

Planar Functionalized Surfaces for Direct Immunoaffinity Desorption/Ionization Mass Spectrometry

Petr Pompach,^{1,2,3} Jana Nováková,¹ Daniel Kavan,^{1,2} Oldřich Benada,¹ Viktor Růžička,⁴ Michael Volný,^{2,3} and Petr Novák^{1,2,3*}

BACKGROUND: Recent studies show that the haptoglobin phenotype in individuals with diabetes mellitus is an important factor for predicting the risk of myocardial infarction, cardiovascular death, and stroke. Current methods for haptoglobin phenotyping include PCR and gel electrophoresis. A need exists for a reliable method for high-throughput clinical applications. Mass spectrometry (MS) can in principle provide fast phenotyping because haptoglobin α 1 and α 2, which define the phenotype, have different molecular masses. Because of the complexity of the serum matrix, an efficient and fast enrichment technique is necessary for an MS-based assay.

METHODS: MALDI plates were functionalized by ambient ion landing of electrosprayed antihaptoglobin antibody. The array was deposited on standard indium tin oxide slides. Fast immunoaffinity enrichment was performed *in situ* on the plate, which was further analyzed by MALDI-TOF MS. The haptoglobin phenotype was determined from the spectra by embedded software script.

RESULTS: The MALDI mass spectra showed ion signals of haptoglobin α subunits at m/z 9192 and at m/z 15 945. A cohort of 116 sera was analyzed and the reliability of the method was confirmed by analyzing the identical samples by Western blot. One hundred percent overlap of results between the direct immunoaffinity desorption/ionization MS and Western Blot analysis was found.

CONCLUSIONS: MALDI plates modified by antihaptoglobin antibody using ambient ion landing achieve low non-specific interactions and efficient MALDI ionization and are usable for quick haptoglobin phenotyping.

© 2015 American Association for Clinical Chemistry

Haptoglobin (Hp)⁵ plays an important role in scavenging of hemoglobin, which induces oxidative stress and tissue damage. Monomeric Hp consists of α and β subunits. Partial duplication of the α chain creates 2 alleles on the gene level, which results in formation of 3 Hp variants, Hp (1-1), Hp (2-1), and Hp (2-2). Phenotype 1-1 is an α -1/ β dimer, phenotype 2-1 is a mixture of an α -1/ β dimer and α -2/ β multimers, and phenotype 2-2 is an α -2/ β multimer (1, 2). Prior to the introduction of modern DNA-based approaches, haptoglobin phenotyping was used for paternity exclusion. Currently, haptoglobin phenotyping serves as a clinically relevant biomarker to predict complications in patients with diabetes mellitus (3–5). To determine Hp phenotype, several biochemical and molecular biological methods have been introduced (6–8). Mass spectrometry (MS) techniques for Hp phenotyping have been previously developed (9, 10) as well. The initial method relied on measurement of albumin-depleted human serum, but only the α 1 form of haptoglobin was successfully detected (9). To overcome this limitation, Tubbs et al. (10) developed a procedure for Hp phenotyping in which haptoglobin was enriched by affinity chromatography and both α forms were characterized using MALDI-TOF MS. This approach was successful in detecting both α forms of haptoglobin, but the number of steps and multiple affinity tips washes made this assay time-consuming and not suitable for high-throughput clinical diagnostics.

Recent developments in biological MS have enabled analytically sensitive qualitative and quantitative analyses of complex biological samples. By combining MS and immunoaffinity techniques, it is currently possible to unambiguously detect a protein antigen of interest in a few microliters of human body fluid, usually plasma or serum. One of the approaches that provide sufficiently high-throughput analysis of patient samples can be based on online coupling of MS with immunoaffinity enrichment.

¹ Institute of Microbiology, v.v.i., Czech Academy of Sciences, Prague, Czech Republic;

² Faculty of Science, Charles University in Prague, Prague, Czech Republic; ³ AffiPro, s.r.o., Mratín, Czech Republic; ⁴ BioVendor, a.s., Brno, Czech Republic.

* Address correspondence to this author at: Institute of Microbiology, Vídeňská 1083, 14220 Prague 4, Czech Republic. Fax +420-2-41062156; e-mail pnovak@biomed.cas.cz.

Received May 29, 2015; accepted August 25, 2015.

Previously published online at DOI: 10.1373/clinchem.2015.244004

© 2015 American Association for Clinical Chemistry

⁵ Nonstandard abbreviations: Hp, haptoglobin; MS, mass spectrometry; DIADI, direct immunoaffinity desorption/ionization; ITO, indium tin oxide; TCEP, tris(2-carboxyethyl)phosphine; DHAP 2,5-dihydroxyacetophenone; SEM, scanning electron microscope.

ment. Initial experiments date back to the early 1990s, when surface-enhanced laser-desorption/ionization was introduced (11). This pioneering work encouraged several research groups to develop affinity MS techniques with primary applications in clinical diagnostics. For example, immunoassay was used for selective enrichment and MS detection of snake venom in human blood (12). This assay was based on detection of myotoxin α by MALDI-TOF MS after immunoaffinity enrichment. H-myotoxin α was used as a concentration standard, providing the quantitative element of the assay. This initial concept was later extended for quantification of proteins in yeast cell lysates by addition of the isotopically labeled peptide standard to samples with subsequent hydrolysis of the antigen of interest and cell lysates by trypsin (13). The ability of this technique to quantify an analyte in human plasma was demonstrated on analysis of angiotensin (14). In parallel to MALDI approaches, electrospray-based stable isotope standards and capture by antipeptide antibodies methods were demonstrated for peptide quantification in complex digest (15). Nanoaffinity columns with antipeptide antibodies were used to specifically enrich peptides along with spiked stable-isotope-labeled internal standards of the same sequence. Selectively captured peptides were separated by reversed phase and analyzed by electrospray-ionization MS. Its potential was further extended to MALDI MS and demonstrated on identification of prostate cancer recurrence (16).

Previous approaches for modification of MALDI plates by antibodies to create affinity probes for MS were based on covalent bonding through gold coatings or an organic interlayer (17, 18). Here we present a technology that allows direct modification of MALDI surfaces by antibodies without the necessity to use any interlayer between antibody and the MALDI surface. The technique uses direct processing of MALDI surfaces by ion soft landing (19–23) in a variant recently referred to as ambient ion landing (24, 25). Soft and reactive ion landing are known surface modification techniques that can achieve unique surface functionalization (26–30). Because of low transport efficiency (31) ion landing in a vacuum seemed impractical, but Cooks' group (24) introduced the ambient ion landing approach. Simultaneously, protein ions were previously deposited by electrospray techniques on surfaces that could not be used as MALDI plates (32–35). Using ambient reactive ion landing, we have described modification of metal and metal oxide MALDI plates by titanium and zirconium dioxides for the purpose of in situ phosphopeptide enrichment prior the MALDI analyses of tryptic phosphopeptides (36, 37). Here we demonstrate that ambient ion landing can be used for permanent deposition of antibodies, which results in protein functionalization of metal and metal oxide surface, analogous to vacuum-

based soft and reactive landing (21, 32, 38). Antibody-functionalized MALDI surfaces, modified by ambient ion reactive landing, allow efficient immunoaffinity enrichment of antigen from complex samples (e.g., serum) directly on the plate. This new method is part of the previously described "laboratory on a plate" concept (38), which we now call direct immunoaffinity desorption/ionization (DIADI) MS (Fig. 1).

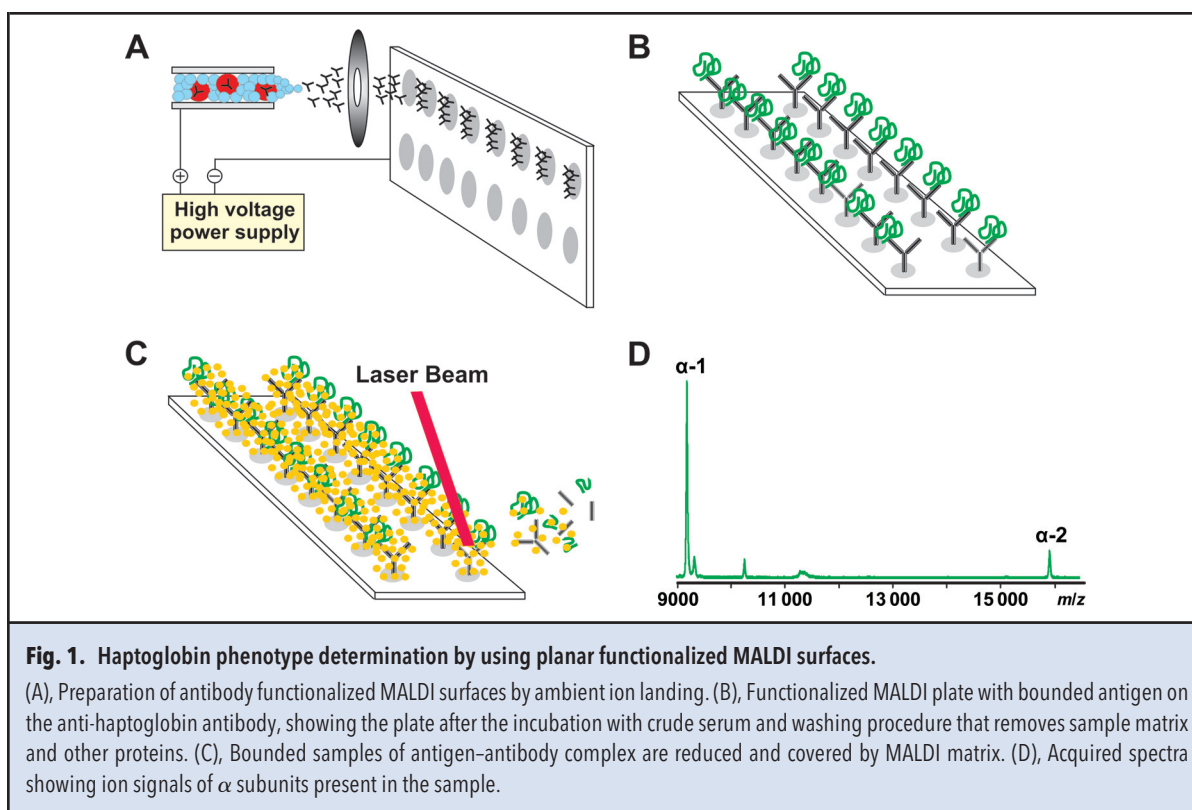
Materials and Methods

SAMPLES

Healthy white individuals were enrolled under protocols approved by the Pilsen Hospital Ethics Board. Samples of venous blood were collected using the VACUETTE blood collection system (Greiner Bioone Company). Blood was centrifuged for 10 min at 1700g and serum samples were immediately removed and frozen to -80°C . Samples were kept frozen and then thawed at room temperature before analysis.

IMMUNOAFFINITY SURFACE PREPARATION

MALDI plates were functionalized by ambient ion landing using the modified lab-made apparatus that was previously described by Krásný et al. (36). A $1\text{-}\mu\text{mol/L}$ solution of polyclonal antihaptoglobin antibody (70R-7558; Fitzgerald) in 20 mmol/L ammonium bicarbonate buffer (Sigma-Aldrich), pH 7.8, was used as an immunoaffinity modifier. The solution was delivered by the syringe pump (Hamilton) operating at a flow rate of $1\text{ }\mu\text{L/min}$. The $20\text{-}\mu\text{m}$ capillary was connected to a $20\text{-}\mu\text{m}$ -diameter nanoelectrospray emitter. A positive voltage (+1.5 kV) generated by a high-voltage power supply was applied on the liquid junction (metal union). Preheated nitrogen gas (40°C) was used as the nebulizer gas. The charged aerosol was dried by passing through the 10-cm-long desolvation tube (4-mm diameter) that was kept on ground potential and externally heated to 45°C . The dried aerosol consisted of multiply charged antibody ions presumably at different stages of desolvation. This beam of charged species was deposited on a vertically mounted indium tin oxide (ITO) glass slide (Bruker Daltonics) or homemade (dimensions are equal to the ITO slide) stainless steel slide that was kept on a high voltage of the opposite polarity with respect to the spray voltage (-1.5 kV). The apparatus for ambient ion landing was equipped with remotely controlled stepper electromotors. The movement of the stage was preprogrammed; the relevant parameters were number of rows (2) and columns (8), position coordinates, and spraying time per position (5 min, 1 min, and 20 s). These conditions resulted in antibody consumptions of 700 ng, 140 ng, and 28 ng per 1 position. Lower antibody depositions were used only to determine method limits. The array deposited on ITO/stainless steel slides had 16 positions



(2 × 8) with equivalent distance of 9 mm. The reproducible round spots of 2 mm in diameter were achieved by using an in-house-made mask from paper sticker sheet taped on the surface. The mask geometry of the 16 positional protein chips followed the standard MTP 384 Bruker MALDI plate (Bruker Daltonics). After deposition, the mask was removed and the surface was rinsed in deionized water for 5 min. The MALDI plates functionalized by anti-haptoglobin antibody using ambient ion landing were stable over time and were stored at 4 °C until used.

SCANNING ELECTRON MICROSCOPY

Functionalized ITO slides were glued by colloidal carbon onto standard aluminum stubs. The surface of the ITO slide was imaged using an annular Circular Backscatter detector (B+C sectors) on a Nova NanoSEM scanning electron microscope (SEM) (FEI) operating at 5 kV and spot size 5. To record the large area occupied by the deposited antibodies, a navigation function of the Nova NanoSEM software combining 9 × 9 frames was used.

ANTIGEN ENRICHMENT AND SAMPLE PREPARATION

One microliter of serum was applied onto the anti-haptoglobin spot on the nonrecyclable functionalized slide and incubated for 1 h at ambient temperature in a humidity chamber. After the incubation, the entire ITO slide/stainless steel slide was washed 3 times in PBS

(137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, pH 7.4) and once in water. After complete drying, 1 μ L of 50 mmol/L tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich) was added onto the spot and incubated for 30 min at ambient temperature in the humidity chamber. The MALDI matrix was prepared as follows: 7.6 mg of 2,5-dihydroxyacetophenone (Bruker Daltonics) was resuspended in 375 μ L of 100% ethanol and 125 μ L of diammonium hydrogen citrate (Sigma-Aldrich) at a concentration of 18 g/L. The matrix solution was further mixed with 2% trifluoroacetic acid in a ratio of 1:1. One microliter of the matrix solution was premixed directly on the spot with the sample and allowed to dry at ambient temperature.

MS ANALYSIS

An MTP Slide Adapter II for MALDI imaging (Bruker Daltonics) was used as a holder for the modified ITO slide/stainless steel slides. One microliter of protein calibration solution containing cytochrome C (C2506; Sigma-Aldrich) at a concentration of 0.3 pmol/ μ L, hemoglobin (H7379; Sigma-Aldrich) at a concentration of 1 pmol/ μ L, and myoglobin (M1882; Sigma-Aldrich) at a concentration of 1 pmol/ μ L was added between each sample spot on the modified ITO/stainless steel slides and covered with a 2,5-dihydroxyacetophenone (DHAP)

matrix. The Ultraflex III MALDI-TOF/TOF (Bruker Daltonics) with a smart beam laser was operated in the linear positive mode. Automated sample analysis with a randomly moving holder and accumulation of 1500 laser shots from each spot was used to acquire spectra of intact proteins. The laser frequency was set to 200 Hz, detector gain to 1620 V, sample rate to 1.0, and gating to 6000. The instrument was externally calibrated by using average ion masses of myoglobin $[M+2H]^{2+}$ at m/z 8477, cytochrome C $[M+H]^+$ at m/z 12 361, hemoglobin A $[M+H]^+$ at m/z 15 126, and myoglobin $[M+H]^+$ at m/z 16 952.

Data were processed using the centroid peak detection algorithm, signal-to-noise threshold set to 5, and TopHat baseline subtraction. A Savitzky-Golay algorithm was used for spectra smoothing (width 0.2, 1 cycle). Average isotopic peaks were annotated in the acquired spectra in flexAnalysis 3.3 (Bruker Daltonics). Script for automated data interpretation, and visualization was written in embedded SAX Basic (SaxSoftware). This script exported all results into a single spreadsheet-compatible file with colored signification of different haptoglobin phenotypes corresponding to the color scheme of spectra in flexAnalysis.

WESTERN BLOT ANALYSIS

Individual sera samples (0.25 μ L) were loaded onto SDS protein electrophoresis, which was run using a Protean III apparatus (Bio-Rad) with a constant voltage of 100 V. Separated proteins were blotted onto a nitrocellulose membrane (Pall Corporation) using a Trans-Blot SD semi-dry apparatus (Bio-Rad). Membranes with blotted proteins were blocked for 1 h in Tris-buffered saline supplemented with 5% milk (Bio-Rad) and 0.05% Tween-20 (Sigma-Aldrich). Membranes were then washed with Tris-buffered saline/0.05% Tween-20 and incubated with anti-Hp polyclonal antibody (70R-7558; Fitzgerald) and subsequently with secondary antibody horseradish peroxidase conjugate (SC-2768; Santa Cruz Biotechnology) according to the manufacturer's protocols. Finally, the membranes were visualized using PierceTM ECL Plus Western-blotting substrate (Life Technologies) and a ChemiDocTM MP system (Bio-Rad).

Results

FUNCTIONALIZED MALDI PLATES

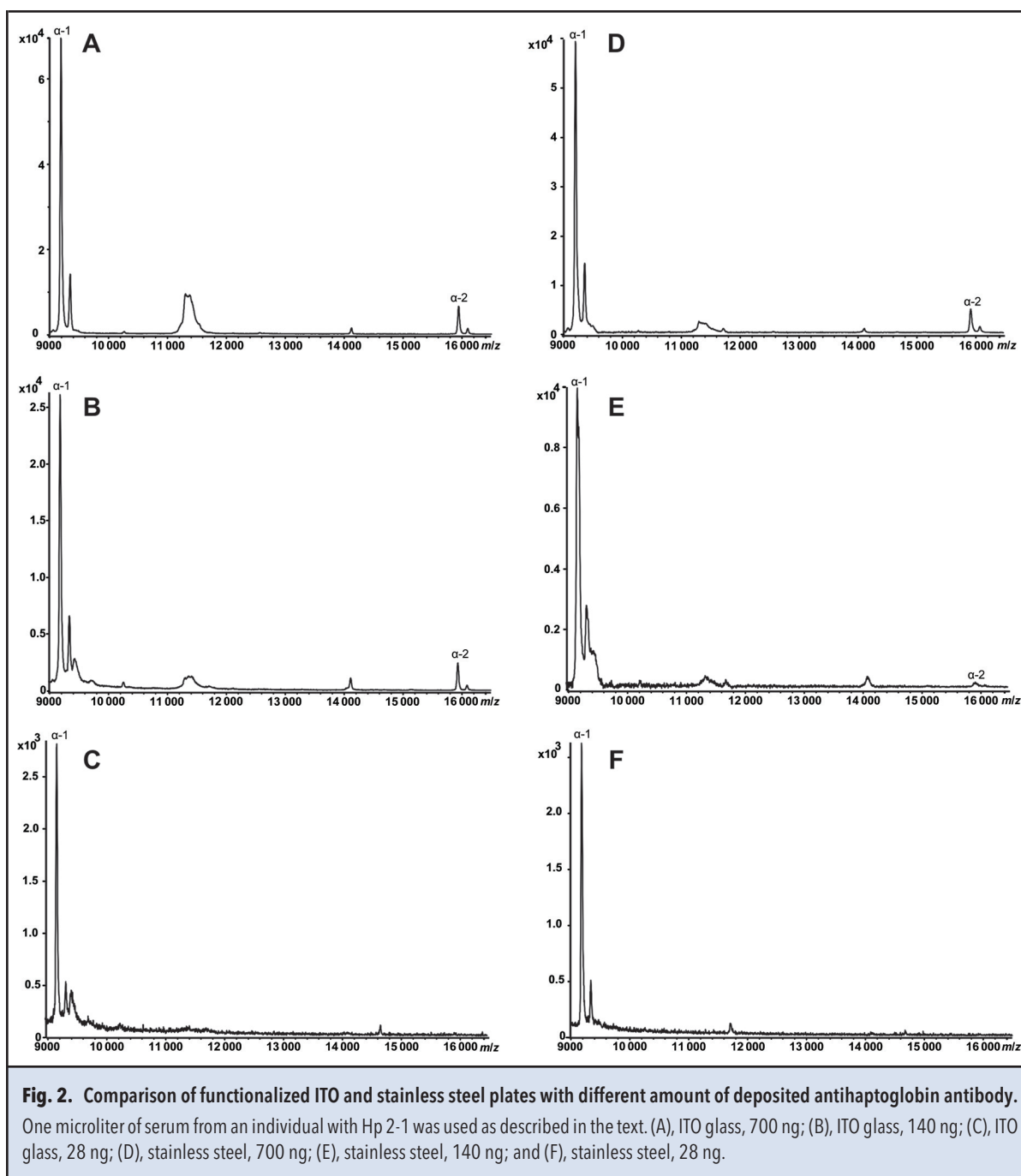
The MALDI slides were functionalized by different amounts of antihaptoglobin polyclonal antibody using the ambient ion landing procedure as described earlier. The desolvation temperature was kept under 45 °C, which still allowed for efficient solvent removal while avoiding temperature denaturation of sprayed antibody. MALDI slides made of 2 different materials were tested: ITO glass and stainless steel. The results on both types of

surfaces were comparable at high (700 ng/spot) and low (28 ng/spot) amounts of antibody deposition, but ITO slides showed better performance for moderate (140 ng/spot) amount of used antibody. Additionally, low antibody consumption (28 ng/spot) was not sufficient to detect the α -2 chain of haptoglobin (Fig. 2). Different arrays of choice could be deposited by ambient ion landing. We tested whether it was possible to deposit up to 80 positions onto an array with 4.5 mm raster on an ITO slide (75 \times 25 mm). However, the arrays used in this study were composed of 16 spots (8 \times 2 positions) with a 9-mm raster and 2-mm immunoaffinity area. MALDI calibration solution was spotted onto the available intact positions in between the modified positions of the antibody array.

To visualize the deposited antibodies on the ITO slides before and after the rinsing procedure, the surfaces modified by ambient ion landing were first analyzed by SEM. The images of sample spots were recorded using back-scattered electrons and thus no conductive sample coating was used before the SEM measurement. The areas with deposited organic material (protein) reflected fewer electrons. That is why the intensity of detected back-scattered electrons from these areas was lower than from the nonmodified areas with intact ITO slides only. The pixels that correspond to protein-modified areas are thus pictured darker in the scans in Fig. 3. Note that the intensity and abundance of dark pixels, which correspond to the presence of organic material with less intense electron back-scattering, decreased after the washing. This resulted from the partial removal of the deposited antibody from the ITO surface by washing. The SEM images proved that antibody was deposited on the ITO surface and that washing with polar solvents removed only the uppermost layers, whereas the bottom layers, which interact with the surface directly, survived the washing. This finding explains why antibody-modified MALDI surfaces maintained immunoaffinity capabilities even after the washing, in accordance with the previous literature on protein and polysaccharide reactive landing on conductive and semiconductive surfaces (29, 30).

SAMPLE PREPARATION AND MS ANALYSIS

The enrichment procedure was first optimized using serum samples from 3 donors with previously detected haptoglobin phenotypes, Hp 1-1, Hp 2-1, and Hp 2-2. The optimal volume capacity of sample applied on the spot was determined to be 1 μ L. Higher sample volumes did not keep the droplet on the spot and the liquid had a tendency to spread out from the position. A 1-h incubation time was adopted from ELISA-type assays based on the same immunoaffinity principle. Sample evaporation was avoided by placing the entire MALDI surface into the humidity chamber. After the washing procedure de-



scribed in the Methods section, the surface was rinsed in deionized water to remove salts from the PBS.

The haptoglobin molecule is composed of α subunits linked to each other and to the β subunit by disulfide bridges. To detect the α subunits by MALDI MS, it was necessary to reduce the protein after the enrichment, which was achieved by applying the TCEP reagent directly on the spots. The DHAP matrix, which was mixed

on the spot with the sample, was selected because in our experience it forms fewer adducts compared to sinapinic acid and exhibited better peak shape and ionization yield (see Fig. 1 in the Data Supplement that accompanies the online version of this report at <http://www.clinchem.org/content/vol62/issue1>). Interestingly, the DHAP matrix enabled better charging of the proteins and thus highly intense double- and triple-charged ions were observed in

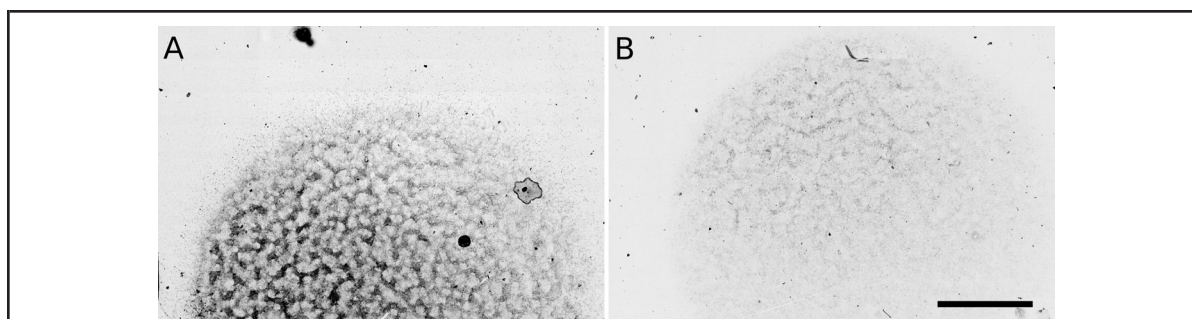


Fig. 3. Scanning electron microscopy of antibody functionalized ITO surface.

(A), SEM image of deposited antibody prior to the ITO surface being washed. (B), the surface after being washed with water-based buffer. Back-scattered electron images were taken from intact non-coated samples. Organic matter appears darker because of the lower electron scattering compare to ITO. Scale bar represents 1 mm.

the spectra (data not shown). For the haptoglobin phenotype Hp 1-1, an ion at m/z 9192, corresponding to the molecular mass of an α -1 subunit, was observed in the spectra. The singly charged ion at m/z 9192 represents the truncated form of the α -1 subunit with missing C-terminal arginine. The ion at m/z 9348 corresponds to the intact form of the α -1 subunit and is also observed in the spectra, but with much lower intensity. The spectrum of haptoglobin phenotype Hp 2-1 contains ions at m/z 9192 (truncated α -1 subunit) and at m/z 15 945 (α -2 subunit). Again, 2 forms of α -2 subunits, truncated, with missing C-terminal arginine (m/z 15 945), and nontruncated at m/z 16 102, are detected in the spectrum. The spectrum of haptoglobin phenotype Hp 2-2 contains the

dominant ion at m/z 15 945, corresponding to the truncated form of the α -2 subunit and the low-intensity ion of the full-length form (m/z 16 102). Fig. 4 shows the primary amino acid sequences of full-length forms of both α subunits and the schematic drawing of multimer formation for each phenotype. The mass spectra in Fig. 4 demonstrate the differences between individual haptoglobin phenotypes as determined by enrichment on anti-haptoglobin antibody-functionalized MALDI plates.

The assay was tested using 116 human serum samples. As a validation method, SDS electrophoresis followed by Western blot with chemiluminescent immunoassay was used to analyze the same samples (Fig. 5; also see online Supplemental Figs. 2 and 3). The same pri-

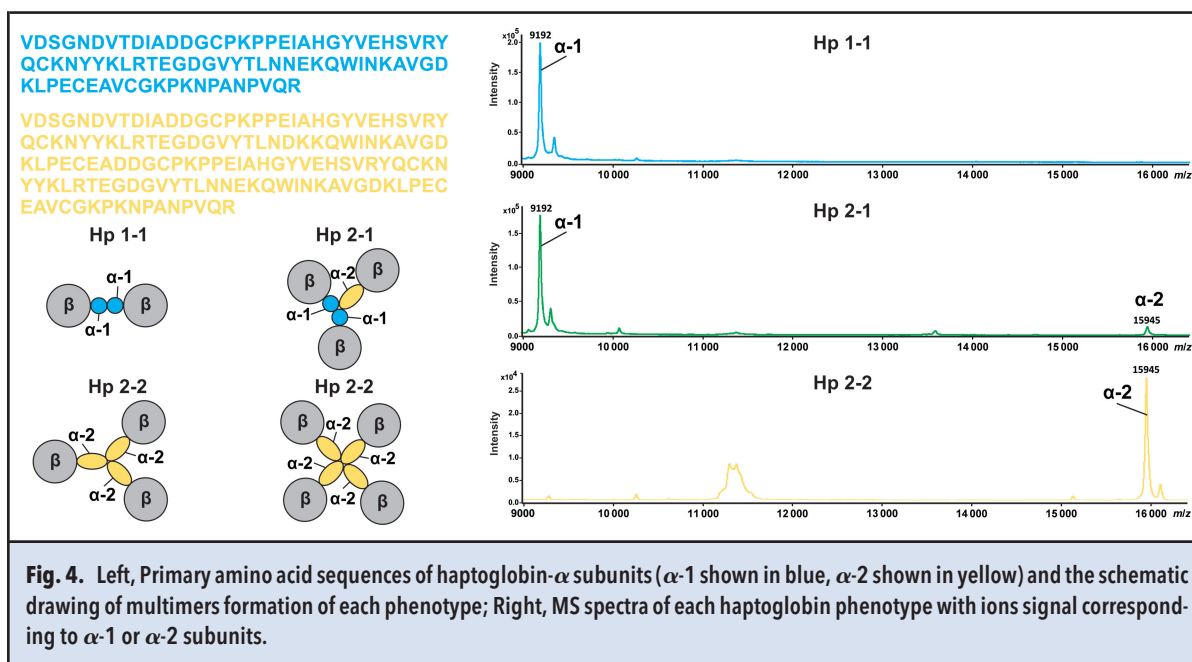
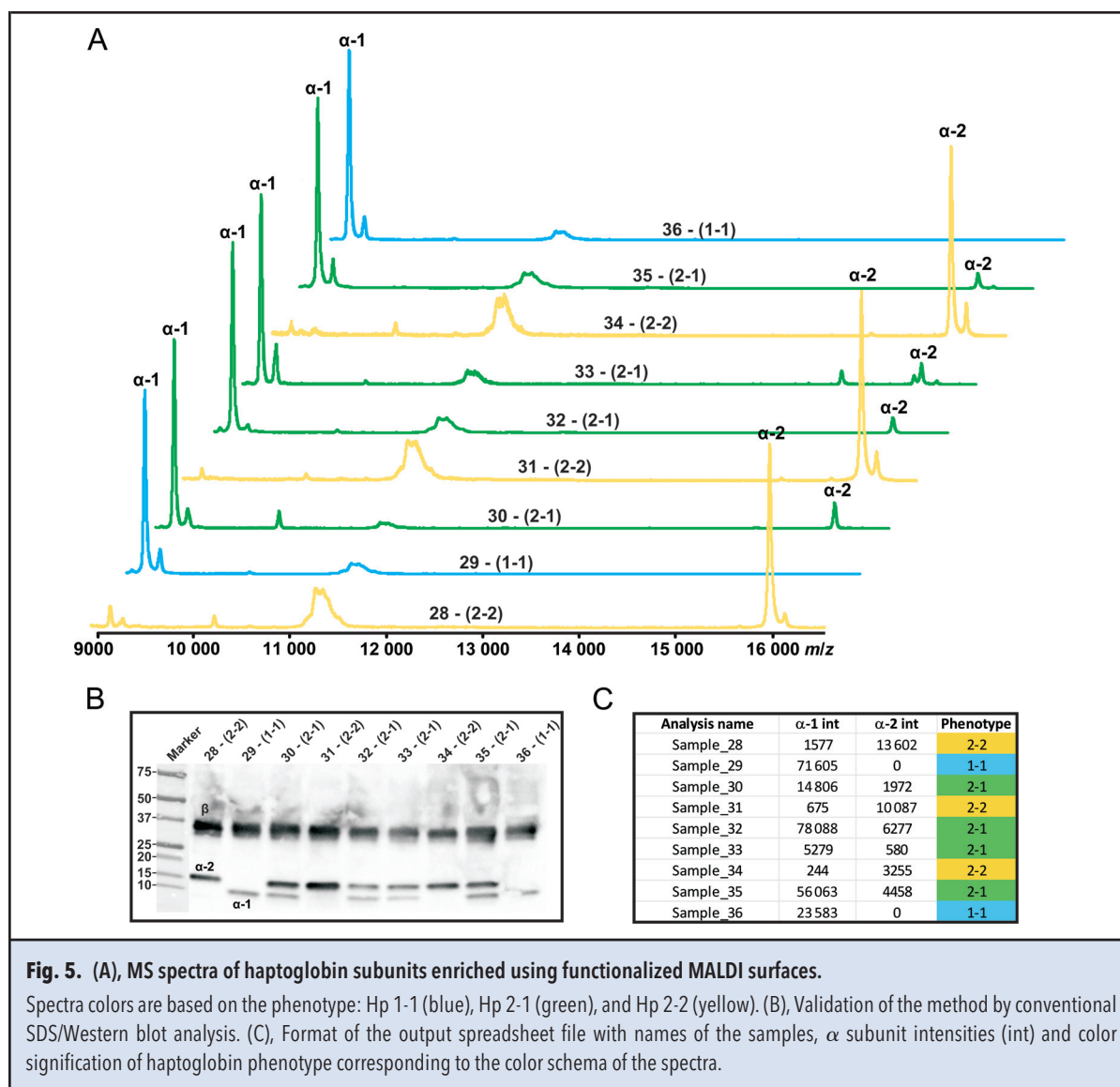


Fig. 4. Left, Primary amino acid sequences of haptoglobin- α subunits (α -1 shown in blue, α -2 shown in yellow) and the schematic drawing of multimers formation of each phenotype; Right, MS spectra of each haptoglobin phenotype with ions signal corresponding to α -1 or α -2 subunits.



mary polyclonal antibody used for MALDI plate functionalization, which detected both α subunits and the β subunit of the haptoglobin (Fig. 5), was used for Western blot as well. A SAX Basic script was implemented in the flexAnalysis processing software to output sample names, intensities of α subunits, and haptoglobin phenotypes (Fig. 5) into a spreadsheet-compatible external data file. The analysis of the 116-patient cohort found 52 individuals (44%) with phenotype Hp 2-2, 47 (41%) with Hp 2-1, and 17 (15%) with Hp 1-1, and the results obtained by Western blot were in 100% agreement.

Discussion

This work presents a new MS-based approach for determination of haptoglobin phenotype from human serum

samples. The simple work flow uses in situ immunoaffinity enrichment on antibody functionalized MALDI plates. The MALDI mass spectra of haptoglobin-α chains enriched on the MALDI plates by the specific interaction with the antihaptoglobin antibody show ion signals of α subunits at m/z 9192 and at m/z 15 945, which allows immediate and straightforward determination of the haptoglobin phenotype. Previously published data (10) showed 2 isoforms of α subunits having additional alanine residues at N-termini (m/z 9263 and 16 012) of sequences due to improper proteolysis entering the endoplasmic reticulum. Although our results are in agreement with the phenotype distribution in the northwestern European population (3), we did not observe different processing of haptoglobin entering the endoplasmic reticulum. A cohort of 116 human sera was

analyzed by the DIADI approach with no sample pre-treatment and with only minimal sample manipulation. This makes the work flow easy to automate and usable in a high-throughput or clinical chemistry environment. In this work we have demonstrated automation of data handling and reporting, and our previous report showed that enrichment on modified MALDI plates was compatible with automated sample deposition and matrix spotting (37). We validated the results obtained by the DIADI technique using standard SDS electrophoresis followed by Western blot with immunodetection. The Western blot confirmed the phenotyping determined by the DIADI-MS technique for all 116 tested samples. The success of the technique is based on use of ambient ion reactive landing for functionalization of MALDI plates by antibodies. The absence of any interlayer between the conductive MALDI surface modified by ion landing and antibody affinity molecules reduces the nonspecific interactions of other proteins in the sample and maintains the original conductivity of the MALDI plate, which provides efficient ionization.

In conclusion, the presented method for phenotyping of haptoglobin by immunoaffinity enrichment combined with MALDI MS is rapid, does not rely on external purification steps, can be automated, and consumes amounts of antibody comparable with those for standard ELISA. It has a potential not just for haptoglobin phenotyping but also for high-throughput MS determination of other clinically relevant antigens.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: M. Volny, AffiPro, Mratin, Czech Republic

Consultant or Advisory Role: M. Volny, AffiPro

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: The Institutional Research Concept of the Institute of Microbiology RVO61388971; grants from the Ministry of Education, Youth and Sports of the Czech Republic (NPU LO1509) and European Regional Development Funds (CZ.1.07/2.3.00/30.0003 and CZ.1.05/1.1.00/02.0109) to The Institute of Microbiology, Charles University (project UNCE 204025/2012) to Charles University.

Expert Testimony: None declared.

Patents: P. Pompach, patent number PV 2014–631; V. Ruzicka, patent number PV 2014–631; M. Volny, patent numbers US 20130260050 A1 and CZ PV2014–631; P. Novak, patent numbers PV2014–631 and PV2015–405.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

Acknowledgments: We acknowledge Dr. Radek Kucera (Pilsen Faculty Hospital) for sera collection.

References

- Boonyapranai K, Tsai HY, Chen MC, Sriyam S, Sinchaikul S, Phutrakul S, et al. Glycoproteomic analysis and molecular modeling of haptoglobin multimers. *Electrophoresis* 2011;32:1422–32.
- Andersen CB, Torvund-Jensen M, Nielsen MJ, de Oliveira CL, Hersleth HP, Andersen NH, et al. Structure of the haptoglobin-haemoglobin complex. *Nature* 2012;489:456–9.
- Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 1996;42:1589–600.
- Sadrzadeh SM, Bozorgmehr J. Haptoglobin phenotypes in health and disorders. *Pathol Patterns Rev* 2004;121:97–104.
- Leclerc JL, Blackburn S, Neal D, Mendez NV, Wharton JA, Waters MF, et al. Haptoglobin phenotype predicts the development of focal and global cerebral vasospasm and may influence outcomes after aneurysmal subarachnoid hemorrhage. *Proc Natl Acad Sci* 2015;112:1155–60.
- Hansson L, Einarsson R, Johansson J, Hjalmarsson K. Typing of haptoglobin using PhastSystem. Application Note 373. https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314723116657/litdoc18101969_20140706230024.pdf (Accessed August 2015).
- Mikkat S, Koy C, Ulbrich M, Ringel B, Glocker MO. Mass spectrometric protein structure characterization reveals cause of migration differences of haptoglobin alpha chains in two-dimensional gel electrophoresis. *Proteomics* 2004;4:3921–32.
- Soejima M, Koda Y. TaqMan-based real-time PCR for genotyping common polymorphisms of haptoglobin (HP1 and HP2). *Clin Chem* 2008;54:1908–13.
- Tolson J, Bogumil R, Brunst E, Beck H, Elsner R, Humeny A, et al. Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid alpha in renal cancer patients. *Lab Invest* 2004;84:845–56.
- Tubbs KA, Kiernan UA, Niederkofer EE, Nedelkov D, Bieber AL, Nelson RW. High-throughput MS-based protein phenotyping: application to haptoglobin. *Proteomics* 2005;5:5002–7.
- Hutchens TW, Yip TT. New desorption strategies for the mass spectrometric analysis of macromolecules. *Rapid Commun Mass Spectrom* 1993;7:576–80.
- Nelson RW, Krone JR, Bieber AL, Williams P. Mass spectrometric immunoassay. *Anal Chem* 1995;67:1153–8.
- Warren EN, Elms PJ, Parker CE, Borchers CH. Development of a protein chip: a MS-based method for quantitation of protein expression and modification levels using an immunoaffinity approach. *Anal Chem* 2004;76:4082–92.
- Reid JD, Holmes DT, Mason DR, Shah B, Borchers CH. Towards the development of an immuno MALDI (iMALDI) mass spectrometry assay for the diagnosis of hypertension. *J Am Soc Mass Spectrom* 2010;21:1680–6.
- Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res* 2004;3:235–44.
- Razavi M, Johnson LD, Lum JJ, Kruppa G, Anderson NL, Pearson TW. Quantification of a proteotypic peptide from protein C inhibitor by liquid chromatography-free SISCAPA-MALDI mass spectrometry: application to identification of recurrence of prostate cancer. *Clin Chem* 2013;59:1514–22.
- Liang X, Lubman DM, Rossi DT, Nordblom GD, Barksdale CM. On-probe immunoaffinity extraction by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chem* 1998;70:498–503.
- Scherl A. Clinical protein mass spectrometry. *Methods* 2015;81:3–14.
- Franchetti V, Solka BH, Baitinger WE, Amy JW, Cooks RG. Soft landing of ions as a means of surface modification. *Int J Mass Spectrom Ion Phys* 1977;23:29–35.
- Miller SA. Soft-landing of polyatomic ions at fluorinated self-assembled monolayer surfaces. *Science* 1997;275:1447–50.
- Ouyang Z, Takáts Z, Blake TA, Gologan B, Guymon AJ, Wiseman JM, et al. Preparing protein microarrays by soft-landing of mass-selected ions. *Science* 2003;301:1351–4.
- Blake TA, Ouyang Z, Wiseman JM, Takáts Z, Guymon AJ,

- Kothari S, et al. Preparative linear ion trap mass spectrometer for separation and collection of purified proteins and peptides in arrays using ion soft landing. *Anal Chem* 2004;76:6293–305.
23. Cooks RG, Jo SC, Green J. Collisions of organic ions at surfaces. *Appl Surf Sci* 2004;231:13–21.
 24. Badu-Tawiah AK, Wu C, Cooks RG. Ambient ion soft landing. *Anal Chem* 2011;83:2648–54.
 25. Badu-Tawiah AK, Cyriac J, Cooks RG. Reactions of organic ions at ambient surfaces in a solvent-free environment. *J Am Soc Mass Spectrom* 2012;23:842–9.
 26. Takáts Z, Wiseman JM, Gologan B, Cooks RG. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* 2004;306:471–3.
 27. Gologan B, Takáts Z, Alvarez J, Wiseman JM, Talaty N, Ouyang Z, et al. Ion soft-landing into liquids: protein identification, separation, and purification with retention of biological activity. *J Am Soc Mass Spectrom* 2004;15:1874–84.
 28. Gologan B, Green JR, Alvarez J, Laskin J, Cooks RG. Ion/surface reactions and ion soft-landing. *Phys Chem Phys* 2005;7:1490–500.
 29. Volný M, Elam WT, Branca A, Ratner BD, Turecek F. Preparative soft and reactive landing of multiply charged protein ions on a plasma-treated metal surface. *Anal Chem* 2005;77:4890–6.
 30. Volný M, Elam WT, Ratner BD, Turecek F. Enhanced in-vitro blood compatibility of 316L stainless steel surfaces by reactive landing of hyaluronan ions. *J Biomed Mater Res Part B Appl Biomater* 2007;80B:505–10.
 31. Volný M, Turecek F. High efficiency in soft landing of biomolecular ions on a plasma-treated metal surface: are double-digit yields possible? *J Mass Spectrom* 2006;41:124–6.
 32. Morozov VN, Morozova TY. Electrospray deposition as a method to fabricate functionally active protein films. *Anal Chem* 1999;71:1415–20.
 33. Avseenko N V, Morozova TY, Ataullakhanov FI, Morozov VN. Immobilization of proteins in immunochemical microarrays fabricated by electrospray deposition. *Anal Chem* 2001;73:6047–52.
 34. Morozov VN. Electrospray deposition of biomolecules. *Adv Biochem Engin/Biotechnol* 2010;119:115–62.
 35. He Q, Badu-Tawiah AK, Chen S, Xiong C, Liu H, Zhou Y, et al. In situ bioconjugation and ambient surface modification using reactive charged droplets. *Anal Chem* 2015;87:3144–8.
 36. Krásný L, Pompach P, Strohalm M, Obsilova V, Strnadová M, Novák P, et al. In-situ enrichment of phosphopeptides on MALDI plates modified by ambient ion landing. *J Mass Spectrom* 2012;47:1294–302.
 37. Krásný L, Pompach P, Strnadová M, Hynek R, Vališ K, Havlíček V, et al. High-throughput workflow for identification of phosphorylated peptides by LC-MALDI-TOF/MS coupled to in situ enrichment on MALDI plates functionalized by ion landing. *J Mass Spectrom* 2015;50:802–11.
 38. Volný M, Elam WT, Ratner BD, Turecek F. Preparative soft and reactive landing of gas-phase ions on plasma-treated metal surfaces. *Anal Chem*. 2005;77:4846–53.