High Throughput Peptide Mass Fingerprinting and Protein Macroarray Analysis Using Chemical Printing Strategies*

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We describe a chemical printer that uses piezoelectric pulsing for rapid, accurate, and non-contact microdispensing of fluid for proteomic analysis of immobilized protein macroarrays. We demonstrate protein digestion and peptide mass fingerprinting analysis of human plasma and platelet proteins direct from a membrane surface subsequent to defined microdispensing of trypsin and matrix solutions, hence bypassing multiple liquid-handling steps. Detection of low abundance, alkaline proteins from whole human platelet extracts has been highlighted. Membrane immobilization of protein permits archiving of samples pre-/post-analysis and provides a means for subanalysis using multiple chemistries. This study highlights the ability to increase sequence coverage for protein identification using multiple enzymes and to characterize N-glycosylation modifications using a combination of PNGase F and trypsin. We also demonstrate microdispensing of multiple serum samples in a quantitative microenzyme-linked immunosorbent assay format to rapidly screen protein macroarrays for pathogen-derived antigens. We anticipate the chemical printer will be a major component of proteomic platforms for high throughput protein identification and characterization with widespread applications in biomedical and diagnostic discovery. Molecular & Cellular Proteomics 1: 490–499, 2002.

The ability to accurately define protein expression in relationship to physiological changes associated with healthy or diseased states and the potential to discover novel drug targets are emerging themes of proteomic programs (1, 2). Understanding these dynamics is rendered complex given there is often no correlation between mRNA expression levels and protein expression (3), and the paradigm of one gene-one protein is known not to hold (4).

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* The abbreviations used are: 2-DE, two-dimensional gel electrophoresis; BSA, bovine serum albumin; CFR, curved field reflectron; ESI, electrospray ionization; FITC, fluorescein isothiocyanate; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; OGP, octyl D-glucopyranoside; PBS-TAC, phosphate-buffered saline containing 0.1% (v/v) Tween 20, 0.05% (w/v) NaN₃, and 0.5% (w/v) casein, pH 7.4; pmf, peptide mass fingerprinting; PSL, Proteome Systems Limited; PVDF, polyvinylidene fluoride; TB, tuberculosis.

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A Western blot of 2-DE separated proteins onto a membrane such as polyvinylidene fluoride (PVDF) or nitrocellulose represents a protein chip, albeit a macroarray. The protein macroarray differs from a protein chip microarray, because the coordinates of each protein are determined by the physical attributes of the isoelectric point and apparent molecular weight of the protein. Once the coordinates of each protein within the protein macroarray are identified by an image-capture device, each protein spot then has a defined X and Y position and can be manipulated by robotic platforms. Here we present technology that combines the advantages of both protein chips and 2-DE, which we have described as a chemical printer (Fig. 1). The chemical printer uses a microjet device utilizing piezoelectric drop-on-demand-type ink-jet technology for rapid liquid microdispensing (31–33). The ability of ink-jet printing to dispense minimal amounts of rare fluids and permit parallel processing of large numbers of tests means assay sizes can be decreased whereas assay density is increased (34, 35). These qualities have lead to increased applications for dispensing of bioactive materials. Immunodiagnostics and antibody/antigen dispensing (36), synthesis and deposition of oligonucleotides in microarray formats (29, 38, 39), protein and peptide analysis (40), and drug discovery (41) are a number of applications utilizing ink-jet printing technologies. We demonstrate in situ proteinase digests of membrane-immobilized protein macroarrays and subsequent MALDI-TOF MS analysis directly from the membrane surface. This approach bypasses the multiple liquid-handling steps associated with in-gel digestion procedures. Importantly, the ability to analyze immobilized proteins allows for both archiving of samples pre-/post-analysis and for multiple chemical reactions to be performed at different locations on an individual protein spot. We demonstrate identification of N-linked glycosylation sites by MALDI-TOF MS using sequential PN-Gase F and trypsin digestion, as well as preparation and extraction of oligosaccharides from PVDF membrane for structural analysis by LC-ESI MS.

An exciting new high throughput assay also uses the chem-
ical printer to microdispense antibodies onto membrane-immobilized proteins to rapidly define immunoreactivity and quantitate signals in a solid phase enzyme-linked immunosorbent assay-like format. The chemical printer thus represents a powerful tool for identification of novel protein targets for biomedical and diagnostic purposes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Standard laboratory chemicals were obtained from Sigma unless specified otherwise. Human serum and plasma samples and 38 kDa tuberculosis (TB) protein were gifts from AP Clinical (Sydney, Australia). Where stipulated in the text, human serum albumin was depleted from plasma using methods described previously (42, 43). Human platelets were purchased from the Red Cross Blood Bank (Sydney, Australia). Contaminating red blood cells were removed from the platelets by centrifugation at 200 × g for 10 min at 4 °C. The platelet-rich plasma was then centrifuged at 1500 × g for 20 min at 4 °C. The platelet component of the pellet was removed gently and then resuspended in 50 mM Tris-HCl, 5 mM EDTA, pH 7.4. The platelets were washed similarly two more times. Whole platelets were finally solubilized using a ProteoPrep™ sample extraction kit (Sigma) using the supplied cellular and organelle membrane solubilizing reagent, to a final protein concentration of 4 mg/ml. Purified immunoglobulin was obtained from CSL (Parkville, Australia).

**Chemical Printing**

Solutions were dispensed using an α-version chemical printer being developed by Proteome Systems Ltd. (PSL) (Sydney, Australia) in collaboration with Shimadzu Biotech (Kyoto, Japan). Solutions were pre-filtered through either 0.22- or 0.45-μm membrane filters (Millipore). Glass capillary piezoelectric microjet devices (Microfab Technologies, Inc., Plano, TX) were used to dispense all solutions. X and Y coordinates of target protein spots were determined using ImagepIQ™, an in-house image-capture product (PSL).

**Two-dimensional Gel Electrophoresis**

**Sample Preparation**—36 μl of whole human plasma was made up to a final volume of 490 μl in 7 m urea, 2 m thiourea, 2% (w/v) CHAPS,
and 5 mM Tris, pH 10.2. The sample was then ultrasonicated for 30 s, reduced with 3 mM tributylphosphine for 2 h, and then alkylated with 15 mM iodoacetamide for 1 h. Before rehydration of immobilized pH gradient strips, sample was ultrasonicated for 2 min and then centrifuged at 21,000 × g for 5 min. The supernatant was collected, and 10 μl of Orange G was finally added as an indicator dye. Sample pre-fractionation into narrow range pI fractions was performed with an ElectrophoretIQ™ multicompartment electrolyzer (8) (PSL).

First Dimension—Dry 11-cm immobilized pH gradient strips (Amersham Biosciences) were rehydrated for 6 h with 200 μl of protein sample. Rehydrated strips were focused on a Protean IEF cell (Bio-Rad) or PSL prototype IsoElectrIQ™ electrophoresis equipment for 120 kV-h at a maximum of 10 kV. Focused immobilized pH gradient strips were equilibrated for 20 min in 6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl, pH 7.0.

Second Dimension—Equilibrated strips were inserted into loading wells of 6–15% (w/v) polyacrylamide ProteoGel™ (Sigma) and the conditions described above. Proteins were finally electrotransferred onto an Immobilon P™ PVDF membrane as described above.

On-membrane Protein Digestions
Immobilon P™ PVDF membranes were first adhered to an Axima-CFR MALDI-TOF target plate (Kratos, Manchester, United Kingdom) using 3M™ electrically conductive tape 9703 (St. Paul, MN). Porcine FIG. 3. On-membrane tryptic digestion of low abundance, alkaline whole-platelet proteins. Reduced and alkylated whole-platelet proteins were separated by 2-DE, pl 3–10, and then electroblotted onto an Immobilon P™ PVDF membrane. Proteins were visualized by staining with Direct Blue 71. The blot was then adhered to an Axima-CFR MALDI-TOF MS plate as described previously. On-membrane tryptic digestion using the chemical printer and subsequent MALDI-TOF MS analysis directly from the membrane surface were then performed as described under “Experimental Procedures.” Peptide mass analysis for the six proteins (spots 15–20) is shown in Table I.

### Table I

Comparison of pmf analysis of proteins identified from on-membrane versus in-gel tryptic digestions

Protein spots 1–14, as shown in Fig. 2, and spots 15–20, as shown in Fig. 3, represent a range of proteins present in variable amounts as indicated by Direct Blue 71 staining intensity in human plasma and platelets, respectively. A comparison of pmf results for these proteins, digested either in-gel or on a PVDF membrane surface, is shown. Peptide hits include peptides containing carboxyamidomethylated cysteine and oxidized methionine modifications.
TABLE II

<table>
<thead>
<tr>
<th>BSA amount (fmol)</th>
<th>Approximate amount of BSA digested (fmol)</th>
<th>Peptide hits</th>
<th>% Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500</td>
<td>250</td>
<td>14 ± 4</td>
<td>26.8 ± 5.9</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>20 ± 5</td>
<td>34.4 ± 8.0</td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td>13 ± 2</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>250</td>
<td>25</td>
<td>10 ± 3</td>
<td>19.8 ± 6.5</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>9 ± 3</td>
<td>17.5 ± 6.1</td>
</tr>
</tbody>
</table>

Chemical Printing for Analysis of Protein Macroarrays

**In-gel Tryptic Digestion**

Protein gel pieces were excised using a prototype Xcite™ system (PML and Shimadzu Biotech) and then washed with 25 mM NH₄HCO₃, pH 8.5. Gel pieces were then dehydrated under vacuum for 15 min and digested with 10 μl of 20 μg/ml porcine trypsin in 25 mM NH₄HCO₃, pH 8.5, for 3 h at 37°C. Peptides were extracted from gel pieces with 10 μl of 50% (v/v) acetonitrile, 0.5% (v/v) formic acid and sonication for 10 min. Prior to MALDI-TOF MS analysis, peptides were concentrated and purified using a C18 ZipTip® (Millipore) and eluted onto a target plate in 2 μl of matrix solution and allowed to dry.

**N-Linked Oligosaccharide Release**

N-linked oligosaccharides were cleaved on the PVDF membrane, which was adhered to a MALDI-TOF plate by printing 50 × 10-nl iterations of 5 units/μl PNGase F (Roche Molecular Biochemicals) per protein spot and incubation for 3 h at 37°C in a humidified environment. Released oligosaccharides were extracted into 1 μl of water by pipette for LC-ESI MS analysis. The membrane was then washed with water using a transfer pipette. PNGase F-treated protein was subsequently digested on the membrane with trypsin using chemical print followed by MALDI-TOF MS printing.

**LC-ESI MS**

Sample was loaded onto a ThermoHyprosil 5-μm Hypercarb column (Keystone Scientific Operations, Bellefonte, PA) using a Surveyor autosampler connected to an LCO Deca mass spectrometer (ThermoFinnigan, San Jose, CA). Oligosaccharides were separated and eluted using a 30-min 0–25% (v/v) acetonitrile gradient in 10 mM NH₄HCO₃. Tandem MS analysis was performed in negative ion mode over a m/z range of 320 to 2000 Da. Oligosaccharide structures were predicted using the GlycoSuite™ database (PSL).

**Antigen Screening**

Human serum was diluted 1:3 with phosphate-buffered saline containing 0.1% (v/v) Tween 20, 0.05% (v/v) NaNO₃, and 0.5% (v/v) casein, pH 7.4 (PBS-TAC), and then filtered through a 0.22-μm membrane (Millipore). Five applications of 10 ml of serum were printed onto proteins immobilized on a nitrocellulose membrane after blocking nonspecific binding sites with PBS-TAC for 15 min at room temperature. The membrane assay area was clamped onto a layer of absorbent tissue paper to prevent dispersal of fluid over the membrane surface during microdispensing. Excess antibody was removed by washing twice with 2 × 50-μl drops of PBS-TAC using a transfer pipette. Bound antibody was detected by pipetting 20 μl of goat anti-human IgG conjugated to fluorescein isothiocyanate (FITC) (Zymed Laboratories Inc., San Francisco, CA) diluted 1/10 with PBS-TAC. Fluorescence was detected using a Bio-Rad FluorS™ system.

**RESULTS**

Microdispensing Trypsin for High Throughput On-membrane pmf Analysis—Conventional pmf analysis of purified proteins involves isolation of individual protein spots from a gel (or membrane) followed by in-gel tryptic digestion, peptide extraction, peptide clean up, and finally loading the extracts onto a MALDI-TOF target (46). As an alternative rapid high throughput approach, we demonstrate dispensing of trypsin onto a macroarray of human plasma proteins on a PVDF membrane that had been adhered to a MALDI-TOF target plate (Fig. 2A). The most efficient digestion conditions were achieved by jetting 200 μg/ml trypsin in 50 × 1-nl iterations onto each protein spot. The small amount of drying time between each iteration increased the digestion efficiency by preventing excessive diffusion of trypsin solution across the membrane surface. Digestion sites on the hydrophobic PVDF membrane surface were pre-wet by printing either 4.5 nl of the non-ionic detergent n-octyl β-D-glucopyranoside or 5 nl of PVP40. This also prevented nonspecific binding of proteinase to the membrane. After digestion, matrix solution was dispensed directly onto the tryptic peptides prior to MALDI-TOF MS analysis directly from the membrane surface. Fig. 2A illustrates the relative size of the digestion zones (~200–
300-μm diameter). Of 14 protein spots representing a range of proteins present in various amounts (Fig. 2A) all were identified successfully by pmf analysis after on-membrane digestion (Table I). Fig. 2B shows a representative spectrum of apolipoprotein E generated after on-membrane digestion. Detection of low abundance proteins is often a limitation in proteomic studies. Furthermore, alkaline proteins are even more difficult to identify given the difficulty in focusing such highly positive charged proteins. For this reason we have analyzed several low abundance alkaline platelet proteins to demonstrate that identification of such proteins is achievable using on-membrane digestion methods with the chemical printer (see Fig. 3 and Table I). Positive identification of nucleoside diphosphate kinase B (Table I) was confirmed following post-source decay analysis of the 1175.76 ion (data not shown).

The sequence coverages from peptides generated by on-membrane digestion were usually less than those from in-gel digests (Table I). This was not unexpected given the in-gel digests were each purified using a C18 ZipTip®, and the excised area of the in-gel digest was 1.2 mm in diameter, more than five times the area digested on-membrane. In some cases increased numbers of peptide hits were observed off the membrane, but lower sequence coverages were obtained relative to in-gel digests. This was a result of the higher abundance of smaller peptides extracted from the membrane surface (Table I). Nevertheless, with respect to sensitivity of peptide detection, analysis of on-membrane tryptic digests of...
BSA demonstrated that a targeted protein amount of 10 fmol of BSA was still sufficient for generating pmf data that enabled reliable protein identification (Table II).

**Multiple Chemistry Subanalysis of PVDF Membrane-immobilized Proteins**—The ability to dispense small volumes of reagents to specific locations permitted multiple chemical analyses of a single protein spot. Here we demonstrate release of N-linked oligosaccharides from human α1-antitrypsin after chemical printing and on-membrane digestion with the endoglycosidase PNGase F. Released oligosaccharides were subsequently extracted from the membrane surface. LC-ESI MS analysis of these extracted oligosaccharides identified four different carbohydrate moieties on α1-antitrypsin (Fig. 4A). Deglycosylation of Asn results in deamidation converting the Asn to an Asp and consequently increasing the m/z of the parent protein by 1.0 Da. This enables identification of sites of N-linked glycosylation following tryptic digestion and pmf analysis. This study revealed one of three predicted peptides, peptide 70–101, with an observed m/z 3692.8 Da, implicating Asn-83 as an N-linked glycosylation site (Fig. 4B). This is the largest of the expected tryptic peptides of α1-antitrypsin, which contained N-linked glycosylation. The other two glycosylation sites occur on peptide 40–69 (predicted m/z 3181.6 Da) and peptide 244–259 (predicted m/z 1755.9 Da). It is possible that sites Asn-46 and Asn-247 were not deglycosylated and hence not desorbed in reflectron mode. The peptide containing Asn-46 was definitely cleaved by trypsin as both peptides N- and C-terminal to peptide 40–69 were identified in the MALDI-TOF spectrum. No peptides between residues 218 and 274 were identified, so it is possible that the tryptic peptide containing Asn-247 was never cleaved.

The ability to dispense multiple enzymes for increasing protein sequence coverage, and thus increase confidence in protein identification, has also been demonstrated (Table III). On-membrane digestion of a single protein spot, apolipoprotein A1 (a different sample from that described in Table I), with both Glu-C and trypsin, resulted in sequence coverages of 41.6 and 46.1%, respectively. The combined sequence coverages represented 66.7% of the total sequence of apolipoprotein A1.

**Mapping Immunodiagnostic Markers**—From a biomedical perspective we have demonstrated the ability to identify immunoreactive pathogen-derived antigens in human plasma and simultaneously compare immunoreactivity of multiple patient sera. Sera from six different patients (a–f) were sequenced onto a macroarray of plasma proteins that had been spiked with a 38-kDa mycobacterium TB protein. The results from two protein spots (1, a human (control) protein and 2, the TB protein) are shown (Fig. 5A). Antigen recognition was determined after subsequent application of anti-human IgG conjugated to FITC in reaction times of less than 3 min. Patients d and e demonstrated strong recognition of the 38-kDa TB antigen whereas patients c and f reacted weakly. Patients a and b were TB-negative controls. These findings demonstrate how potentially new pathogen-specific antigens can be identified using patient serum antibodies. In additional studies, titrated amounts of human immunoglobulin antigen were printed onto nitrocellulose. Subsequent to detection using excess anti-human IgG–FITC-conjugated antibody, comparison of relative signal intensities versus antigen concentration demonstrated a linear relationship, thus highlighting the potential for quantitating signal responses derived from chemical printing strategies (Fig. 5B).

**DISCUSSION**

Although DNA microarrays have been heralded as a revolution in genomic studies, the role of protein microarrays in proteomic studies is not as simple, principally because of the challenges of developing high throughput strategies for preparing proteins prior to their deposition onto chip surfaces. Generally, protein arrays use recombinant proteins that are arrayed robotically onto surfaces. However, a fundamental problem with this approach is that the recombinant proteins are not unique, lacking the myriad co- and post-translational modifications that lead to protein isomers, many of which are cell type-specific.

The strategy here is to array authentic
samples using 2-DE gels as the primary protein array technology rather than robotic printing of recombinant proteins. This approach ensures that the proteins modified authentically are arrayed properly to a coordinate that is determined by the isoelectric point and apparent molecular weight of the protein. Depending on the sample preparation technique, hundreds to thousands of proteins can be arrayed using this “GelChip” approach.

The ability to miniaturize classical technologies like protein digestion and immunoblotting as a protein chip approach has been demonstrated in this report using chemical printing strategies. This printer technology can reproducibly dispense picoliter volumes of reagents to defined locations thereby permitting microscale chemical analyses. Printing is a non-contact process ensuring the fluid source is not contaminated by substrate during a printing event. Furthermore, the accuracy of dispensing is not affected by how the fluid wets a substrate as can be the case in other well established high throughput technologies where positive displacement or pin transfer systems “touch off” or stamp fluid onto a substrate during dispensing (14). The ability to free-fly fluid droplets over 0.5 mm or more allows fluid dispensing into wells or onto other substrate features such as those created by controlled wetting and chemical deposition.

Using human plasma and platelets as model systems, we have demonstrated how on-membrane tryptic digests can be generated rapidly after accurately dispensing trypsin directly onto a protein target. Adherence of the membrane to a MALDI-TOF plate during this procedure permits easy transition to the mass spectrometer for pmf analysis. The ability of chemical printing strategies to bypass the more time consuming procedures of in-gel digestion, peptide extraction, and C18 ZipTip® clean-up steps, without significantly compromising protein identification, provides both a much more rapid approach to pmf for high throughput mapping of complex protein solutions and the ability to archive samples for future analysis. Furthermore, these approaches enable identification of both high and low abundance proteins, and future studies will aim to further characterize the human platelet protein map.

The sensitivity of on-membrane digestion using the chemical printer has also been highlighted in this study by digestion
and subsequent successful pmf identification of ~10 fmol of BSA immobilized on an Immobilon P\textsuperscript{SG} PVDF membrane. This level of sensitivity is equal to, if not higher than, other reported in-gel digestion procedures (47, 48).

Another emerging technology for automated pmf analysis is the molecular scanner, which simultaneously digests and electrotransfers peptides onto a PVDF membrane (49). The strategy of the molecular scanner is not to visualize the polypeptides on the electroblot but to rapidly scan the membrane to detect the presence of peptides. The process is inefficient, because digested proteins only occupy a low percentage of the entire area that is scanned. Unlike the chemical printer, the molecular scanner cannot control delivery of different amounts of trypsin to different protein spots. Furthermore, the digestion of all of the sample on the membrane abolishes the ability to archive untreated samples. Subsequent analyses using alternate chemistries are therefore limited. The ability to archive and also subject a protein to multiple analyses are advantages of the chemical printer approach. Furthermore, the option to specifically select individual proteins for subanalysis using multiple chemistries increases both time efficiency and conservation of valuable reagents and samples.

Protein transfer onto a membrane is an inherent component of the experimental strategies presented here. Importantly, we have found routinely that protein transfer of a specific sample onto a particular membrane type is a highly reproducible process. However, it must be appreciated that buffer conditions should be optimized for analysis of a particular proteome (50). The studies presented here have used the transfer conditions described by Kyhse-Andersen (44) that were optimized for proteins with molecular mass less than 200 kDa and pl 4–7. Thus, other buffer systems, as well as membrane types, should be considered if particular proteins with specific characteristics, such as size, antigenicity, pl, or hydrophobicity are required for analysis (51).

Although other studies have demonstrated remarkable examples of large scale protein identification (52), the ability for multiple analyses on a specific protein(s) within the same sample, such as mapping post-translational modifications, is limited in these approaches. We have demonstrated successful on-membrane digestion by sequentially delivering PNGase F and trypsin for mapping sites of N-glycosylation on human \alpha\textsubscript{1}-antitrypsin. With the increasing interest in post-translational modifications, particularly glycosylation (37), this technology also represents a powerful tool, particularly when coupled with LC-ESI MS, for structural analyses of oligosaccharides released from a solid-phase membrane. Furthermore, the ability to analyze a single protein spot using multiple endoproteinases has demonstrated the ability to increase markedly the sequence coverage of a protein, thus increasing confidence of a successful identification. In principle it should be possible to achieve complete characterization of proteins with such an approach. This is a significant advantage, particularly when considering characterization of low abundance proteins or proteins that contain minimal cleavage sites for a single enzyme.

One of the major outcomes of the proteomic era will be the identification of novel protein targets useful for biomedical applications, particularly in the diagnostic arena. In this study we have printed nanoliter volumes of serum from different patients onto individual plasma protein spots of a macroarray in a quantitative miniaturized enzyme-linked immunosorbent assay format. Unlike Western blotting protocols practiced currently that routinely require at least 3 h per analysis, rapid dispensing of multiple antibodies can be used to screen for antigens in several minutes. Using chemical printing, Western blotting becomes a rapid and quantitative user-independent technology. We have thus demonstrated one strategy that permits mapping immunoreactive antigens using human sera for subsequent identification by pmf analysis with implications for identifying vaccine and diagnostic targets.

Understanding the dynamic and complex nature of the proteome of an organism will require the development of high throughput multi-tasking proteomic platforms to cope with this enormous task. The chemical printer represents a technology that can function as a component of a proteomic platform working in conjunction with the powerful resolving tools of 2-DE and MS or as a standalone work station.

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