Research Article

Rapid Communications in Mass Spectrometry

Received: 12 May 2015 Revised: 15 July 2015 Accepted: 13 August 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2015**, *29*, 2057–2060
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7314

Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to detect monoclonal immunoglobulin light chains in serum and urine

David R. Barnidge[1]*, Thomas P. Krick[2], Timothy J. Griffin[2] and David L. Murray[1]

[1]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA
[2]Center for Mass Spectrometry and Proteomics, University of Minnesota, St. Paul, MN

RATIONALE: Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) to monitor serum and urine samples for endogenous monoclonal immunoglobulins. MALDI-TOFMS is faster, fully automatable, and provides superior specificity compared to protein gel electrophoresis (PEL).
METHODS: Samples were enriched for immunoglobulins in 5 min using Melon Gel™ followed by reduction with dithiothreitol for 15 min to separate immunoglobulin light chains and heavy chains. Samples were then desalted using C4 ZipTips, mixed with sinapinic acid matrix, and analyzed on a Bruker Biflex III MALDI-TOF mass spectrometer.
RESULTS: Monoclonal immunoglobulin light chains were identified in serum and urine samples from patients with a known monoclonal gammopathy using MALDI-TOFMS with minimal sample preparation.
CONCLUSIONS: MALDI-TOFMS can identify a monoclonal immunoglobulin in serum and urine samples. The molecular mass of the monoclonal immunoglobulin light chain is obtained providing unprecedented specificity compared to PEL. In addition, the methodology can be automated, making it a practical alternative to PEL.

Monoclonal gammopathies are caused by a population of clonal plasma cells that secrete a monoclonal immunoglobulin (M-protein) into the bloodstream at concentrations typically ranging from 1 to 5 g/dL (10 mg/mL or 70–350 μM) disrupting hematopoiesis and causing peripheral organ damage.[1] In some cases a patient will progress from a benign form of the disorder called monoclonal gammopathy of undetermined significance (MGUS) to a malignant form called multiple myeloma (MM). During the transition period, which can take decades, patients that have presented in the past with an elevated monoclonal immunoglobulin are routinely screened in case an increase in the monoclonal immunoglobulin concentration occurs indicating treatment may be necessary.[2] The prevalence of MM is roughly 14% of all B cell malignancies and the symptoms at onset are often nondescript. The first clinical laboratory assay that is done when MM is suspected is nondenaturing serum protein gel electrophoresis (PEL). A positive PEL gel has a discrete dark band in the region where immunoglobulins focus which appears above the dispersed polyclonal background. While PEL only provides a low-resolution description of a monoclonal immunoglobulin, clinical chemists have used the technology for decades thus creating a large knowledge-base of gel banding patterns allowing for efficient diagnosis.

* *Correspondence to*: D. R. Barnidge, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA. E-mail: barnidge.david@mayo.edu

In this study we demonstrate that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) can be used to detect a monoclonal immunoglobulin light chain in serum and urine at levels greater than the normal polyclonal background. The principle of the method is built upon the fact that clonal populations of malignant plasma B cells secrete a monoclonal immunoglobulin with heavy and light chains that have the same amino acid sequence and therefore the same molecular mass. An elevated level of a clonal light chain is observed as a distinct peak in the mass spectrum. Since the light-chain portion of a monoclonal immunoglobulin is lower in molecular mass (average roughly 22,400 Da for lambda and 23,400 Da for kappa) than the heavy chain (50,000 Da to 65,000 Da), and does not contain post-translational modifications (PTMs) such as glycosylation, it is the ideal portion of the monoclonal immunoglobulin to monitor by MALDI-TOFMS.

MALDI-TOFMS has been used extensively as a tryptic peptide mapping tool and has recently been applied as a methodology for peptide quantification.[3,4] However, it has seen limited use in the clinical laboratory except for recent advances in identifying microorganisms and in imaging.[5,6] A number of benefits are associated with using MALDI-TOFMS for qualitative assays compared to liquid chromatography/mass spectrometry (LC/MS) including fast acquisition speeds, individual patient sample spotting eliminating carry-over, and easier spectral interpretation due to singly or doubly charged analyte ions. Our previous work demonstrated that Melon Gel™ is a fast and inexpensive way to enrich for immunoglobulins and that monoclonal immunoglobulins purified in this way can be

easily identified and quantified using microLC/ESI-QTOFMS.[7] We postulated that MALDI-TOFMS could be used in the same manner to detect a monoclonal immunoglobulin in serum samples from patients with a high concentration of a monoclonal immunoglobulin. Here we show that MALDI-TOFMS can be used to identify a monoclonal immunoglobulin using a simple Melon Gel™ purification coupled with reduction by dithiothreitol (DTT) and background reagent removal using a C4 ZipTip. This purification process results in a mass spectrum with predominant $[MH^+]^{+1}$ and $[MH_2^{+}]^{+2}$ immunoglobulin light-chain ions with a lesser response from heavy-chain ions. Mass spectra collected using this approach clearly showed a monoclonal immunoglobulin light chain above the normal polyclonal background in patients that tested positive for a monoclonal immunoglobulin by PEL. These findings demonstrate the utility of MALDI-TOFMS to detect and provide accurate molecular mass for monoclonal immunoglobulins in patient samples.

EXPERIMENTAL

Serum and immunoglobulin reagents

Adalimumab (Humira) was supplied by the Mayo Clinic pharmacy. Both control and patient sera and urine were waste samples from the clinical laboratory.

Reagents

Ammonium bicarbonate, dithiothreitol (DTT), formic acid, and sinapinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melon Gel™ was purchased from Thermo-Fisher Scientific (Waltham, MA, USA). Water and acetonitrile were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA).

Serum and urine preparation

A volume of 20 µL of serum was enriched for immunoglobulins using 200 µL Melon Gel™ following the manufacturer's instructions. After immunoglobulin enrichment 20 µL of sample was reduced by combining with 10 µL of 200 mM DTT and 20 µL of 50 mM ammonium bicarbonate then incubated at 55 °C for 15 min. Excess reagents and salts were removed from the samples using C4 ZipTip. A volume of 10 µL of 80% acetonitrile/20% water + 0.1% trifluoroacetic acid was used to elute the immunoglobulins off the ZipTip. A volume of 1 µL of sample was then mixed with 1 µL of sinapinic acid dissolved in 50% acetonitrile/50% water at a concentration of 10 mg/mL. Urine was prepared in the same manner without Melon Gel™ purification. Mass spectra were obtained using a Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics) operated in linear mode with delayed extraction. All MALDI-TOFMS spectra were acquired in positive ion mode from a total of 400 summed laser shots.

RESULTS

The recombinant therapeutic monoclonal immunoglobulin adalimumab (Humira) was used to optimize sample preparation and instrument conditions. Figure 1 shows the MALDI-TOF mass spectrum observed for 500 µg/mL adalimumab in 50 mM ammonium bicarbonate buffer reduced with DTT, ZipTip cleaned, and then mixed with sinapinic acid matrix. The predominant peaks in the spectrum over the mass/charge range of 10,000 to 30,000 are the singly charged ion from the kappa light chain of adalimumab at an m/z of 23,407 Da and the doubly charged ion at an m/z of 11,703. The calculated m/z for a protonated adalimumab kappa light

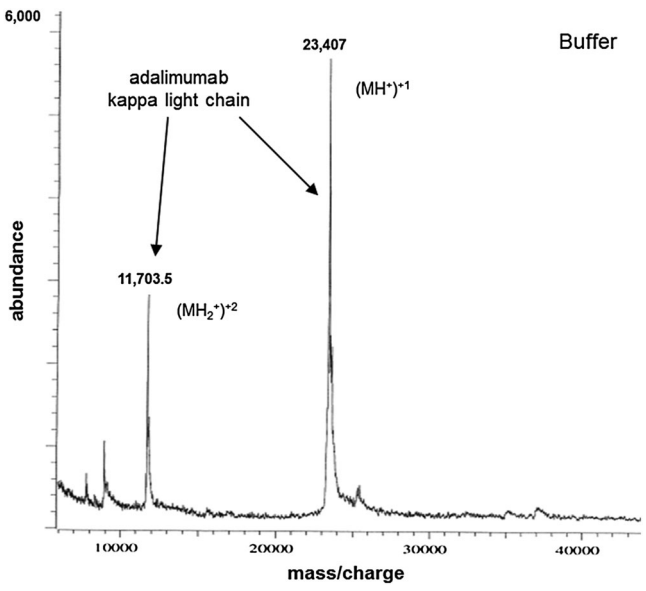

Figure 1. MALDI-TOF mass spectrum observed for 500 µg/mL adalimumab in 50 mM ammonium bicarbonate buffer reduced with DTT, ZipTip cleaned, and then mixed with sinapinic acid matrix. The singly charged kappa light chain of adalimumab is observed at an m/z of 23,406 Da and the doubly charged ion is observed at m/z 11,703 Da.

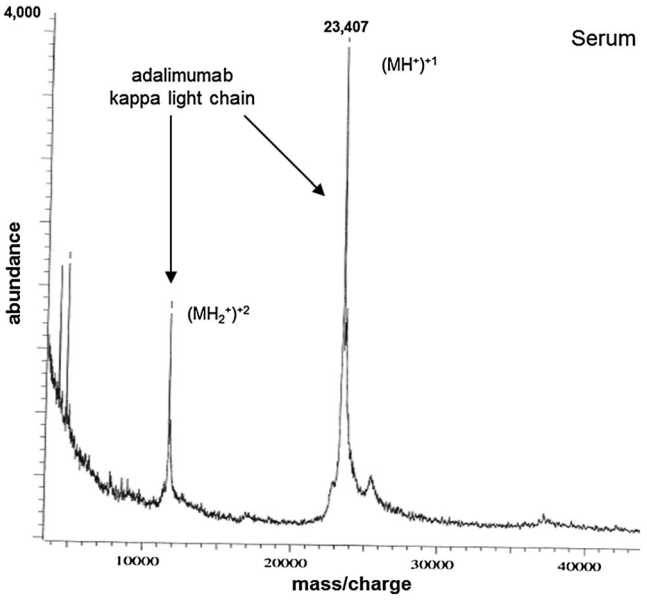

Figure 2. MALDI-TOF mass spectrum of adalimumab diluted into normal serum at a concentration of 500 µg/mL. The spectrum matches the spectrum shown in Fig. 1 without serum where the singly charged kappa light chain of adalimumab is clearly observed at an m/z of 23,406 Da and the doubly charged ion is observed at an m/z of 11,703 Da.

Rapid Communications in Mass Spectrometry

chain is 23,411 which is in good agreement with the observed m/z of the light chain in Fig. 1. The difference of 4 Da is likely due to the incomplete reduction of the intra-disulfide bonds in the light chain. The heavy-chain portion of adalimumab was observed but at a 10-fold lower abundance than the light-chain ion (data not shown); therefore, we decided to optimize conditions for the light chain only.

Figure 2 shows the MALDI-TOF mass spectrum observed for adalimumab stock diluted into normal serum at a concentration of 500 µg/mL. The sample was then purified using Melon Gel™, reduced with DTT, and ZipTip cleaned prior to mixing with the matrix. The predominant peak in the spectrum is the singly charged ion from the kappa light chain at an m/z of 23,407 matching the spectrum shown in Fig. 1. After conditions had been optimized we began comparing the performance of MALDI-TOFMS to PEL for identifying a monoclonal light chain in patient serum and urine. Figure 3 shows the results found by PEL for normal serum and serum from a patient with multiple myeloma (top) and the responses observed for the same samples by MALDI-TOFMS (bottom). The left-hand side of both PEL gels shows a dark band which represents albumin. The right-hand side of each PEL gel is labeled as the immunoglobulin, or gamma region, portion of the gel. The normal serum PEL gel immunoglobulin region does not contain distinct bands but instead shows a broad non-descript staining pattern. The MALDI-TOF mass spectrum also does not contain distinct mass peaks. In contrast, the PEL gel from the patient with multiple myeloma shows a distinct dark band that represents a monoclonal immunoglobulin. This monoclonal immunoglobulin is also observed in the corresponding MALDI-TOF mass spectrum. These initial findings matched our observations obtained from serum samples taken from patients with multiple myeloma that we analyzed using microLC/ESI-QTOFMS.[7] We have also reported using microLC/ESI-QTOFMS to identify monoclonal light chains in urine from patients with a monoclonal gammopathy.[8] Using the same simple sample preparation where the sample is simply diluted 1:1 with buffer containing DTT, we analyzed urine from patients with a confirmed monoclonal light chain in their urine. An example of a mass spectrum from such a patient is shown in Fig. 4. The figure clearly shows a kappa light-chain peak at an m/z of 23,327 demonstrating that a monoclonal free light chain can be isolated directly from urine and analyzed by MALDI-TOFMS. Currently, urine PEL involves concentrating the urine using molecular weight cut-off filters and centrifugation. The method demonstrated here uses a ZipTip, not only to desalt the sample, but also to increase the concentration of light chains to be spotted on the MALDI target. This methodology can be easily automated compared to concentrating with cut-off filters which is more labor intensive.

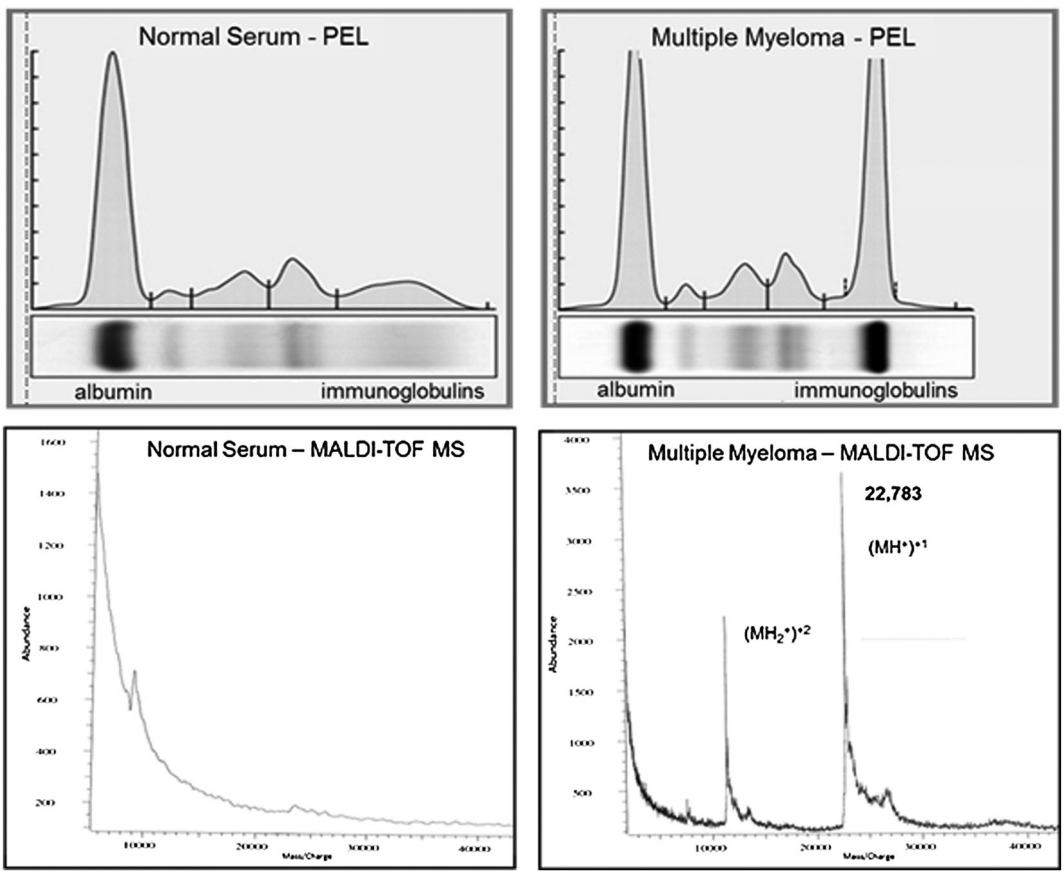

Figure 3. Comparison of serum analyzed by PEL (top) and MALDI-TOFMS (bottom). PEL results from normal serum (left) and serum from a patient with multiple myeloma (right) are shown above their corresponding MALDI-TOF mass spectra. The monoclonal lambda light chain (m/z 22,783) is clearly visible in the mass spectrum from the serum taken from a patient with multiple myeloma while no peak is observed in the spectrum from the normal serum.

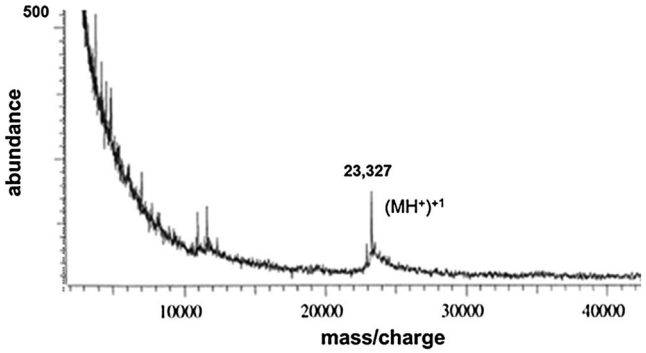

Figure 4. MALDI-TOF mass spectrum of urine from a patient with a known urine kappa free light chain. The spectrum clearly shows a peak at m/z 23,327; however, the abundance is much lower than in serum as a result of the lower levels of light chains in urine.

DISCUSSION

The results presented demonstrate that MALDI-TOFMS can be used to screen serum and urine samples for a monoclonal immunoglobulin with minimal sample cleanup. MALDI-TOFMS has been used previously as a tool for tryptic peptide mapping of monoclonal light chains and for determining the intact molecular mass of recombinant monoclonal light chains.[5,9,10] Our previous work employed the use of microLC to separate the immunoglobulins followed by positive ion ESI.[11] Positive ESI under acidic LC conditions typically generates immunoglobulin light-chain ions with charge states ranging from +25 to +10; therefore, accurate molecular mass determination requires spectral deconvolution. In contrast, MALDI-TOF mass spectra of monoclonal immunoglobulins are dominated by the +1 and +2 charge states. The lower charge states produced by MALDI makes interpretation much simpler compared to ESI and eliminates the need for spectral deconvolution.

The use of Melon Gel™ to enrich serum samples combined with ZipTip removal of buffer salts is efficient; however, albumin is still observed in some patient samples. The +3 charge state of albumin has been seen at roughly m/z 22,000, just outside the low end of the lambda light-chain m/z range. The peak is very broad and has only been seen in low abundance, but has not interfered with interpretation of mass spectra. Another broad peak that was routinely observed in patients with a monoclonal IgG at an m/z range of roughly 25,000 is assumed to be the +2 charge state of the heavy chain. We also prepared a dilution series mixing serum from a patient with a 2.0 g/dL IgG kappa monoclonal immunoglobulin with normal serum and we were able to detect the kappa light chain down to 0.2 g/dL. In addition, we have observed the response for a monoclonal kappa light chain in a patient with a 1.3 g/dL monoclonal IgG kappa with a signal/noise of approximately 6 (data not shown). These observations and the data collected on patient serum samples to date suggest that the sensitivity of MALDI-TOFMS is similar to PEL. However, additional work needs to be done to define how the methodology would be used in the clinical lab compared to PEL since samples that have ambiguous results by PEL are reflexed to the more sensitive but more complex method of immunofixation (IFX). We anticipate that ambiguous results by MALDI-TOFMS will be reflexed to the longer and more complex LC/MS analysis on an instrument that is more sensitive and has higher resolution and better mass measurement accuracy. Such an operational structure would reduce the likelihood of false positives and enable the identification of post-translationally modified light chains.

As the use of mass spectrometry for identifying and quantifying monoclonal immunoglobulins matures, MALDI-TOFMS shows promise as an alternative to PEL gels. The methodology is simple to perform and lends itself to being fully automated, including identification of the peak corresponding to the monoclonal light chain.

REFERENCES

[1] R. A. Kyle, S. V. Rajkumar. Treatment of multiple myeloma: a comprehensive review. *Clin. Lymphoma Myeloma* **2009**, *9*, 278.
[2] R. A. Kyle, F. Buadi, S. V. Rajkumar. Management of monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM). *Oncology* **2011**, *25*, 578.
[3] V. Egelhofer, K. Bussow, C. Luebbert, H. Lehrach, E. Nordhoff. Improvements in protein identification by MALDI-TOF-MS peptide mapping. *Anal. Chem.* **2000**, *72*, 2741–50.
[4] M. Razavi, L. D. Johnson, J. J. Lum, G. Kruppa, N. L. Anderson, T. W. Pearson. 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.
[5] D. Murray, D. Barnidge. Characterization of immunoglobulin by mass spectrometry with applications for the clinical laboratory. *Crit. Rev. Clin. Lab. Sci.* **2013**, *50*, 91.
[6] K. Schwamborn, R. M. Caprioli. Molecular imaging by mass spectrometry – looking beyond classical histology. *Nat. Rev. Cancer* **2010**, *10*, 639.
[7] D. R. Barnidge, S. Dasari, C. M. Botz, D. H. Murray, M. R. Snyder, J. A. Katzmann, A. Dispenzieri, D. L. Murray. Using mass spectrometry to monitor monoclonal immunoglobulins in patients with a monoclonal gammopathy. *J. Proteome Res.* **2014**. DOI:10.1021/pr400985k.
[8] C. M. Botz, D. R. Barnidge, D. L. Murray, J. A. Katzmann. Detecting monoclonal light chains in urine: microLC-ESI-Q-TOF mass spectrometry compared to immunofixation electrophoresis. *Br. J. Haematol.* **2014**, *167*, 437.
[9] F. Lavatelli, F. Brambilla, V. Valentini, P. Rognoni, S. Casarini, D. Di Silvestre, V. Perfetti, G. Palladini, G. Sarais, P. Mauri, G. Merlini. A novel approach for the purification and proteomic analysis of pathogenic immunoglobulin free light chains from serum. *Biochim. Biophys. Acta* **2011**, *1814*, 409.
[10] P. Rognoni, F. Lavatelli, S. Casarini, G. Palladini, L. Verga, P. Pedrazzoli, G. Valentini, G. Merlini, V. Perfetti. A strategy for synthesis of pathogenic human immunoglobulin free light chains in E. coli. *PLoS One* **2013**, *8*, e76022.
[11] D. R. Barnidge, S. Dasari, M. Ramirez-Alvarado, A. Fontan, M. A. Willrich, R. C. Tschumper, D. Jelinik, M. R. Synder, A. Dispenzieri, J. A. Katzmann, D. L. Murray. Phenotyping polyclonal kappa and lambda light chain molecular mass distributions in patient serum using mass spectrometry. *J. Proteome Res.* **2014**, *13*, 5198.

