresponding to a specific fragment of that peptide. The LINAC® collision cell minimizes the cross-talking effect in very fast MRM experiment (up to 5 ms per transition).

During a single reaction monitoring measurement one precursor ion was mass-selected by the first quadrupole being set in such a way that ions were transmitted within a narrow window (typically 0.7 Da). The precursor ion of interest underwent fragmentation in the collision cell generating product ions. The collision energy was tuned to optimize the intensity of the fragment ions of interest, one of which was selected and transmitted through the second analyzer (set with a mass window of 0.7 Da). In the multiple reaction monitoring mode, a series of single reactions (precursor/fragment ion transitions) were measured sequentially, and the cycle (typically 1–2 s) was looped throughout the entire time of the HPLC separation. MRM transitions were determined from the MS/MS spectra of the existing peptides. Typically doubly charged precursors (or triply charged in some instances) were selected. Two transitions per peptide, corresponding to high intensity fragment ions, were selected, and the collision energy was optimized to maximize signal strength (see “Results and Discussion”). The instrument parameters, e.g. declustering potential and collision energy, can be optimized automatically using automation software to get maximal intensity of MRM transitions. For the scheduled MRM experiments a β -version of the Analyst software (version 1.4.1) was used.

RESULTS AND DISCUSSION

Assessment of Sample Quality—The objective of the study was to identify and quantify selected plasma proteins at high sensitivity by applying the MRM technique to populations of N-glycosite peptides isolated from the plasma proteome. N-Glycosylation is widely represented within the human proteome and characterized by the NXS or NXT sequence motif (where X is any amino acid except proline) (16, 17). N-Glycosites were prepared according to a solid-phase extraction protocol described previously (10). A typical LC-MS map is shown in Fig. 1 using the Pep3D visualization tool (18). Peptides that were selected for CID in data-dependent acquisition mode are marked in *blue*, whereas peptides that were iden-

FIG. 1. Three-dimensional representation of an LC-MS analysis of one plasma *N*-glycosite fraction (gray scale reflects the intensity of the signals). Peptides selected for CID in data-dependent acquisition mode are marked in blue; peptides identified with a high confidence are marked in red.



tified with a high confidence are marked in red. Using very conservative parameters for peak detection ($S/N > 3$; clear peptide isotopic pattern) approximately 2500 features could be reliably detected. Among the identified peptides a very large fraction were fully tryptic peptides with the D*X(S/T) consensus sequence in which D* represents an aspartic acid residue that was formed during the peptide-*N*-glycosidase-catalyzed deglycosylation of a glycopeptide (conversion of asparagine into aspartic acid). These data indicate that the isolated *N*-glycosite samples were highly enriched for the target peptide population. Although the majority of proteins in the plasma proteome are *N*-glycosylated, the complexity of plasma samples after glyco-capture is significantly reduced compared with a tryptic digest of whole plasma.

Selection of Target Peptides and Synthesis of Isotopically Labeled Reference Peptides—To establish the method for detecting and quantifying *N*-glycosites in plasma samples by MRM, a number of isotopically labeled reference peptides were synthesized. The peptides selected, their respective protein of origin, and the isotopically labeled amino acid are shown in Table I. Each peptide selected as a reference for an MRM experiment was first validated to ensure that it fulfils the criteria of a proteotypic species (19), *i.e.* that it is observable (ionizes well and is within the practical mass range of the spectrometer) and unambiguously associated to a single protein (or closely related protein set).

The design of the reference peptides was important for the success of the MRM measurements. The incorporation of stable isotopes at or near the C terminus ensured retention of the label in larger *y*-fragment ions of the reference peptide, thus facilitating precise quantification (20). Non-degenerate fragments, *i.e.* ions with distinct masses, also minimized

cross-talking between the MRM transitions of the internal standard and the endogenous analyte.

The synthetic reference peptides were also useful for confirming the identity of the target peptide through perfect co-elution and by exhibiting identical fragmentation patterns. Specifically the peptides showed the same MRM transitions and a constant ratio of the corresponding fragment ion signals across the entire elution profile. A minimum mass difference of 6 daltons was appropriate to properly separate the isotope clusters of analyte and reference.

Optimization of MRM Transitions—The choice of the transitions is critical for the success of MRM experiments. Ideally a large fraction of the precursor ions current should be converted into a few specific fragments that are indicative for the target peptide. Although the masses of peptide fragments are predictable, their observed intensities needed to be derived from empirical data because algorithms that reliably predict fragmentation patterns are still lacking. Therefore, the reference synthetic peptides were used to optimize the collision conditions.

Aliquots of the reference peptides were injected into the LC-MS/MS system, and fragment ion spectra were recorded under different collision energies (ranging from 10 to 50 eV). Doubly or triply charged peptides were typically chosen to ensure proper separation by the first analyzer of the precursor of the co-eluting heavy labeled internal standards. Singly charged ions were disqualified due to their usually poor fragmentation. Higher charge state ions (four and higher) were excluded to avoid overlapping isotope clusters that may result in cross-talk between the isotope cluster of the reference and endogenous peptides. Results from the MRM optimization are illustrated in Fig. 2 for peptide 1 where the relative abundance

Fig. 2. A, MS/MS (CID) spectrum of the LDVDQALDR peptide after collision energy optimization (arrows indicate precursor and fragment ions selected for MRM analysis). B, pseudo-breakdown curves showing optimization of fragmentation conditions (the dotted line indicates optimal collision energy for MRM). C, selection of MRM transitions. *Rel. Int.*, relative intensity; *P*, *P**, precursor ions of endogenous and labeled peptides, respectively.

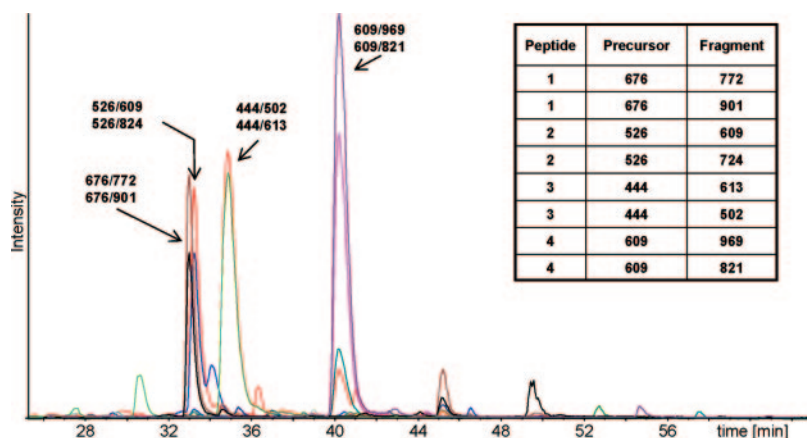
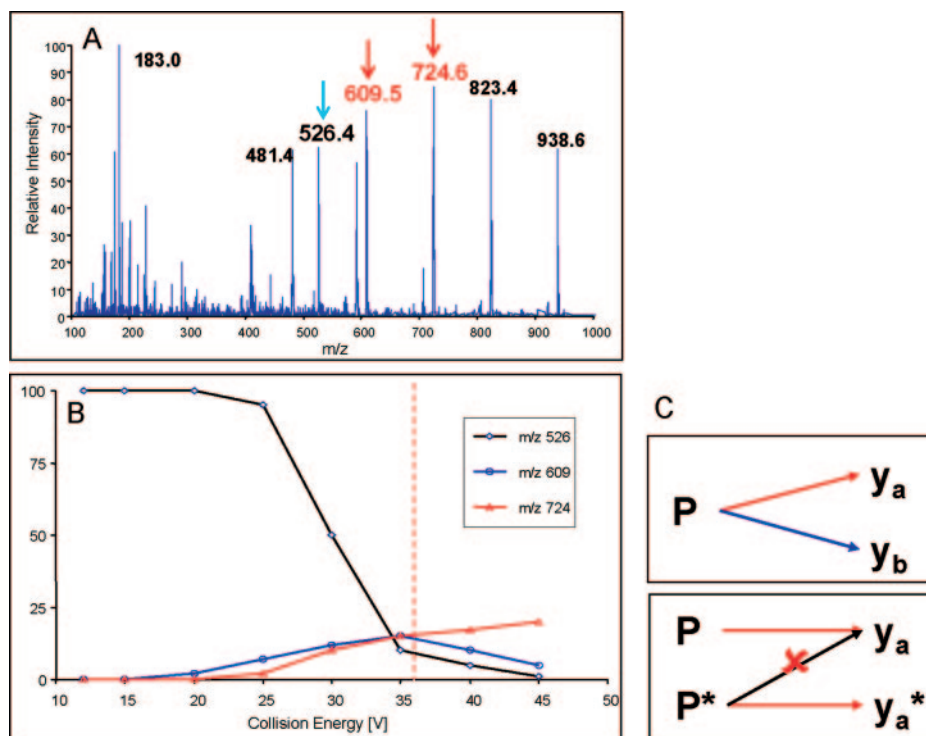


FIG. 3. Ion chromatogram corresponding to the 16 MRM transitions associated with peptides 1–4 (endogenous and isotopically labeled forms).

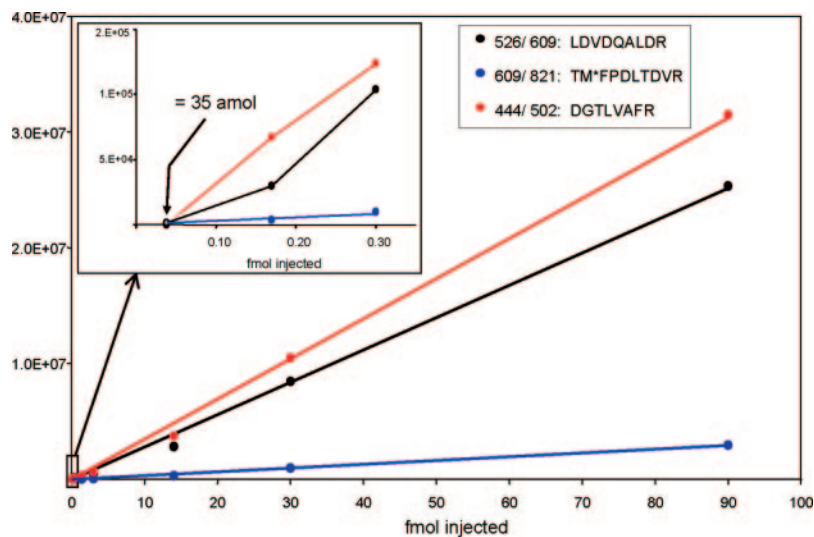
of the precursor ions and specific *y*-fragments were measured at various collision energies. The voltage was considered optimal when a major part of the precursor ion was converted into high mass *y*-ions, whereas secondary (internal) fragments remained lowest. The optimized conditions for each peptide as well as the chromatographic coordinates were recorded for use in the experiments described below.

Detection Limits of Isotopically Labeled Peptides in *N*-Glycosite Samples Isolated from Plasma—To assess the quantitative performance of the technique for specific peptides in a complex sample we used isotopically labeled peptides for which the MRM conditions had been optimized and determined their respective limit of detection (LOD) and limit of quantification. Aliquots of four isotopically labeled synthetic peptides (Table I, peptides 1–4) were spiked into *N*-glycosite

samples that had been derived from human plasma. The peptide amounts added spanned a concentration range of 5 orders of magnitude (5 amol/ μ l to 500 fmol/ μ l). For each analyte (endogenous and reference peptides), the doubly charged precursor ion and two optimized MRM transitions were measured, corresponding to a total of 16 transitions (see Fig. 3). Each transition was monitored for 50 ms. Fig. 4 shows the dilution curves for three representative transitions, 526/609, 609/821, and 444/502, corresponding to the peptides LDVDQALDR, TM*FPDLTDVR, and DGTLVAFR, where M* is Met oxide.

A linear response was observed over a range of concentrations that exceeded 4 orders of magnitude. The limit of quantification (where the S/N exceeds 10) in favorable cases was as low as 50 amol of peptide injected onto the HPLC column.

FIG. 4. Dilution curves of three peptides spiked into *N*-glycosites isolated from serum samples analyzed by MRM. Black, 526/609: LDVDQALDR; blue, 609/821: TM*FPDLTDVR (where M* is Met oxide); red, 444/502: DGTLVAFR.



For the four peptides tested the LOD (with a S/N exceeding 3) ranged from 10 to 30 amol. Considering that $\frac{1}{10}$ of the *N*-glycosite sample was injected onto the column, these results translate into an actual protein concentration in plasma in the range of 0.1 ng/ml assuming a 100% efficiency of the glyco-capture procedure. The results thus demonstrate that the intrinsic limit of quantification using this approach is significantly lower than the limit observed for the commonly used “full-scan” LC-MS methods (21). The achieved detection limit is also lower than the 0.5 μ g/ml limits reported after affinity enrichment of selected peptides from whole plasma digests using the stable isotope standards and capture by anti-peptide antibodies (SISCAPA) method or by analyzing plasma samples depleted for the six highest abundance proteins (9). This level of sensitivity was achievable because the number of transitions was limited, and thus a high duty cycle (approximately 1–2 s) and sufficient sampling time for each transition (typically 20–50 ms) could be obtained. Although the conventional approaches were only able to analyze the classical plasma proteins, the MRM technique combined with a drastic reduction of the sample complexity lowers the limit of detection to a range that includes proteins originating from tissue leakage and the level of validated plasma biomarkers.

Detection Limit for *N*-Glycosylated Protein Spiked into Human Plasma—Although the detection limits achieved with isotopically labeled peptides spiked into *N*-glycosite samples clearly demonstrated the advantages of combining MRM with the selective isolation of an information-rich subproteome, it is conceivable that some of these advantages would be mitigated by poor yields in the protocols used to isolate the *N*-glycosites. We therefore measured the limit of detection of a glycoprotein spiked at defined amounts into plasma samples.

To generate a reliable source of a protein modified with human glycan structure we expressed a glycosylated form of the recombinant protein TMEM27 in HeLa cells. The concentration of TMEM27 in a cell extract was determined by MRM

(data not shown) using a synthetic isotopically labeled form of one proteotypic TMEM27 peptide as calibrant (EATEISHVLL-C*DVTQR where C* is carbamidomethylcysteine; see Table I, peptide 5). Subsequently this calibrated protein solution was spiked into human serum aliquots to reach a final TMEM27 concentration of 10 ng/ml. The combined sample was processed using the glyco-capture protocol described above. Prior to LC-MS MRM analysis, the isotopically labeled TMEM27 reference peptide was spiked into the sample to a concentration corresponding to 50 ng/ml TMEM27 in serum. By MRM analysis the concentration of TMEM27 present in the *N*-glycosite fraction was determined to correlate to 5 ng/ml TMEM27 (Fig. 5) in serum, translating into an overall recovery rate of 50%. The presence of endogenous TMEM27 in the plasma sample used for this study could not be detected at a concentration level above 2 ng/ml. To establish the robustness and the reproducibility of the entire analytical process four independent dilution series were performed in which variable amounts of recombinant TMEM27 protein ranging from 5 to 750 ng/ml were spiked into aliquots of the glycosite fraction. The samples were processed and measured independently. The results of the MRM analyses are shown in Fig. 6 and indicate excellent linearity in the range of concentrations measured down to a limit in the low ng/ml range. The reproducibility of the entire process, as indicated by an average coefficient of variance below 20% (15% in most cases), is well within acceptance considering the multiple steps of the sample preparation (see Supplemental Table 2). These results indicate that the glycopeptide isolation procedure combined with MRM technology enables the highly reproducible isolation of glycosylated proteins from serum samples and the quantitative determination down to the low ng/ml concentration range.

Scheduled MRM for Multiplexed Peptide Detection—There is a physical limit to the number of transitions that can be monitored reliably in a single LC-MS/MS run and thus the number of peptides that can be detected and quantified pre-

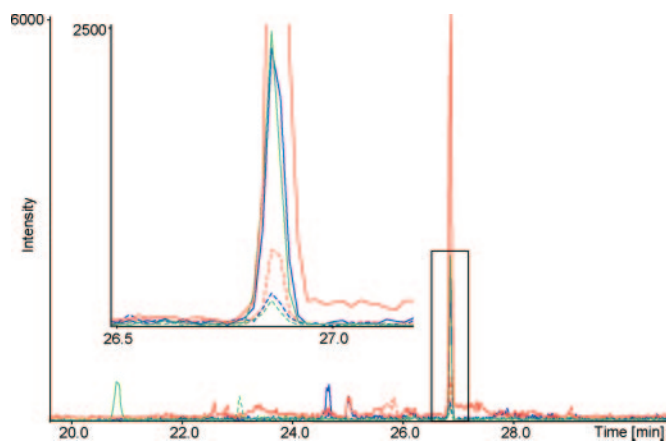


FIG. 5. **Detection of TMEM27 protein in serum.** 10 ng/ml TMEM27 (dashed lines) was spiked into serum and subjected to *N*-glycosite isolation. Preceding specific analysis by MRM, the heavy labeled reference peptide (solid lines) EATEISHVLLC[#]DVTQR (where C[#] is carbamidomethylcysteine and bold indicates the isotopically labeled amino acid) was added to the sample. Transitions are: solid red, 627.7/788.4; dashed red, 624.3/778.4; solid blue, 627.7/901.4; dashed blue, 624.3/891.4; solid green, 627.7/1014.5; dashed green, 624.3/1004.5.

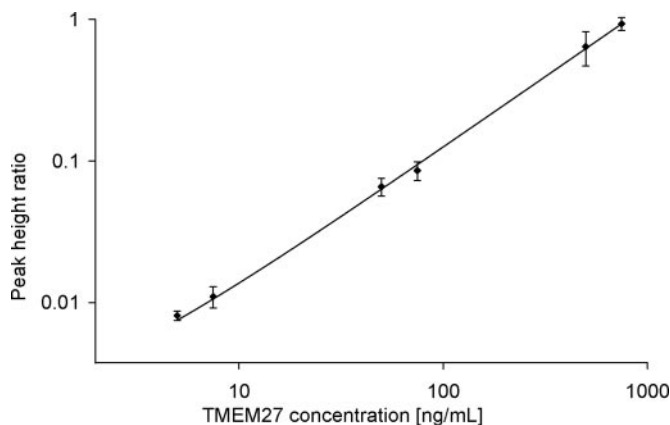


FIG. 6. **Dilution curve of TMEM27 protein spiked into serum.** Four serum samples per dilution point where subjected to *N*-glycosite isolation and subsequently analyzed by MRM. Heavy labeled reference peptide EATEISHVLLC[#]DVTQR (where C[#] is carbamidomethylcysteine and bold indicates the isotopically labeled amino acid) was added at a concentration corresponding to 200 ng/ml TMEM27. ($n = 4$; error bars indicate coefficient of variation.)

cisely. This limit arises from the fact that the duty cycle is directly proportional to the number of transitions included in the experiment. Consequently there exists a tradeoff between the number of transitions (*i.e.* dwell time multiplied by the number of transitions) and the limit of detection. A large number of transitions results in insufficient sampling of data points across the chromatographic elution profile of a peptide and in inaccurate quantification. Conversely a reduction of the dwell time results in a reduction of the signal-to-noise ratio and therefore decreases the limit of detection.

To determine the maximal number of transitions that could be measured in one single run without compromising the

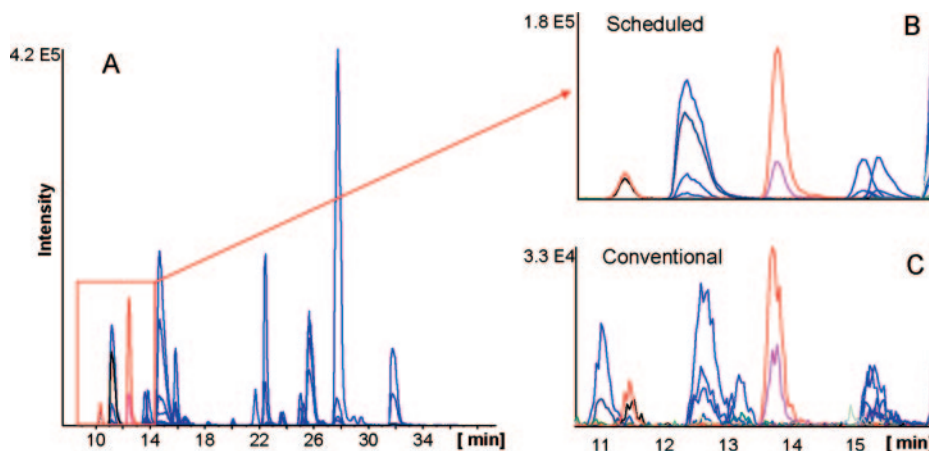
LOD, we systematically increased the number of transitions and related this number to the actual sensitivity. The data (not shown) indicated that 50–100 transitions with an overall cycle time of some 5 s or less per full cycle yielded results without appreciable loss of sensitivity.

To further and significantly increase the total number of transitions that can be measured in a single LC-MS/MS experiment a time constraint was added, effectively scheduling the expected transitions within a defined time window. Using a modified version of the instrument control and data acquisition software, the overall retention time range was restricted in segments of typically 3–5 min, and MRM transitions for peptides expected to elute in each segment were programmed to be measured under optimized parameters. The number of MRM transitions per LC-MS/MS run was gradually increased to 600. Fig. 7 shows an experiment in which 220 transitions were measured with time constraints (Fig. 7, A and B); for comparison measurements without time constraints are shown in Fig. 7C in which each transition was monitored during the entire LC-MS run.

Several beneficial effects of scheduling MRM experiments were immediately apparent. First, the longer dwell time resulted in an increase of the signal quality as reflected by an improved signal-to-noise ratio. Second, the selectivity was also improved as nonspecific biochemical background was reduced as the elution time acted as a further filter. The use of time constraints also considerably expands the field of application of MRM experiments. The analysis of over 500 transitions within one single experiment is possible, and this number is expected to be expanded further with additional improvement of the instrument control and data acquisition software. The scheduled MRM technique used in conjunction with very reproducible HPLC separations is anticipated to increase drastically the number of transitions that will enable screening of a larger number of analytes within one single experiment.

Conclusion—In this study we have demonstrated the performance of a platform integrating an effective reduction of sample complexity through selective isolation of *N*-glycosite peptides and a sensitive mass spectrometry technique, which enables precise quantification of peptides at very low concentrations in plasma. The reduction of sample complexity was essential to lessen the biochemical background to reach a low limit of detection. The recovery of the *N*-glycosite peptides was determined to be 50% despite the involved sample preparation protocol as demonstrated by spiking known amounts of a specific protein into the plasma prior to its processing. Triple quadrupole (and triple quadrupole-derived) instruments allow the analysis of complex samples with high selectivity due to two levels of mass selection, and the non-scanning nature of the technique enabled higher sensitivity and a wider dynamic range. It permits precise quantification when used in conjunction with stable isotope dilution using ¹³C/¹⁵N-labeled standard peptides. Limits of detection in

FIG. 7. Example of an experiment that included 220 scheduled transitions: A, full chromatogram; B, scheduled MRM; C, unscheduled transitions.



plasma were observed in the range below 1 ng/ml corresponding to the level at which tissue leakage proteins are present. In addition, the development of instrument control and data acquisition software that incorporates time constraints to monitor peptides only during their elution window enables the measurement of a large number of MRM transitions (500 or more), and thus larger number of analytes, during one LC-MS experiment without compromising sensitivity.

As demonstrated in this study, the structural selectivity of MRM transitions allowed the detection with an increased degree of confidence of analytes with low abundances in plasma-derived samples despite biochemical background. MRM-based strategies are gaining momentum for biomarker evaluation as they allow analyzing in a generic way a large panel of biomarker candidates in dozens of samples very rapidly (days). It compares very favorably to more conventional approaches (such as ELISA, which necessitates several months to produce the antibodies and develop an assay). The actual rate-limiting step is the synthesis of the isotopically labeled internal standards, which has become routine. The addition of internal standard at the beginning of the analysis would take into account losses that could occur during the entire sample preparation process. In that context, the approach using chimeric recombinant proteins that concatenate all the peptides to be quantified in a single construct (QCONCAT) represents an attractive option (22). The scheduling of elution times allows detection and quantification of a large number of analytes in complex mixtures and thus expands the scope of proteomics applications. This technique has prompted a major shift of paradigm for future proteomics studies by addressing some of the limitations of the current shotgun approach, which tends to analyze only the abundant components of complex mixtures. None of the proteomics approach in use today, including directed sequencing strategies, provides the level of depth required to discover relevant biomarkers as the detection of a signal in the full spectrum remains mandatory to trigger an MS/MS data acquisition. Thus low concentration analytes, often hidden by the high chemical background of complex samples, remain undetec-

ted and unidentified.

An alternative strategy for future proteomics studies consists in hypothesis-driven screens of larger sets of peptides. In analogy to high throughput genomics screens, a two-stage approach can be adopted: first, the creation of an inventory of all the elements present in a proteome (e.g. PeptideAtlas (19)), and second, screening for each element in numerous samples (in a way similar to arrays used for genomics studies). With an established catalog of peptides, the scheduled MRM technique can be used to detect and quantify targeted peptides (including low abundance components) to overcome the limitation of classical shotgun strategies. As comprehensive peptide and protein databases become available, hypothesis-driven mass spectrometric analyses combined with an effective sample preparation to drastically reduce complexity (and thus the chemical background) are likely to become more routine as they enable detection of peptides at concentrations several orders of magnitude lower than current methods.

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§ Both authors contributed equally to this work.

§§ To whom correspondence should be addressed. Tel.: 41-44-633-2088; Fax: 41-44-633-2052; E-mail: domon@imsb.biol.ethz.ch.

REFERENCES

- Polanski, M., and Anderson, N. L. (2006) A list of candidate cancer biomarkers for targeted proteomics. *Biomarker Insights* **2006**, 1–48

2. Anderson, N. L., and Anderson, N. G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* **1**, 845–867
3. Aebersold, R., and Goodlet, D. R. (2001) Mass spectrometry in proteomics. *Chem. Rev.* **101**, 269–295
4. States, D. J., Omenn, G. S., Blackwell, T. W., Fermin, D., Eng, J., Speicher, D. W., and Hanash, S. M. (2006) Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nat. Biotechnol.* **24**, 333–338
5. Liu, T., Qian, W., Gritsenko, M. A., Xiao, W., Moldawer, L. L., Kaushal, A., Monroe, M. E., Varnum, S. M., Moore, R. J., Purvine, S. O., Maier, R. V., Davis, R. W., Tompkins, R. G., Camp, D. G., and Smith, R. D. (2006) High dynamic range characterization of the trauma patient plasma proteome. *Mol. Cell. Proteomics* **5**, 1899–1913
6. Rifai, N., Gillette, M. A., and Carr, S. A. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* **24**, 971–983
7. Omenn, G. S., States, D. J., Adamski, M., Blackwell, T. W., Menon, R., Hermjakob, H., Apweiler, R., Haab, B. B., Simpson, R. J., Eddes, J. S., Kapp, E. A., Moritz, R. L., Chan, D. W., Rai, A. J., Admon, A., Aebersold, R., Eng, J., Hancock, W. S., Hefta, S. A., Meyer, H., Paik, Y. K., Yoo, J. S., Ping, P., Pounds, J., Adkins, J., Qian, X., Wang, R., Wasinger, V., Wu, C. Y., Zhao, X., Zeng, R., Archakov, A., Tsugita, A., Beer, I., Pandey, A., Pisano, M., Andrews, P., Tammen, H., Speicher, D. W., and Hanash, S. M. (2005) Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* **5**, 3226–3245
8. Picotti, P., Lee, H., Domon, B., and Aebersold, R. (2006) Novel approach to identify low abundance biomarkers in serum, in *Proceedings of the 54th ASMS Conference on Mass Spectrometry, Seattle, May 28–Jun 1, 2006*, MOB-6, American Society for Mass Spectrometry, Santa Fe, NM
9. Anderson, N. L., and Hunter, C. (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **5**, 573–588
10. Zhang, H., Li, X. J., Martin, D. B., Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* **21**, 660–666
11. Spiro, R. G. (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implication of glycopeptide bonds. *Glycobiology* **12**, 43R–56R
12. Pan, S., Zhang, H., Rush, J., Eng, J., Zhang, N., Patterson, D., Comb, M. J., and Aebersold, R. (2005) High throughput proteome screening for biomarker detection. *Mol. Cell. Proteomics* **4**, 182–190
13. Zhang, H., Liu, A. Y., Loriaux, P., Wollscheid, B., Zhou, Y., Watts, J. D., and Aebersold, R. (2007) Mass spectrometric detection of tissue proteins in plasma. *Mol. Cell. Proteomics* **6**, 64–71
14. Le Blanc, J. C. Y., Hager, J. W., Ilisiu, A. M. P., Hunter, C., Zhong, F., and Chu, I. (2003) Unique scanning capabilities of a new hybrid linear ion trap mass spectrometer (Q TRAP) used for high sensitivity proteomics applications. *Proteomics* **3**, 859–869
15. Domon, B., Stahl-Zeng, J., and Aebersold, R. (2006) Novel strategy for rapid screening and quantification of biomarkers in serum, in *Proceedings of the 54th ASMS Conference on Mass Spectrometry, Seattle, May 28–Jun 1, 2006*, TOC-1135, American Society for Mass Spectrometry, Santa Fe, NM
16. Ben-Dor, S., Esterman, N., Rubin, E., and Sharon, N. (2004) Biases and complex patterns in the residues flanking protein N-glycosylation sites. *Glycobiology* **14**, 95–101
17. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta* **1473**, 4–8
18. Li, X. J., Pedrioli, P. G., Eng, J., Martin, D., Yi, E. C., Lee, H., and Aebersold, R. (2004) A tool to visualize and evaluate data obtained by liquid chromatography-electrospray ionization-mass spectrometry. *Anal. Chem.* **76**, 3856–3860
19. Deutsch, E. W., Eng, J. K., Zhang, H., King, N. L., Nesvizhskii, A. I., Lin, B., Lee, H., Yi, E. C., Ossola, R., and Aebersold, R. (2005) Human Plasma PeptideAtlas. *Proteomics* **5**, 3497–3500
20. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6940–6945
21. Perchalski, R., Yost, R., and Wilder, B. (1982) Structural elucidation of drug metabolites by triple-quadrupole mass spectrometry. *Anal. Chem.* **54**, 1466–1471
22. Pratt, J. M., Simpson, D. M., Doherty, M. K., Rivers, J., Gaskell, S. J., and Beynon, R. J. (2006) Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. *Nat. Protoc.* **1**, 1029–1043