Research Article

Analysis of monoclonal immunoglobulins from bone marrow plasma cells using immunopurification and LC-MS

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A B S T R A C T

Introduction: Clonal plasma cells secrete immunoglobulins, each with the exact same amino acid sequence, that are referred to as monoclonal immunoglobulins. The monoclonal heavy chain and light chain secreted from clonal plasma cells have the same molecular mass prior to the addition of post-translational modifications (PTMs) since their amino acid sequences are the same.

Objective: To examine the molecular masses of monoclonal light chains and heavy chains isolated directly from the cytoplasm of bone marrow (BM) plasma cells and compare them to the serum derived monoclonal heavy and light chains.

Methods: Using immunopurification and LC-MS we compared the molecular masses of immunoglobulins immunopurified from a patient's serum to those immunopurified from the cytoplasm of their BM plasma cells.

Results: Our findings demonstrate that the light chain molecular masses were identical whether they were obtained from serum or plasma cell cytoplasm. However, the heavy chain molecular masses did not match in bone marrow and serum due to differences in glycosylation, a common post-translational modification (PTM) found on the heavy chain.

Conclusion: The data presented here show that by using LC-MS to analyze monoclonal immunoglobulins (also referred to as miRAMM) additional phenotype information is obtained at the cellular level which is complementary to other more common techniques such as flow cytometry and histopathology.

Introduction

Terminally differentiated bone marrow plasma cells secrete immunoglobulins into circulation. When a monoclonal immunoglobulin (also referred to as an M−protein) is observed in circulation at a higher concentration than other immunoglobulins (referred to as the polyclonal background) it indicates that clonal plasma cells are secreting a monoclonal immunoglobulin at an elevated level, possibly due to a plasma cell dyscrasia. Clinical laboratory tests that quantify and isotype monoclonal immunoglobulins in serum are currently based on electrophoretic methods, such as gel-based protein electrophoresis (PEL) and immunofixation (IFE), and column-based methods, such as capillary zone electrophoresis (CZE). These techniques are robust and have been used to identify M−proteins in serum for decades. However, they do not offer the resolution needed to classify unique M−protein−specific glycoforms that may only differ by a hexose monomer and are known to exist in plasma cell dyscrasias, such as light chain amyloidosis and multiple myeloma [1,2]. Screening patient sera for the presence of a monoclonal immunoglobulin is now being performed in a limited number of clinical laboratories by combining isotype specific immunopurification and mass spectrometry [3,4]. The goal of this work was to determine if the immunoglobulins obtained from the cytoplasm of plasma cells residing within a highly localized region where bone marrow sampling occurred would contain monoclonal immunoglobulins with the same phenotype as those in circulation. Immunopurification provides a simple and effective way to isolate monoclonal immunoglobulins from various matrices. In this work we investigate immunopurifying immunoglobulins directly from BM plasma cell lysates and then analyzing the purified material using microLC-ESI-Q-TOF MS (miRAMM) [5,6]. While miRAMM is not currently in widespread use in the clinical laboratory, it provides the high resolution and excellent mass measurement accuracy needed to make unambiguous mass assignments of monoclonal immunoglobulins with and without post-translational modifications (PTM's). The results from cell lysates can then be compared to the monoclonal phenotype patterns observed in circulation, something that is not possible with intact native Ig gel electrophoresis methods used in the clinical laboratory. This approach provides an additional level of specificity in the phenotype profiling of

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Table 1

M-protein detials for the plasma cell disorders for each patient used in the study.

|	M−protein Isotype	M−protein Conc. (g/L)	Purified Cell Count	Cell Lysate (BM)	Serum	Cell Lysate (BM)	Serum
Patient 1	Kappa FLC	1	5.0E + 06	23,437.8	23,437.5	n/a	n/a
Patient 2	Lambda FLC	1.2	5.0E + 06	22,808.2	22,809.4	n/a	n/a
Patient 3	IgA Kappa	44	4.0E + 06	23,937.5	23,937.0	55,896	heterogenous
Patient 4	IgA Lambda	43	5.0E + 06	22,760.5	22,760.3	54,231	heterogenous
Patient 5	IgG Kappa	48	3.0E + 06	23,312.8	23,312.7	50,971	50,586
Patient 6	IgG Lambda	64	5.0E + 06	22,526.1	22,526.3	51,457	51,234
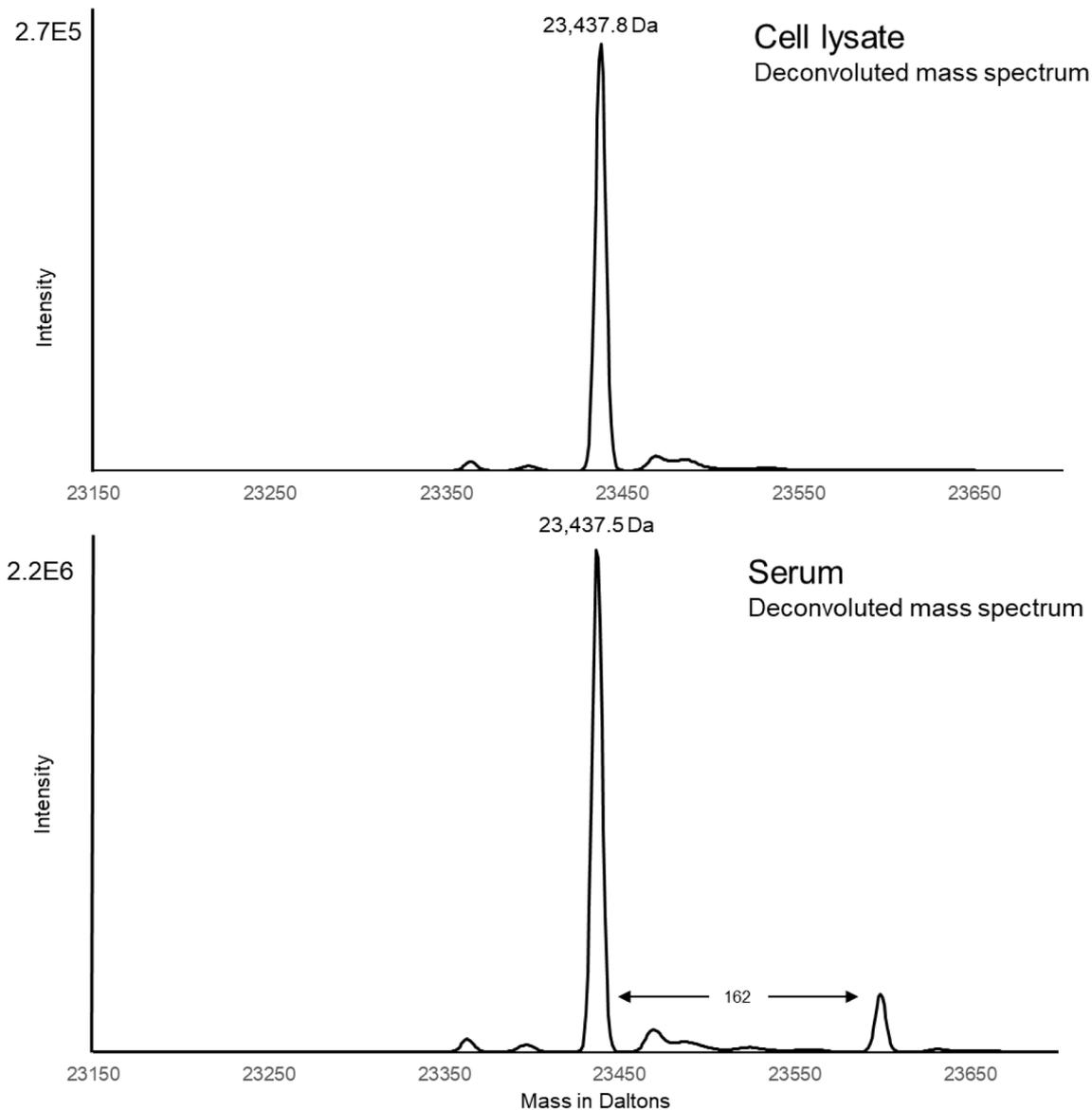
Fig. 1. Patient with a confirmed monoclonal kappa light chain only disease immunopurified using anti-kappa nanobody beads. The deconvoluted mass spectrum from the CD138 + plasma cell lysate is shown on the top of the figure and the matching deconvoluted mass spectrum from the serum is shown on the bottom. The monoclonal kappa light chain molecular mass observed in the cell lysate and in the serum are listed. An additional peak is observed in the mass spectrum from the serum at a higher molecular mass of 23,599.4 Da which is thought to be a glycated form of the monoclonal kappa light chain having a mass difference between the two peaks is 162 Da which is equivalent to the mass of a hexose.

clonal CD138 + plasma cells isolated from a bone marrow biopsy and serves as a complementary technique to techniques such as flow cytometry and histopathology.

Materials and methods

Plasma cells and serum

Plasma cells from a bone marrow biopsy, along with matching serum, were previously obtained under patient informed consent in accordance with the Declaration of Helsinki in a study approved by the
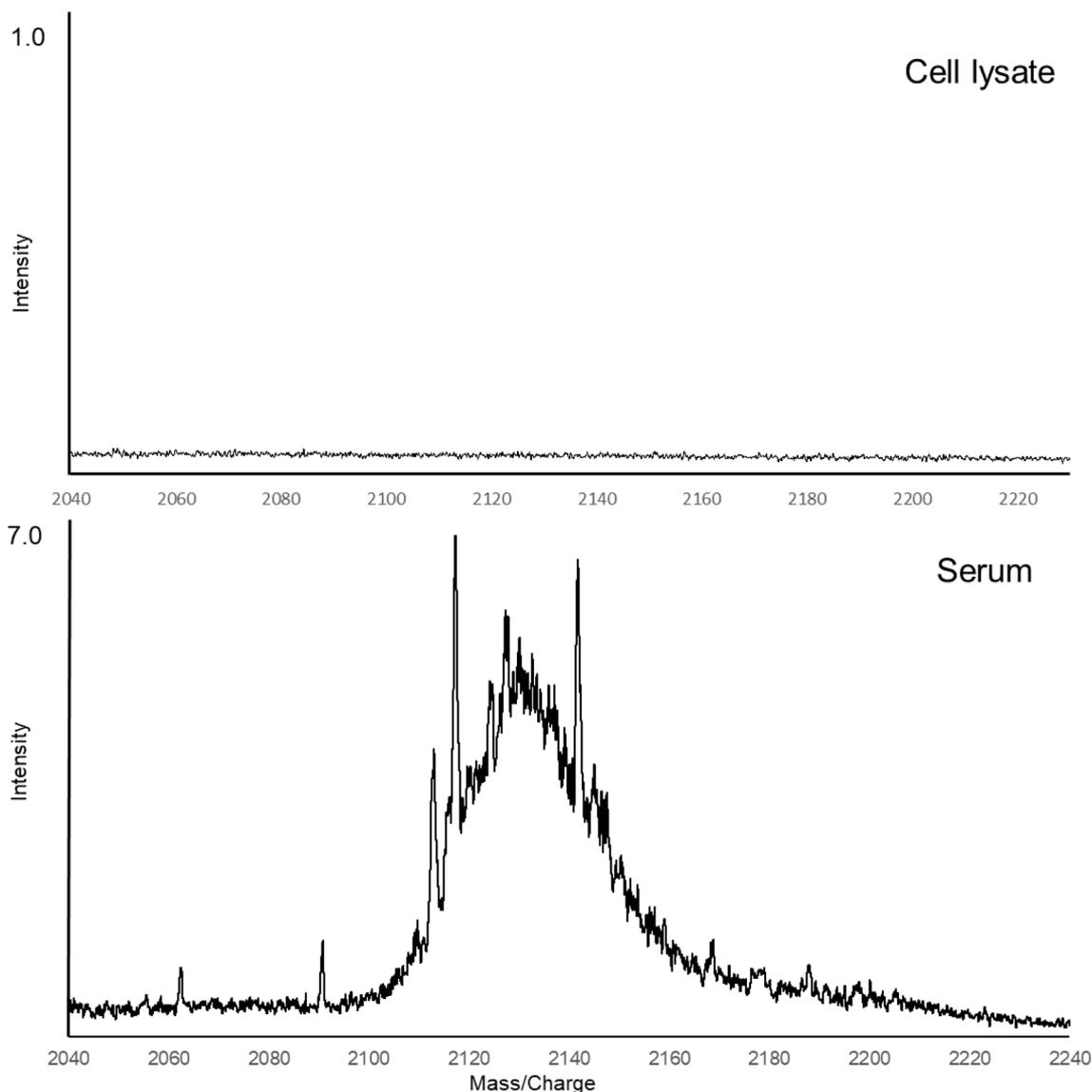
Fig. 2. Summed mass spectra from outside the retention time window for the monoclonal kappa light chain (4.5 to 9 min). In this retention time window other polyclonal kappa light chains would be expected to elute off the LC column. The mass spectrum from serum shows a Gaussian shaped molecular mass distribution obtained from the polyclonal kappa light chain background originating from normal plasma cells secreting immunoglobulins into circulation that is not observed in the cell lysate sample.

Institutional Review Board of the Mayo Clinic. For the current study, as only anonymized patient samples from the above referenced study were used, which were not obtained through investigator intervention or interaction with the individuals, and the investigators cannot readily ascertain the identity of the individual(s) to whom the coded specimens pertain because there are agreements, IRB-approved policies and procedures, and/or legal requirements in place that prohibit the release of the key to the code to the investigators under any circumstances until the individuals are deceased, the IRB committee deemed the study as minimal risk and patient consent was not necessary..

Each patient had serum samples analyzed using standard serum protein electrophoresis to determine the concentration of the M−protein and immunofixation to determine the isotype of the heavy chain and light chain. Concentrations of the M−protein and the isotype are provided in Table 1.

Reagents

Anti-human CD138 beads were purchased from Miltenyi-Biotec

(Auburn, CA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). CaptureSelect camelid nanobody beads and TCEP and RNALater were purchased from Thermo-Fisher Scientific (Waltham MA). Water, acetonitrile, and 2-propanol were purchased from Honeywell Burdick and Jackson (Muskegon, MI).

Methods

Plasma cells were isolated from a bone marrow biopsy aspirate using an automated magnetic bead isolation system (AutoMACS; Miltenyi-Biotec, Auburn, CA) and anti-CD138 antibodies bound to magnetic beads. CD138 + plasma cells (roughly 5x10^6 cells) were then stored in RNA Later and frozen at −80 °C. The sample (approximately 1 mL of buffer + cells in a 1.5 mL centrifuge tube) was thawed and centrifuged at 14,000 × g for 10 min to create a cell pellet. The supernatant was removed for analysis and the cell pellet was gently resuspended in 1 mL of PBS containing protease inhibitor cocktail. The suspended cells were then refrozen at −80 °C for 30 min to freeze-fracture the cells. After 30 min the sample was removed from the freezer and allowed to thaw. The
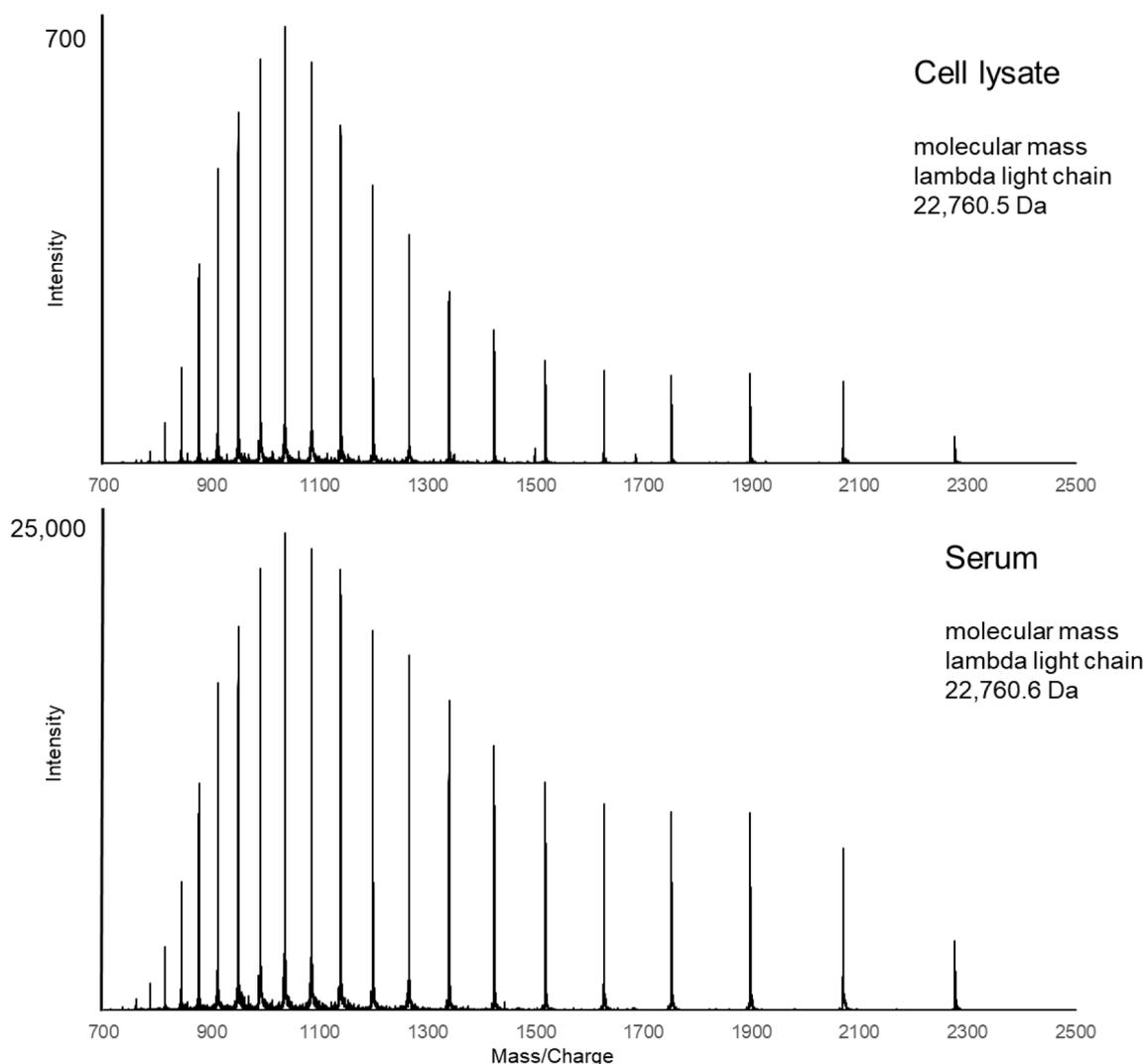
Fig. 3. ESI mass spectra from cell lysates and serum after immunopurification of a patient with an IgA lambda monoclonal immunoglobulin using anti-lambda nanobody beads. Molecular masses for the monoclonal lambda light chain determined after deconvolution are also shown in the figure.

cell suspension was gently homogenized by repeated aspiration with a 1 mL pipette to further disrupt the cells. The sample was centrifuged again at 14,000 × g for 10 min and the supernatant containing the cell lysate was removed for immunopurification. A volume of 100 μL of camelid nanobody bead slurry specific for either the light chain or the heavy chain of the M−protein isotype was added to the cell lysate and allowed to incubate for 30 min at room temperature. After incubation, the supernatant was removed and 1 mL of PBS wash buffer containing protease inhibitor was added to the beads, then the resuspended beads were allowed to mix with the wash buffer for 5 min. The sample was centrifuged at 1,000 × g for 15 s to pellet the beads. The supernatant was removed and discarded, then another 1 mL of PBS wash was added to suspend the beads. A total of three washes using PBS were performed. The same bead washing protocol was then performed three times using water. Bound immunoglobulins were eluted off the beads by adding 50 μL of 5 % acetic acid containing 20 mM TCEP reducing agent. It is assumed that all disulfide bonds are broken after reduction using TCEP and, therefore, all heavy chains and light chains are no longer associated due to the breaking of their inter-chain disulfide bonds. In addition, the TCEP is assumed to break any J-chain to heavy chain bonds that are responsible for generating dimers in the case of IgA immunoglobulins. A volume of 10 μL of patient matched serum was immunopurified using the same type of beads and the same bead washing and elution protocol.

A volume of 2 μL of the total 20 μL extract was injected onto an Eksigent Ekspert 200 microLC for analysis by microLC-ESI-Q-TOF MS. The LC separation conditions consisted of mobile phase A: 100 % water + 0.1 % FA, and mobile phase B: 90 % acetonitrile + 10 % 2-propanol + 0.1 % v/v formic acid. Separation of the heavy and light chains was performed using a 1.0 × 75 mm Poroshell 300SB-C3 column with 5 μm particle size heated to 60 °C and flowing at 25 μL/minute. The gradient used for separations started at 80 % A / 20 % B where it was held for 1 min, then ramped to 75 % A / 25 % B over 1 min, then ramped to 65 % A / 35 % B over 10 min, then ramped to 50 % A / 50 % B over 4 min, then ramped to 95 % A / 5 % B over 2 min and held for 5 min, then ramped to the starting percentage of 80 % A / 20 % B over 1 min, then re-equilibrated at 80 % A / 20 % B for 1 min. Mass spectra were acquired on a Sciex Triple-TOF™ 5600 quadrupole time-of-flight mass spectrometer (Sciex, ON,CA) in ESI positive mode with a Turbo V dual ion source. The source conditions were: ion spray voltage (IS): 5,500, Temp: 500 °C, Curtain gas: 45, GS1 gas: 35, GS2 gas: 30, collision energy (CE) = 10 in full scan mode and CE = 45 in MS/MS mode. TOF MS scans were acquired from *m/z* 600–2,500 with an acquisition time of 100 ms in high sensitivity mode.

Data Analysis: Analyst TF v1.6 was used for instrument control. Data were viewed using Analyst TF v1.6 and PeakView v1.2.0.3. Monoclonal light chain mass spectra used for deconvolution were derived from summing spectra over the elution time of the extracted ion peak for the + 11 charge state. Monoclonal heavy chain mass spectra used for
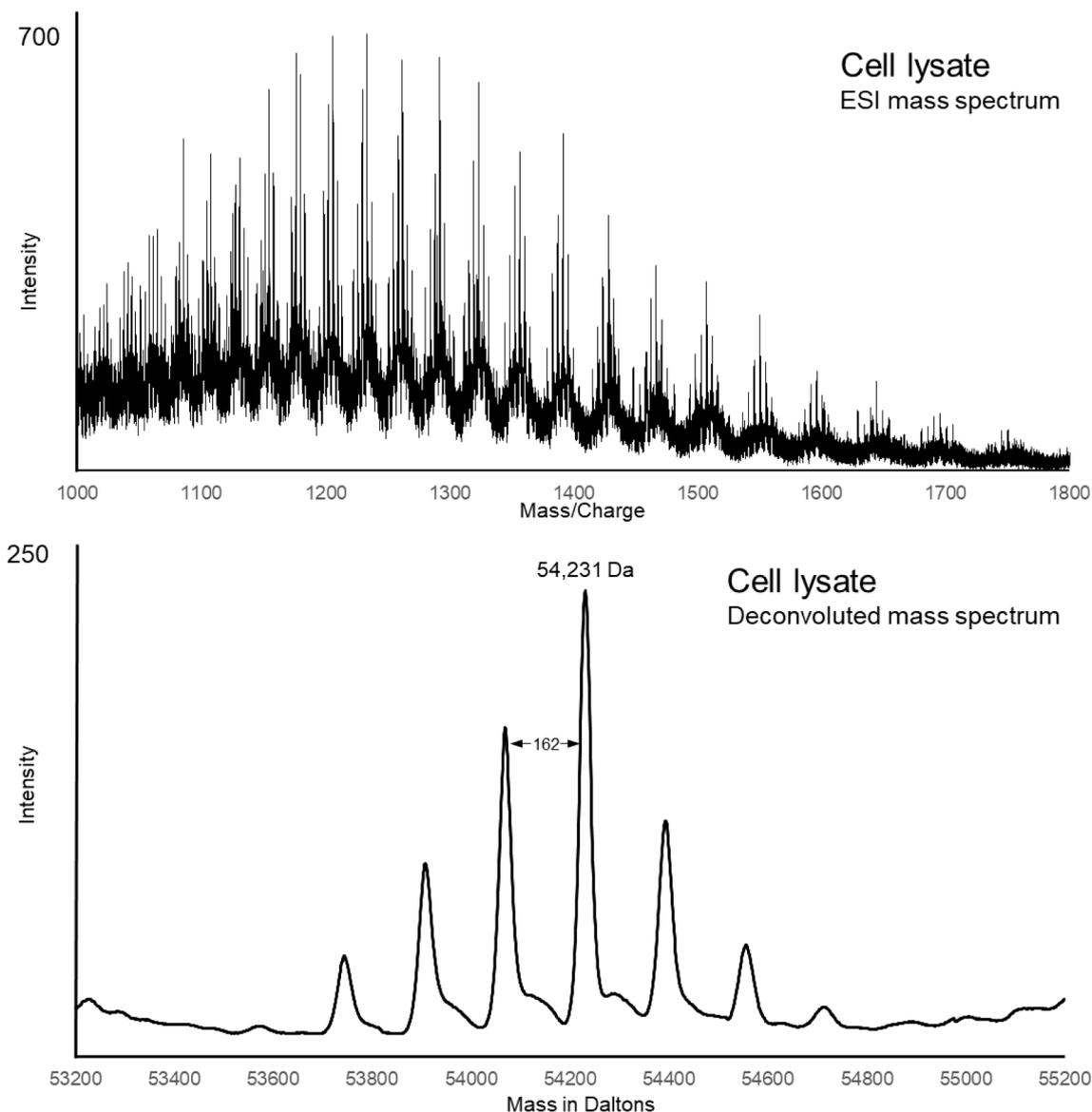
Fig. 4. Monoclonal IgA heavy chain ESI mass spectrum (top) displaying the multiply charged monoclonal IgA heavy chains (+30 to + 54 charge states) from the same IgA lambda positive patient shown in Fig. 3. The mass spectrum on the bottom of the figure is the deconvoluted form of the ESI mass spectrum showing different IgA heavy chain glycoforms of the monoclonal heavy chain that differ in molecular mass by 162 Da matching the mass of a hexose monomer.

deconvolution were derived from summing spectra over the elution time of the extracted ion peak for the most intense heavy chain glycoform peak. Deconvolution of multiply charged light chain ions was performed using the Bayesian Protein Reconstruct program in BioAnalyst. The deconvolution settings for the light chain mass spectra were; 700–2,500 m/z Input range, Output mass range Start mass: 20,000, Stop mass: 30,000 and Step mass: 1.00 with the Input spectrum isotope resolution: Very low and Charge agent: H +. The settings for the heavy chain mass spectra were the same except with the following Start mass: 45,000 and Stop mass: 60,000 and variable m/z Input range depending on the range of the multiply charged ion envelope.

Results

Monoclonal light chain comparison in cell lysates and serum

Purified CD138 + plasma cells were analyzed from six patients with established cases of multiple myeloma and a confirmed monoclonal immunoglobulin in serum. Additional patient sample information is provided in Table 1. LC-MS results start with an example in Fig. 1 from a patient with a confirmed case of monoclonal kappa light chain only disease (Table 1; Patient 1). The deconvoluted mass spectrum from the CD138 + plasma cell lysate is shown on the top of the figure and the matching deconvoluted mass spectrum from the serum is shown on the bottom. Both mass spectra represent summed spectra over a roughly 30 s retention time window from the total ion chromatogram. The spectra clearly show the same monoclonal kappa light chain with a molecular mass of 23,437.8 Da in the cell lysate and 23,437.5 Da in the serum. A difference of 0.3 Da (13 ppm) is within the mass measurement accuracy tolerance for the instrument run in high sensitivity mode. An additional peak is observed in the mass spectrum from the serum at a higher molecular mass of 23,599.4 Da which is thought to be a glycated form of the monoclonal kappa light chain having a mass difference between the two peaks of 162 Da, which is equivalent to the mass of a hexose. Monoclonality of the plasma cell lysate was evaluated by looking for any other kappa light chain clones in spectra summed outside the retention time of the monoclonal kappa light chain, however, none were observed. In contrast, other kappa light chains were observed in the serum sample (i.
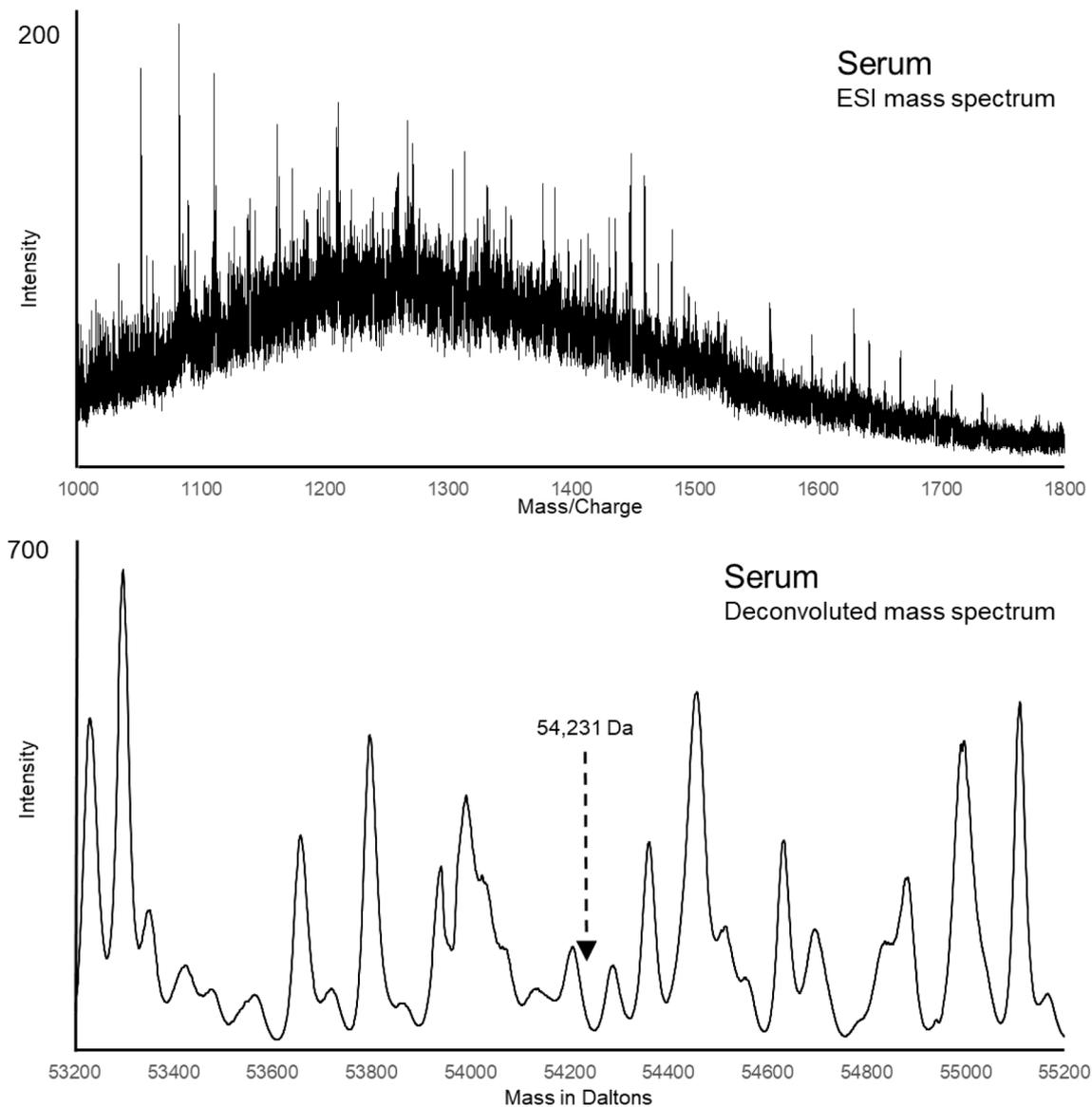
Fig. 5. Serum derived ESI mass spectrum generated by summing mass spectra in the total ion chromatogram at 10.8 min from the same IgA lambda positive patient shown in Fig. 3. No discernable multiply charged ion envelopes that match those shown in the top of Fig. 4 are observed (top). After deconvolution (bottom) no peaks are observed that match the monoclonal heavy chain glycoforms seen in the cell lysate.

e., polyclonal kappa light chains) in spectra summed outside the retention time of the monoclonal kappa light chain. This is clearly presented in Fig. 2 where the + 11 charge state m/z range for kappa light chains observed in mass spectra summed outside the retention time window for the monoclonal kappa light chain (4.5 to 9 min). Within this retention time window other polyclonal kappa light chains would be expected to elute off the LC column. The mass spectrum from the cell lysate summed over this area did not show a signal for any polyclonal kappa light chains (top of Fig. 2). However, the mass spectrum from the serum (bottom of Fig. 2) shows a Gaussian-shaped molecular mass distribution obtained from the polyclonal kappa background that originated from normal plasma cells that secrete their respective immunoglobulins into circulation, as previously described [7]. These findings support the premise that the plasma cells purified from the bone marrow biopsy contained primarily clonal plasma cells since there was no evidence of polyclonal plasma cells, which would have likely created a molecular mass distribution observed from the serum sample in Fig. 2. For reference, the total ion chromatograms from the cell lysate and serum sample used to produce the deconvoluted mass spectra in Fig. 1 and Fig. 2 can be seen in **Supplementary Materials** Fig. 1.

A view of the multiply charged ESI mass spectra obtained using this LC-MS method are shown in Fig. 3. The ESI mass spectra are shown to provide visualization of the charge state distribution associated with monoclonal light chains acquired using this method. The mass spectra are from cell lysates and serum obtained from a patient with a confirmed IgA lambda monoclonal immunoglobulin immunopurified using anti-lambda nanobody beads (Table 1; Patient 4). The ESI mass spectra show the multiply charged monoclonal lambda light chains (+10 to + 29 charge states: acquisition range 700 – 2,500 m/z) from mass spectra summed across a peak in the total ion chromatogram with a 5 min retention time.

We also investigated using middle-down MS/MS fragmentation data from monoclonal light chains to augment the molecular mass of the monoclonal light chains as a means of demonstrating that monoclonal light chains in cell lysates and serum were the same protein. Using middle-down fragmentation conditions previously described [5], we further verified that the monoclonal light chains observed in cell lysates and serum had the same fragmentation spectra. The use of middle-down vs top-down to describe the MS/MS fragmentation experiment stems from the fact that an intact secreted immunoglobulin consists of
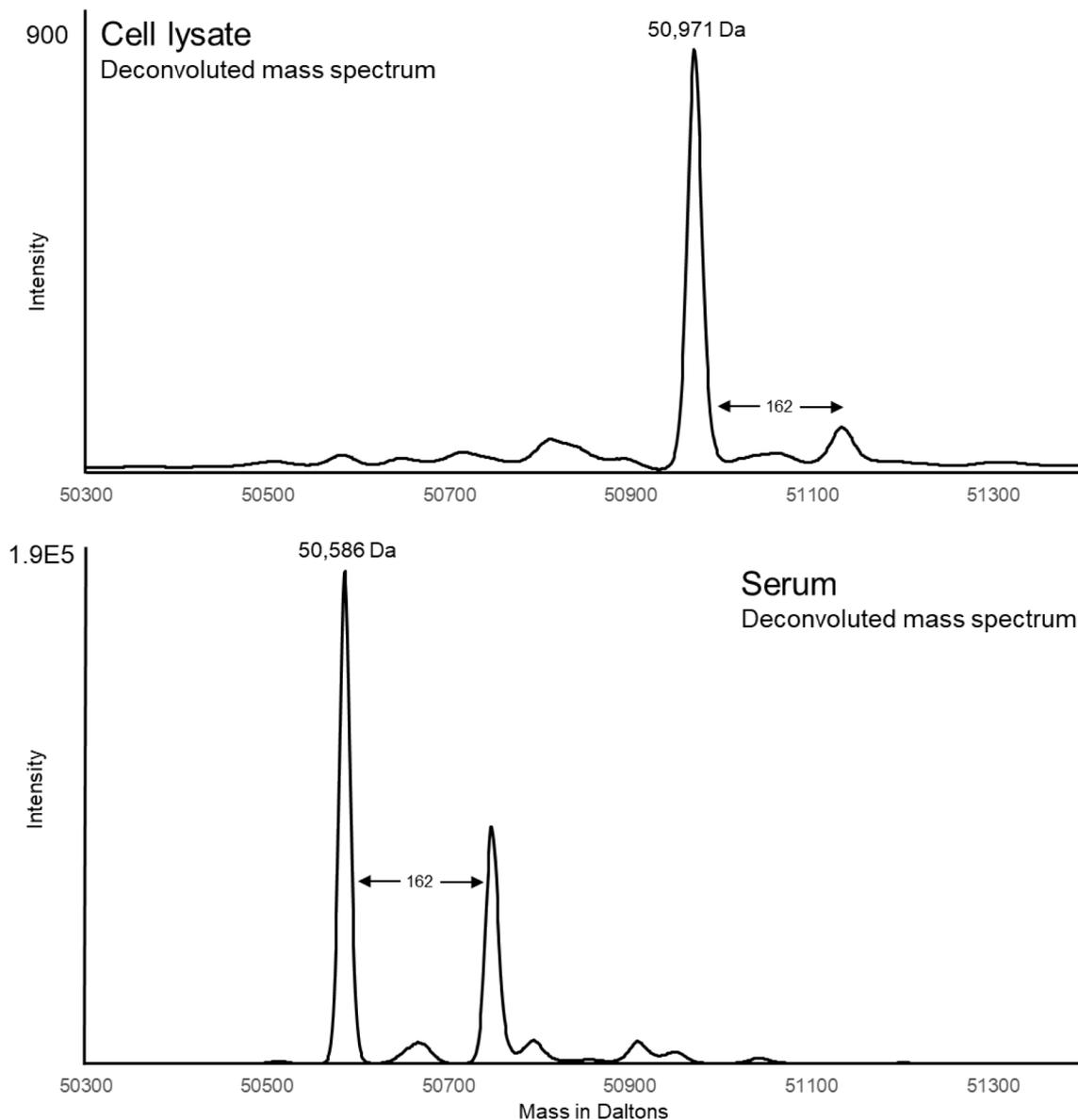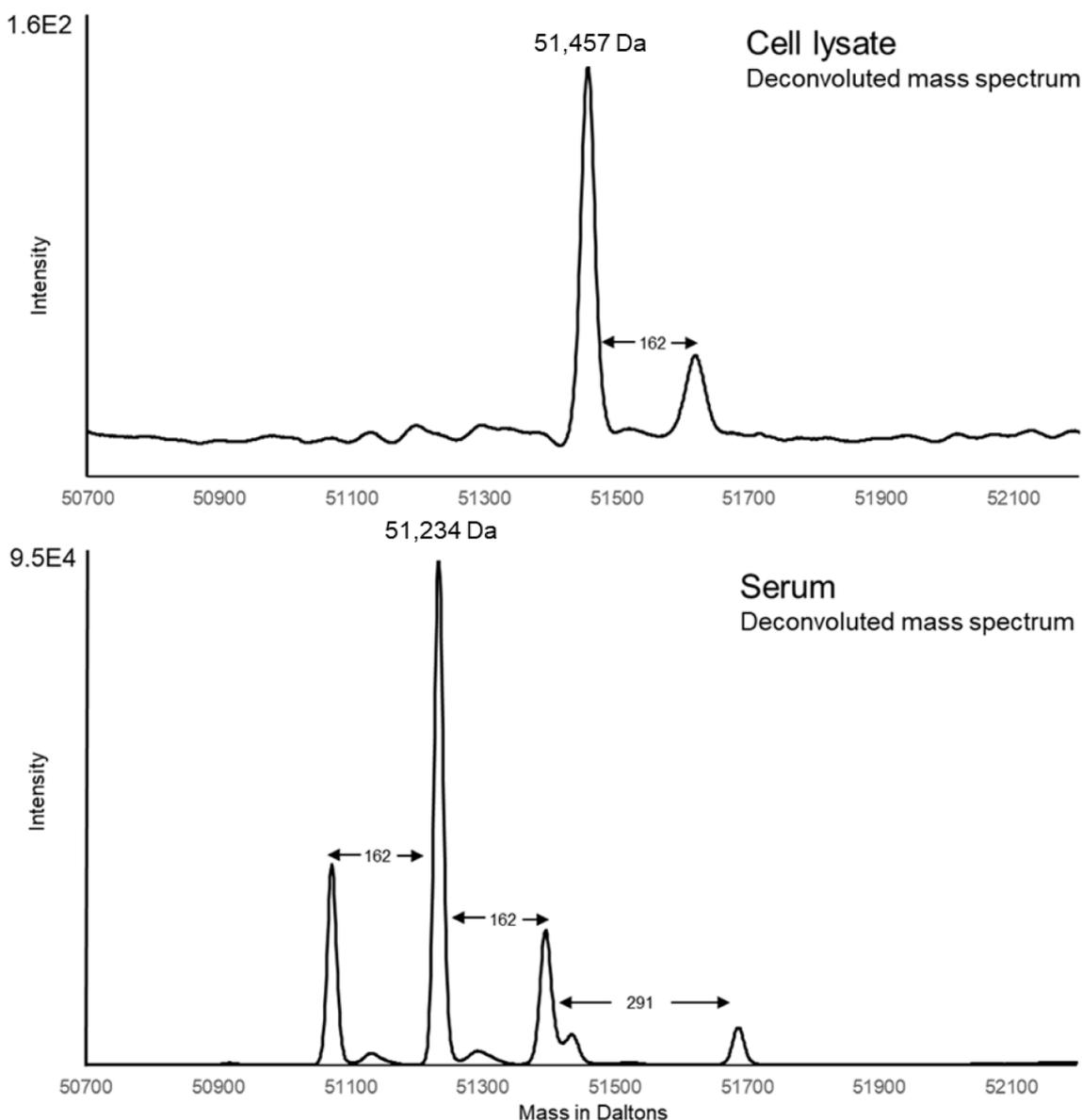
Fig. 6. Deconvoluted IgG heavy chain mass spectra from a patient with a confirmed IgG kappa monoclonal immunoglobulin after immunopurification using anti-human kappa light chain beads. The mass spectrum on the top is from the cell lysate sample and the mass spectrum on the bottom is from the serum sample. The molecular mass of the primary glycoform is labeled in each mass spectrum.

disulfide-bound heavy chains and light chains and the fragmentation was performed on a light chain after the disulfide bond was broken by adding TCEP. An example of matching cell lysate and serum middle-down MS/MS fragmentation spectra from the + 20 charge state from the monoclonal kappa light chain from **Patient 3** in Table 1 is shown in **Supplementary Materials** Fig. 2. Deconvoluted monoclonal light chain mass spectra from Patient 2, Patient 3, Patient 5, and Patient 6 taken from cell lysates and sera are show in **Supplementary Materials** Figs. 3, 4, 5 and 6.

Monoclonal heavy chain comparison in cell lysates and serum

In this study we also explored the use of LC-MS to determine the molecular masses of glycosylated heavy chains from monoclonal IgG and IgA heavy chain isotypes obtained from cell lysates and serum. Informative reviews on the topic of IgG and IgA glycosylation can be found in articles by Ding et al. [8] and Cobb [9] The mass spectra show in Fig. 4 are from Patient 4 in Table 1 and are from cell lysate data focusing on the monoclonal IgA heavy chain. The figure shows an ESI

mass spectra (top) displaying the multiply charged monoclonal IgA heavy chains (+30 to + 54 charge states) across the acquisition range 1,000 – 1,800 *m/z* that was summed over a peak observed at 10.8 min in the total ion chromatogram from the cell lysate. The mass spectrum on the bottom of the figure is the deconvoluted mass spectrum clearly showing a set of IgA heavy chain glycoforms each differing in molecular mass by 162 Da which matches the mass of a hexose monomer. The monoclonal IgA heavy chain glycoform with the greatest intensity is labeled with its molecular mass of 54,231 Da. The mass spectra shown in Fig. 5 were generated by summing spectra from the serum sample using the same retention time as the peak in Fig. 4. However, as shown in the ESI mass spectrum (top) there were no discernable multiply charged ion envelopes that match those shown in the top of Fig. 4. Rather a highly complex mix of unresolved peaks are observed that upon deconvolution (bottom) show no matches for the monoclonal heavy chain glycoforms observed in the cell lysate. This finding illustrates that it is easier to identify the intact monoclonal light chain without any PTMs in cell lysates and serum as opposed to comparing the monoclonal IgA heavy chain due to the additional complexity of a large number of polyclonal

Fig. 7. Deconvoluted IgG heavy chain mass spectra from a patient with a confirmed IgG lambda monoclonal immunoglobulin after immunopurification using anti-human kappa light chain beads. The mass spectrum on the top is from the cell lysate sample and the mass spectrum on the bottom is from the serum sample. The molecular mass of the primary glycoform is labeled in each mass spectrum.

IgA heavy chain glycoforms in serum. LC-MS mass spectra from Patient 3 in Table 1 are shown in **Supplementary Materials** Fig. 7. This patient had a confirmed IgA kappa M−protein and the IgA heavy chain glyco-forms are clearly displayed in the mass spectra. However, as with Patient 4, no matching monoclonal IgA heavy chain glycoforms were observed in the serum sample (data not shown).

We also evaluated the LC-MS results from Patient 5 and Patient 6 in Table 1 where the patients had a confirmed IgG kappa and IgG lambda respectively. Fig. 6 shows an example of IgG monoclonal heavy chain glycoforms from Patient 5 after deconvolution of the multiply charged ESI mass spectrum. It is clear from the mass spectra that after decon-volution two distinct IgG heavy chain glycoforms are observed. How-ever, the highest abundance glycoform in the cell lysate is greater in mass by 385 Da compared to the primary glycoform in serum. The mechanism causing the discrepant molecular masses for the glycosy-lated IgG heavy chain in the cell lysate sample vs the serum sample is not known. In contrast, the monoclonal kappa light chain had the same molecular mass in both cell lysate and serum (see **Supplementary Materials** Fig. 4).

The deconvoluted mass spectra for the IgG heavy chain in cell lysate and serum from Patient 6 are shown in Fig. 7. The deconvoluted mass spectrum from the cell lysate (top) shows the primary IgG heavy chain glycoform in the cell lysate sample with a molecular mass of 51,457 Da with one additional glycoform 162 Da higher in mass. In contrast, the primary IgG heavy chain glycoform observed in serum (bottom) was 51,234 Da. Overall, five IgG heavy chain glycoforms were present in the serum sample two of which differed in molecular mass by 291 Da (presumably an *N*-acetylneuraminic acid monomer) and one by 203 Da (presumably a *N*-acetylhexosamine monomer). Again, the mechanism causing the discrepant molecular masses between cell lysates and serum is not known. Regardless, this limited data set demonstrates that, by using LC-MS, monoclonal IgG heavy chains can be identified in serum. However, the LC-MS method used in the assay is not able to resolve the different IgA heavy chain glycoforms chromatographically or generate mass spectra with the resolving power to deconvolute them. Efforts to increase resolution, both chromatographically and by using a mass spectrometer with greater resolving power, may enable IgA heavy chain glycoforms associated with a specific plasma cell population to be

identified in serum.

Conclusions & discussion

This work represents an extension of the serum based miRAMM LC-MS-based method and is the first time it has been applied to bone marrow plasma cell lysates. In this study we wanted to find out if immunoglobulins, obtained from the cytoplasm of plasma cells present in the localized region where bone marrow sampling occurred, contained monoclonal immunoglobulins with the same phenotype as those in circulation. As expected, the molecular masses of monoclonal light chains were conserved in bone marrow plasma cell lysates and in peripheral blood. Furthermore, middle-down MS/MS fragmentation mass spectra were collected to provide additional evidence that clonal plasma cell lysates contain the exact same monoclonal light chain as serum. In contrast, our observations for the monoclonal heavy chain IgG and IgA glycoforms demonstrate that the plasma cell clones purified from the bone marrow biopsy in these samples appear to have different monoclonal heavy chain glycosylation patterns compared to the monoclonal heavy chains in circulation. The reason for this is unknown, but it could be due to natural variation in the glycosylation of clonal plasma cell populations, additional processing of heavy chain glycosylation at the time of secretion, and/or preanalytical handling of cell lysate samples and serum samples. Little work has been done comparing intact heavy chain monoclonal immunoglobulin glycosylation phenotypes in cell lysates and peripheral blood in the same person. However, studies have been done evaluating the IgG glycosylation patterns of Fc region glycopeptides in patients before and after stem cell transplants [10] and in patients with multiple myeloma compared to healthy controls [11]. In both studies, the glycosylation patterns of the glycopeptides differed from the patients and the donors in the study by de Haan et al. and between patients with multiple myeloma and healthy controls in the study by Zhang et al. Deglycosylation of the immunopurified samples could potentially determine if the heavy chains without carbohydrate had matching molecular masses, but those studies were beyond the scope of this work. The miRAMM methodology presented here is faster to perform than enzymatic-based methods since it does not rely on the extra time needed for enzymatic digestion. Furthermore, the methodology has high mass measurement accuracy allowing for heavy chain glycoforms and their constituent monomers (i.e., sialic acid, pentose, hexose) to be described from a population of plasma cells isolated from a localized region of bone marrow.

Even though this study was conducted on a small number of samples, the findings make it clear that the methodology has the ability to confirm that the molecular masses of monoclonal light chains are conserved in bone marrow plasma cell lysates and in peripheral blood. The study also shows that, in the patients sampled, the heavy chain glycosylation patterns from the population of plasma cells isolated from a restricted bone marrow site are different than the global monoclonal glycosylation patterns observed in peripheral blood. The ability to directly measure these glycoforms from plasma cells originally purified for use in genetic sequencing studies, demonstrates the utility of an LC-MS based miRAMM approach to phenotyping monoclonal immunoglobulins. Monoclonal heavy chain glycoform patterns observed from bone marrow plasma cell lysates vs patterns observed in circulation could potentially be used to determine the heterogeneity of malignant plasma cell populations in a patient, which could, in turn, be used diagnostically and/or prognostically. However, further study is needed to determine just how heavy chain and light chain phenotyping by LC-MS could be used in conjunction with current phenotyping techniques, such as flow cytometry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.04.001.

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