A high-throughput (HT) comprehensive analysis approach was developed for assaying proteins directly from human plasma. Proteins were selectively retrieved, by utilizing antibodies immobilized within affinity pipet tips, and eluted onto enzymatically active mass spectrometer targets for subsequent digestion and structural characterization. Several parameters, including uniform parallel protein elution from 96 affinity pipet tips, proper buffering for on-target digestion, termination of the digestion, and MALDI matrix (re)introduction, were evaluated and optimized.

The approach was validated via parallel, high-throughput analysis of transthyretin (TTR) and transferrin (TRFE) from 96 identical plasma samples. The 96 parallel analyses for each protein were completed in less than 90 min, measured from protein extraction to insertion in the mass spectrometer. Virtually identical mass spectra were obtained from the 96 TTR analyses, characterized by the presence of 14 tryptic fragments that allowed TTR sequence mapping with 100% coverage. Database search returned TTR as the best match for all 96 data sets. In regard to the TRFE analyses, database searching using data from the 96 spectra returned TRFE as the best match for all 96 data sets. Intrinsic Bioprobes Inc., 625 South Smith Road, Suite 22, Tempe, Arizona 85281

the use in future population proteomics

Overall, the peptide fragments and large and small trypsin fragments that fell outside the window of mass analysis. Overall, the combined high-throughput affinity capture-protein digestion approach showed high reproducibility and speed and yielded an exceptional level of protein characterization, suggesting its use in future population proteomics endeavors.

The field of proteomics has been largely focused on the development of technologies and tools that enable detection and analysis of as many proteins as possible in a single analysis. Combinations of 2DE, LC, and affinity isolation, with mass spectrometry, have been successfully used for simultaneous analysis of hundreds of proteins from complex biological media.1–6

However, none of these approaches is simple or robust enough to allow for reproducible and repetitive protein analysis from hundreds to thousands of samples. Such next-phase proteomics undertaking is immediately needed to map the numerous quantitative and qualitative protein modulations, in an effort to define a "standard" for each protein and delineate what is a "normal" human proteome for a specific tissue and biological fluids. Thousands of biological samples will need to be analyzed and the range of protein concentrations, along with the most frequent posttranslational modifications or point mutations, determined and catalogued for each protein. Deviations from the "norm" can then be readily delineated, and, when analyses are performed with disease-associated pool of samples, those variations can be correlated with specific ailments and possibly used as biomarker indicators of disease onset and progression. While approaches such as ELISA, biosensors, and protein arrays are currently being utilized for high-throughput (HT) quantitative protein analysis, an equivalent methodology capable of structural (or both structural and quantitative) HT protein analyses does not yet exist.

We have in the past developed two individual technologies, mass spectrometric immunoassay (MSIA)7,8 and bioreactive mass spectrometer probes (BRPs),9,10 that, when put together, can lead...

[References: 1-10]
to a comprehensive functional and structural analysis of proteins from complex biological samples. M SIA utilizes microliter-volume affinity capture within pipet tips to selectively retrieve proteins from biological solutions, which are then eluted onto BRPs for subsequent digestion via surface-immobilized enzymes. The MS analysis of the resulting peptide fragments reveals structural features (i.e., modifications) of the protein sequence, resulting from posttranslational modifications, point mutations, and even splice variations. The combined approach can also be used for functional protein screening and protein interaction analyses, and quantitation can readily be performed by incorporating internal standards into the M SIA process.10,11

The feasibility of the combined M SIA–BRP approach has already been demonstrated, but on a small scale, in manual mode, and with limited experimental conditions.10,12 Nevertheless, fast and sensitive protein analysis from start (biological media) to finish (computer analysis of the peaks in the mass spectra) was achieved, confirming the benefits of combining the two technologies into one concerted protein characterization approach. The focus of this work is demonstration of the high-throughput combination of affinity protein capture, elution, and on-target enzymatic processing, optimization of critical execution parameters, and evaluation of the overall speed and the reproducibility of the parallel analyses.

EXPERIMENTAL SECTION

Materials. 11′-Carbonyldimidazole-activated affinity pipet tips were prepared and derivatized with anti-transferrin (TRFE) antibodies (DAKO), as previously described.13 Trypsin-derivatized MALDI targets (BRPs) were manufactured as described elsewhere,14 with the exception of using sequencing grade modified trypsin from Promega (Catalog No. V5111), and the omission of the final TRIS-HCl coating step. A single large-volume human plasma sample was obtained from ProMedDX (Norton, MA), diluted 10-fold with HBS physiological buffer (HEPES-buffered saline, 10 mM HEPES, pH 7.4, 150 mM NaCl), and aliquoted into 96-well microtiter plates (200 µL into each well).

Methods. Parallel processing of 96 plasma aliquots from start to finish (i.e., from affinity capture of the target protein in the affinity pipet tips to elution onto the BRP) was performed under robotic control of Beckman Multimak 96 pipetting workstation. The protein extraction process consisted of several repetitive 150-µL aspiration and dispensing steps through 96 antibody-derivatized affinity pipet tips mounted on the robotic head, in the following sequence: HBS buffer (10 times), 10-fold diluted plasma samples (70 times), HBS (10 times), H2O (5 times), acetonitrile–2M ammonium acetate mixture (1:3 v/v, 10 times), and H2O (2 × 10 times). After the final water rinses, the affinity pipet tips were "normalized" using an equilibrating rinse with acetone (a single 150-µL aliquot was aspirated and dispensed through each tip), resulting in complete drying of the porous supports inside the affinity pipet tips. For elution of the captured proteins, 6 µL aliquots of MALDI matrix (saturated aqueous solution of α-cyano-4-hydroxy-cinnamic acid (ACCA), in 33% (v/v) acetonitrile, 0.4% (v/v) trifluoroacetic acid, TFA) were aspirated into each affinity pipet tip and, after a 10-s time delay, dispensed directly onto a 96-well formatted trypsin-derivatized BRP. Following drying, one 10-µL aliquot of 25 mM TRIS, pH 9.1 (with or without 1 mM DTT) was added to each of the 96 spots. Digestions were carried out at a temperature of 50 °C in a humidified enclave. To keep the samples solvated, one 10-µL aliquot of water was added to each spot at ~15 min into the digestion. Digestions were terminated after 25 min by air-drying the BRPs. The sample spots were rehydrated with 5 µL aliquots of 0.8% TFA and allowed to air-dry again. Following matrix recrystallization, MS analysis was performed on a Bruker Biflex III MALDI-TOF mass spectrometer in a reflectron mode using an instrument setting of full accelerating potential of 19.35 kV, an ion mirror voltage of 20 kV, drawout pulse voltage of 2.7 kV, and a 600-ns delay.

RESULTS AND DISCUSSION

Process Optimization. Several parameters were critical to the execution of 96 assays in a high-throughput mode (Figure 1). The first is parallel and uniform protein elution from all 96 affinity pipet tips on the pipetting workstation. During preliminary studies it was observed that ~5% of the affinity pipet tips repeatedly failed to adequately elute/stamp onto the MALDI targets. These failures were attributed to different amounts of residual water present in the tips after the final water rinse, which, due to the slight differences in capillary action, resulted in inconsistencies in the amount of matrix eluant solution drawn into each tip. Hence, a final equilibrating rinse that either completely dried or wetted the affinity pipet tips was needed to achieve uniform matrix aspiration and elution. The tips were exposed to a single pass (150 µL) of either acetone (rapidly evaporating and leaving the tips dry) or 10 mM n-octylglucosid (NOG, uniformly wetting the tip surfaces).

In each instance, uniform matrix aspiration and elution was achieved in the subsequent step. Furthermore, both acetone and
HNO or bases were initially used as elution solutions (e.g., TFA, HCl, can be broken at extreme pH (i.e., pH with the MALDI process. As most antibody destroy the enzymatic activity of the BRP or ultimately interfere of introducing elution compounds into the analysis that will elution solution. Major concern regarding this parameter was that compounds did not have a negative influence on the enzymatic activity, and the matrix could be readily buffered up to the pH optimal for trypsin digestion. Moreover, the matrix had the apparent effect of denaturing the eluted proteins, yielding highly reproducible digests. The elution with the matrix proved to be beneficial in one more way: the matrix molecules served as a good indicator of the pH of digestion, because at neutral pH (7–9, the pH of trypsin digestion) the ACCA matrix molecules had yellow appearance.

The third variable in the high-throughput analysis was that of protein eluate buffering. The matrix/protein eluates had a pH ~2 (the pH of the matrix solution) and needed to be buffered up to pH ~8 for optimal trypsin digestion. In past work, an approach was taken where TRIS was applied and dried onto the BRP to compensate for the low pH of the matrix eluate and adjust the mixture to pH ~8. In this work, however, we found that the reverse process was more effective. By first drying the matrix/protein eluates and then rehydrating/buffering with TRIS, the amount of TRIS needed for efficient buffering was significantly reduced, due to the evaporation of residual TFA in the eluates. The reduced amount of buffer also benefited the MS acquisition process and resulted in improved signal quality in the mass spectra. For the digestion of TRFE, which contains 19 disulfide bonds, 25 mM TRIS containing 1 mM dithiothreitol (DTT) was used for rehydration/buffering and disulfide reduction. This concentration of DTT was effective in reducing all of the disulfides and was readily tolerated during the MALDI-TOF MS.

The final parameters that needed optimization were the digestion termination conditions and MALDI matrix (re)introduction. It was found that highly consistent and homogeneous samples sites were obtained only when the TRIS/matrix/protein digest mixture was first air-dried and then rehydrated with 5xL aliquots of 0.8% TFA. The addition of surplus, volatile TFA converted the already present matrix molecules on the sample spots from the salt form (at neutral pH) to the MALDI-desired acid form. This conversion was monitored by the disappearance of the neutral pH yellow color of the ACCA. On the other hand, rehydration with matrix solution resulted in overall matrix excess on the sample spots and yielded lower quality mass spectra.

The next step of the process optimization was selection of the elution solution. Major concern regarding this parameter was that of introducing elution compounds into the analysis that will destroy the enzymatic activity of the BRP or ultimately interfere. The dry (acetone) rinse was selected for use in all subsequent experiments.

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All 96 assays were completed in less than 90 min, measured from protein extraction with anti-TTR affinity pipet tips to insertion in the mass spectrometer. High-resolution spectra (m/z > 5000) in the range of 1000–4000 Da were acquired from each sample spot. Figure 2 shows eight mass spectra obtained from column 4 of the BRP plate (A4–H4), and Figure 3 shows an expanded view of a single spectrum obtained from sample spot A4 (the other 88 spectra can be found in Supporting Information). Database search using Profound (with monoisotopic mass tolerance of 150 ppm, maximum of four missed cleavages, and all other parameters including species left wide open) returned human TTR as the best match for all 96 data sets. For the A4 sample, TTR was matched with a probability score of 1.0e+000 and a Z-score of 2.41; the next closest match had a probability of 1.5e-022, with no Z-score. A second pass search did not yield any significantly matched proteins. Characteristic of all 96 spectra is the presence of 14 tryptic fragments (labeled with * in Figure 3) that allowed TTR sequence mapping with 100% coverage (Figure 3). All 96 spectra contained an additional signal that corresponded to a cysteinylated peptide fragment shifted by +119 Da from its wild-type analogue (residues 1–15, peptide fragment T16). TTR contains a single cysteine (residue 10), which is partly cysteinylated in vivo and

**Figure 2.** Transthyretin analysis. Shown are eight mass spectra obtained from column 4 (A4–H4) of the trypsin BRP, following the affinity retrieval of transthyretin from human plasma, elution, and on-probe digestion.
more extensively so in plasma samples stored for a prolonged period of time.

**HT Analysis of TRFE.** To show the applicability of the approach to the analysis of disulfide cross-linked proteins, parallel, high-throughput analysis of transferrin from 96 identical plasma sample aliquots was performed. All protocols used in sample preparation and digestion remained the same as those used in the TTR mapping, with the exception of fortifying the 25 mM TRIS digestion buffer with 1 mM DTT in order to reduce the 19 disulfide bonds in TRFE prior to trypsin digestion. High-resolution mass spectra (m/z > 5000) were obtained in the 1000–4000-Da mass range from all 96 sample spots. Eight of the 96 spectra are shown in Figure 4 (column 7 on the BRP, A7–H7), and an expanded view of the spectrum obtained from sample spot B7 is shown in Figure 5 (the other spectra are included in the Supporting Information). Database search using Profound (with the same parameters as for the TTR analyses) returned human TRFE as the best match in all but one spectrum (C7). For the B7 sample shown in Figure 4, TRFE was matched with a probability score of 1.0e+000 and a Z-score of 2.4; the next closest match had a probability of 3.2e-075 with no Z-score. A second pass search did not yield any significantly matched proteins. Overall, TRFE was mapped with 47–69% sequence coverage in all but one spectrum (the C7 sample, which yielded 27% sequence coverage). For the B7 sample, 69% sequence coverage was achieved with 75 (out of the 132) signals observed in the mass spectrum (Figure 5 inset). The gaps in the sequence coverage correspond to the carbohydrate containing regions of the TRFE (residues 381–433 and 603–623) and large (residues 51–88, M W = 3953.010, residues 149–193, M W = 4644.961) and small (residues 117–124, M W = 829.386) trypsin fragments that fell outside the window of mass analysis.

**CONCLUSIONS**

The results presented in this paper illustrate a novel, high-throughput approach for parallel protein analysis, characterized by high reproducibility and unprecedented extent of structural protein characterization. The approach has significant potential as a protein phenotyping method for small-to-moderate sized proteins (with application toward point mutation and PTM analyses) and can readily be applied to the analysis of large, disulfide-bridged glycoproteins. The assays for transthyretin and transferrin developed in this work can immediately be applied to
plasma samples screening, as each protein contains structural modifications that have been associated with a number of ailments. Over 80 different point mutations in transthyretin have been reported, most of which lead to late onset autosomal dominant disorders due to amyloid deposition on the peripheral nerves or the heart. On the other hand, carbohydrate-deficient transferrin (lacking one or both of the N-linked glycans) occurs with high prevalence in chronically increased alcohol consumption and is widely used for laboratory diagnosis of chronic alcohol abuse. Other assays targeting variety of plasma proteins can also be applied to such plasma screening and can be performed in parallel to minimize both the sample and time requirements. Ultimately, this throughput affinity capture-protein digestion approach will find use in larger population proteomics endeavors, where samples from hundreds of individuals will be rapidly analyzed, sets of proteins characterized, and a database of structural modulations established for each protein. Such undertaking would represent a new direction in the field of proteomics research, with significant clinical and diagnostic implication thereafter.

ACKNOWLEDGMENT

This project has been funded in part with Federal Funds from the National Institute of Environmental and Health Sciences, under Contract N43ES-25491. This publication was also supported in part by Grant 4 R44 CA99117 from the National Cancer Institute, National Institutes of Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

SUPPORTING INFORMATION AVAILABLE

All 96 mass spectra from the TTR and TRFE analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review September 19, 2003. Accepted January 9, 2004.

AC035105+