RESEARCH ARTICLE

Analysis and Quantitation of Glycated Hemoglobin by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

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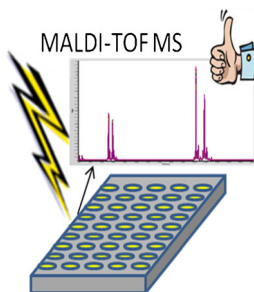
Abstract. Measurement of glycated hemoglobin is widely used for the diagnosis and monitoring of diabetes mellitus. Matrix assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) analysis of patient samples is used to demonstrate a method for quantitation of total glycation on the β-subunit of hemoglobin. The approach is accurate and calibrated with commercially available reference materials. Measurements were linear ($R^2 > 0.99$) across the clinically relevant range of 4% to 20% glycation with coefficients of variation of $\leq 2.5\%$. Additional and independent measurements of glycation of the α-subunit of hemoglobin are used to validate β-subunit glycation measurements and distinguish hemoglobin variants. Results obtained by MALDI-TOF MS were compared with those obtained in a clinical laboratory using validated HPLC methodology. MALDI-TOF MS sample preparation was minimal and analysis times were rapid making the method an attractive alternative to methodologies currently in practice.

Keywords: Hemoglobin, Matrix assisted laser desorption ionization, MALDI-TOF, Diabetes, Glycation, Quantitation

Introduction

MALDI-TOF MS is an established technique for analyzing nonvolatile molecules; however, its use as a tool for making quantitative measurements is not widely accepted. Throughout most of its history, accurate and precise quantitation by MALDI-TOF MS was impractical for routine, high-throughput usage because of limitations in the hardware available for instrument construction. A modern MALDI-TOF mass spectrometer equipped with high repetition rate laser for rapid sample analysis, fast and efficient sample scanning for thorough sample interrogation, and modern computing to keep pace with data acquisition and processing, is well suited for making routine quantitative measurements. This work demonstrates quantitation by MALDI-TOF MS for assessing glycation of hemoglobin, a consequence directly proportional to the blood glucose concentration and currently an important clinical measurement used in the diagnosis and monitoring of diabetes mellitus.

Noncommunicable diseases (NCDs), including cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes, are leading causes of death and disability globally and are estimated to kill more than three in five people worldwide [1–3]. From a medical standpoint, an important feature of many aspects of NCDs is that early and accurate detection can lead to prompt, effective treatment and improved outcomes at a reduced monetary cost to health care.

Type 2 diabetes mellitus [4, 5] (type 2 DM)—characterized by an elevation in blood glucose concentration—is one of the most common and costly of the NCDs. The scope of the type 2

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DM problem is enormous. It is estimated that 380 million people worldwide are living with diabetes and this number is predicted to grow to 550 million by 2030 [6]. Prolonged circulating high blood glucose levels have profound and wide-ranging adverse consequences [7, 8], including damage to the kidneys, heart, eyes, and nervous system. Diabetes is the leading cause of blindness [9] among adults and the leading cause of kidney failure [10]. About 60%–70% of people with diabetes have mild to severe nervous system damage and over 60% of non-traumatic lower-limb amputations [11] occur in diabetics. Among people in the US with diabetes, both heart disease death rates and the risk of stroke are increased by 2- to 4-fold [12].

Currently, two of the main strategies used to diagnose and monitor diabetes [13, 14] are fasting blood glucose determinations and assessment of percent hemoglobin A1c (HbA1c). Measurement of fasting blood glucose levels is straightforward, accurate, and precise, but overnight fasting is required. The alternative strategy targets measurement of HbA1c [15–18]. HbA1c is defined as the nonenzymatic, covalent attachment of glucose to the N-terminal valine of the β-subunit of hemoglobin A (βHb) and correlates with the weighted average blood glucose concentration over the prior 60 to 90 d. HbA1c measurements do not require fasting and are routinely obtained in a single analysis. Current guidelines [19] state that a nondiabetic level of HbA1c is 4%–5% of the total hemoglobin; by contrast, 10%–15% HbA1c is characteristic of levels seen in an unmanaged diabetic.

Measurement of HbA1c is frequently based on absorbance (415 nm; heme moiety) following separation by either liquid chromatography [20–22] (HPLC) or capillary electrophoresis [23, 24] (CE). Glycation of hemoglobin blocks a basic site and reduces the overall molecular charge by one charge. Separation technologies exploit this charge difference to isolate HbA1c from Hb and quantify both species separately. The amount of HbA1c present is reported as a percentage of total hemoglobin, i.e., % HbA1c = 100 * (HbA1c/(βHb + HbA1c)). Hemoglobin molecules modified elsewhere (e.g., other primary amines on lysine side chains) and most hemoglobin genetic variants are detectable using these methods, but often these species display different retention properties, and this can compromise accurate quantification of both the βHb and HbA1c peaks [25–27]. Existing methods are not designed to detect other glycated hemoglobin species and can yield inaccurate results if patients express variant hemoglobin forms [28, 29].

This study reports an approach to quantifying hemoglobin glycation by MALDI-TOF MS. Earlier work done by Biroccio et al. [30] clearly demonstrated the utility of MALDI-TOF MS for quantification of glycated-βHb as well as the potential for measuring the glutathione modification of βHb, but to date the method is not in clinical practice. Recent improvements in detection sensitivity and acquisition speed of MALDI-TOF instrumentation [31, 32] as well as improvements in computer technology and data processing speed suggest that it may be feasible to adapt MALDI-TOF MS technology for routine use as a quantitative assay for βHb glycation. This study was

undertaken to assess the practicality of this application. To be clear, the MALDI-TOF MS signal for glycated-βHb represents total monoglycated hemoglobin. In addition to reacting with the N-terminal amine group, glucose can also react with the amine groups on lysine residues. Both the β and α chains of hemoglobin contain 11 lysine residues with several of these sites being reported to undergo glycation as a function of blood glucose concentration, most notably βHb Lys-66 and αHb Lys-61 [33–35]. MALDI-TOF MS cannot distinguish between these distinct, isobaric, mono-glycated forms. Regardless, the inclusion of these additional species did not significantly impact the correlation between MALDI-TOF derived measurements of βHb glycation with HPLC derived measurements of HbA1c.

In addition to information regarding βHb glycation, the present study extended the analysis to include corroborative information from within the same spectrum that may be used to validate βHb glycation measurements. For example, doubly charged hemoglobin subunit ions were also analyzed as a confirmation to the extent of glycation calculated from the singly charged species. Also, the extent of α-chain glycation (αHb and glycated-αHb) was shown to correlate with the glycated-βHb values in the "normal," non-variant hemoglobin A [36] samples that were analyzed. These measurements provide additional confidence for glycated-βHb determinations. The MALDI-TOF MS method was calibrated by measurement of validated HbA1c standards to relate MALDI-TOF MS derived data to results from a validated HPLC method for quantitation of clinical samples.

A MALDI-TOF MS based assay offers important advantages, such as a low cost of analysis, minimum sample preparation, and the potential for high-throughput. The MALDI-TOF MS technique offers the accuracy of mass selective detection, high sensitivity (low concentration detection threshold), and in this study quantitation and mass calibration were performed using signals inherent to each sample, thereby eliminating the need for addition of external reference materials.

Experimental

Whole blood specimens were collected in phlebotomy at the Veterans Administration (VA) Hospital (San Diego, CA) in purple-top (EDTA) tubes in ~3.5 mL volume. Specimens for MALDI-TOF MS analysis were specifically chosen to span the range of % HbA1c found in the blood of normal, treated, and unmanaged diabetic patients. Prior to MALDI-TOF MS the samples were analyzed by a clincially validated HPLC method. Use of discarded specimens for test development was approved by the VA Subcommittee on Research, VA San Diego Healthcare Systems, Protocol H120059.

MALDI-TOF MS Sample Preparation

Whole blood samples were diluted 1:200 in DI water, mixed, and centrifuged (3000 rpm). An aliquot of the supernatant was

then mixed 1:10 with 10 mg/mL sinapinic acid (30% CH$_3$CN, 0.1% TFA). One µL of this sample/matrix mixture was then spotted onto a disposable stainless steel MALDI target (2600 µm Slide Type (5 × 16 sample array), Hudson Surface Technology, Fort Lee, NJ, USA). For the purposes of assay evaluation and development, all samples were run in 5× technical replication. In one experiment, a broad concentration range hemoglobin dilution series was conducted using a 4 × 12 array disposable MALDI plate (Sony Corporation, Tokyo, Japan) and therefore, for this experiment only, samples were spotted in 4× analytical replication. Disposable plates were chosen because of the potential for adaptation of the analysis to the clinical laboratory; however, the type of MALDI-TOF MS target used had no impact on the quality of the spectra.

Hb/HbA1c Standard Curve Preparation

Lyphochek hemoglobin A1c Linearity Set [37] was purchased from BioRad (Hercules, CA, USA). These blood-based HbA1c reference standards vary systematically in their % HbA1c and were prepared and analyzed in the same manner as clinical blood samples.

HPLC, UV Quantitation

HbA1c was purified from the level 6 Lyphochek standard (16%–22% HbA1c) using a Mono S cation-exchange column (GE Healthcare, Uppsala, Sweden) following the HbA1c analytical protocol [38]. Lyophilized hemoglobin standard purchased from Lee Biosolutions (St. Louis, MN, USA) was weighed and diluted to create a range of protein concentrations (0.20–50 µM [Hb]), and 10 µL of each dilution was injected (3×) through the UV flow cell detector of an Agilent 1100 HPLC system (10 µL injection loop, 415 nm detection of heme). The results were used to create a calibration curve for quantification of hemoglobin samples of unknown concentration. HPLC purified HbA1c and lyophilized Hb standards were mixed together in various concentrations to establish a quantitative MALDI-TOF MS response for the two molecules.

Dilution Study

A dilution study on whole blood samples was used to determine that a concentration range from ~1 to 5 µM of hemoglobin resulted in a MALDI-TOF MS signal that is proportional to concentration. Two independent experiments were conducted. The initial experiment covered a broad concentration range of 0.20–50 µM and a second, follow-up experiment, focused on a narrower region of interest [0.31–10 µM]. The hemoglobin dilution series used for the MS measurements were injected through a UV flow cell to create a [Hb] calibration curve based on the UV absorbance of heme at 415 nm. Triplicate injection of nine independent blood samples established a dilution of 1:2000 as appropriate for bringing the [Hb] into the range required for accurate MALDI-TOF MS quantitation (1–5 µM). The results from this study showed that after 1:2000 dilution the average hemoglobin concentration was ~2.2 µM (n

= 9; range 1.4–2.9 µM; Std. Dev = 0.47, and CV of 22%). The data and results from these experiments are included in the auxiliary file that accompanies this manuscript.

Mass Spectrometry

Mass spectra were generated in linear, positive-ion mode on a SimulTOF 100 [39] (SimulTOF Systems, Sudbury, MA, USA) MALDI-TOF mass spectrometer. Spectra were the average of 100 individual laser shots over the range m/z 5000–20,000. The acquisition parameters were: acceleration voltage 20 kV, focus mass 15000, laser pulse frequency 1 kHz, laser pulse energy 12 µJ, scan rate 1 mm/s at a 100 µm raster to cover each sample position.

Data Processing

Post-acquisition data processing was performed by averaging all spectra that passed a 20 mV minimum signal intensity threshold at each sample position. These spot-averaged spectra (~250 spectra/spot) were calibrated using the known masses for MH^{+1} and MH_2^{+2} ions of hemoglobin alpha (αHb) and beta (βHb) subunits (i.e., m/z = 15127.38, 7564.19, 15868.24, 7934.62) respectively. Estimates of the quantity of both the αHb and βHb, modified by attachment of a single glucose moiety, were derived by comparison of the integrated signal intensity generated by the mass of the unmodified species to that of the signal generated by the m/z value of the unmodified species with an additional mass of +162 (glucose). Results generated are presented as a ratio of the percent total monoglycation of each chain using the following formula: 100 * glycated-αHb/(αHb + glycated-αHb) and 100 * glycated-βHb/(βHb + glycated-βHb), respectively.

Results

The experimental workflow used in following analyses required only dilution of whole blood into MALDI matrix. A final dilution of 1:2000 was chosen because it was the approximate mid-point of the linear portion of a response curve analyzing hemoglobin signal as a function of concentration. Based on the average concentration of hemoglobin in blood, this approximates a final βHb concentration of 2 µM and a final matrix/analyte ratio of ~17,000, values that are both common for MALDI-TOF analyses [40, 41]. All samples were manually spotted by handheld, disposable tip pipetting. Figure 1 shows 80 superimposed spectra derived from a single sample of whole blood that was diluted (1:2000) and spotted across an entire MALDI plate. Figure 1a shows the overlay of spectra that span the full mass range (5–20 kDa) and shows that the most intense peaks in the spectrum are the singly and doubly charged αHb and βHb ions. The region of the singly charged ions (Figure 1b) shows multiple low abundance peaks surrounding the αHb and βHb primary chains. Most of these peaks are modified hemoglobin and are assigned in the figure caption. The peaks of interest to the current study are the unmodified αHb and βHb
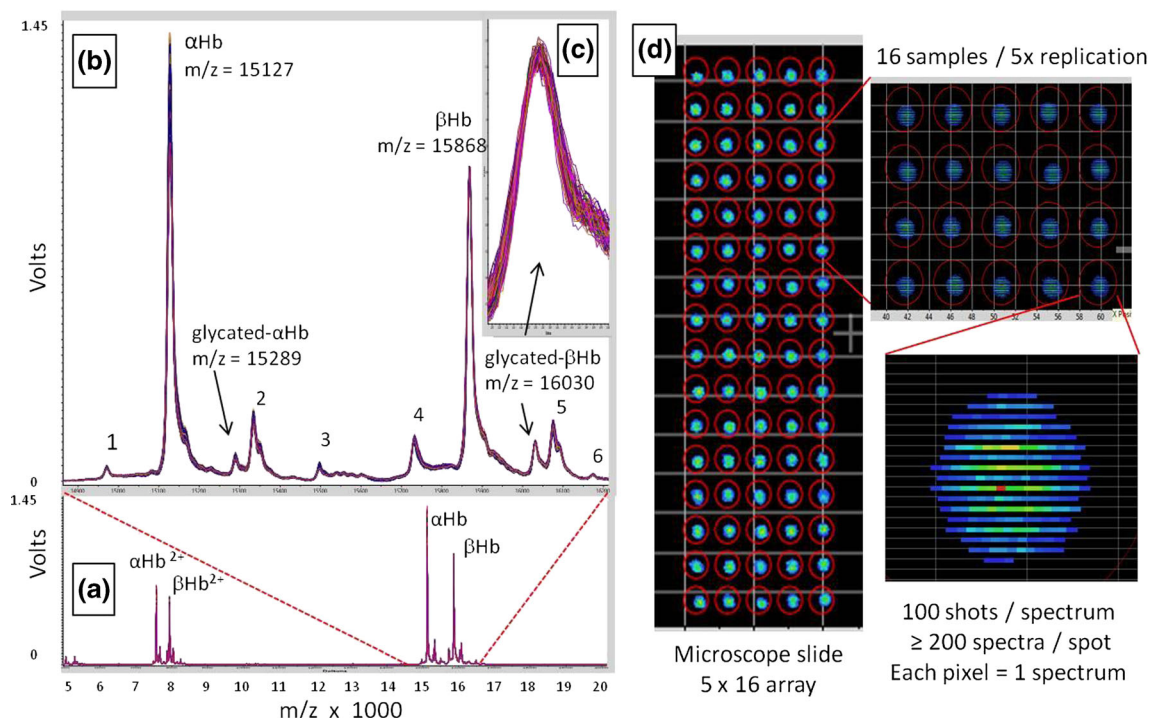
Figure 1. Overlay of 80 replicate analyses of a single whole blood sample diluted to a ratio of 1:2000. The spectra show the dominating Hb1+ and Hb2+ species (used as calibrants). Other lesser abundant peaks in the spectrum (marked 1–6) may also be assigned to hemoglobin by inference based on their masses (included in auxiliary data file). The m/z assignments of these peaks are as follows: 1 = αHb missing C terminal arginine; 2 = sinapinic acid matrix adducts to αHb; 3 = m/z^{2+} species of the α-βHb dimer; 4 = βHb missing C-terminal histidine; 5 = sinapinic acid matrix adducts to βHb; 6 = glutathione modification to βHb; (**d**) displays a graphic demonstrating sample acquisition in regards to data collection across a typical sample spot, sample row, and sample plate

chains and the glycated forms of each, all of which are marked in Figure 1b. The glycated-βHb peak is expanded further (Figure 1c) to highlight the measurement reproducibility. Quantitative analysis of this plate followed the processing protocol by treating each row as five replicates of a distinct sample and resulted in a % glycated-βHb of 13.14% with a CV of 1.22% for the 16 rows and an average CV for intra-sample precision of 0.90% (data included in auxiliary file). The right side of the figure (1d) demonstrates the sample acquisition process. One μL sample depositions result in ~ 2 mm dried spots arranged in a 5 × 16 array (1 row = 1 sample). Figure 1d shows a post-analysis "heat map" of the location and relative intensity of all spectra passing a minimum signal intensity threshold for the presence of targeted analyte. For each spot, all spectra are averaged to create a single "spot-averaged" spectrum. Statistics regarding intra-sample reproducibility are garnered from the five technical replicates that constitute each sample deposition.

Figure 2 shows the overlay of spectra resulting from an analysis of the 6 Lyphochek Hemoglobin A1c Linearity Set calibration standards (Figure 2a) and a plot of the results of 6 separate and independent analyses of these standards (Figure 2b). The table inset into Figure 2a the figure shows the % HbA1c range for each standard as determined by the National Glycohemoglobin Standardization Program (NGSP) [42] by an approved HPLC method. Figure 2a shows an overlay of the five spectra acquired from the technical

replicates for each standard and demonstrates an increase in the glycated-βHb signal relative to the unmodified βHb peak as expected. The integrated peak area (ion count) from the βHb and glycated-βHb peaks are used to calculate the (glycated-βHb/(βHb + glycated-βHb)) ratio. Each of the spectra shown in the figure is an average of ~200–250 individual spectra obtained from each sample spot. The statistical coefficients of variance (CV) calculated for five measurements of % glycated-βHb in each of the standards shown in Figure 2a are 2.90, 2.18, 2.98, 1.59, 0.83, and 1.37% for levels 1–6, respectively.

Figure 2b shows a plot from six separate analyses of Lyphochek HbA1c standards plotted against the mid-range % HbA1c as reported for that particular standard. If the MALDI-TOF MS data were to agree exactly with the mid-range value of each standard, the results would plot to create a line of slope = 1; this idealized result is plotted as NGSP (blue diamond) in the figure. The error bars associated with this idealized result represent the range of expected % HbA1c measurement values for each sample. As the plot shows, the MALDI-TOF MS measurements are elevated in comparison to reported % HbA1c values. Each separate MALDI-TOF MS trial (T1–T6) represents an independent sample preparation and the error bars associated with the MS data equate to ±2× the standard deviation of the average value for the six measurements. The calibration curve shown is constructed using the average value of the six analyses for each standard and is drawn through the MALDI-TOF MS data. This curve defines the relationship between the % HbA1c
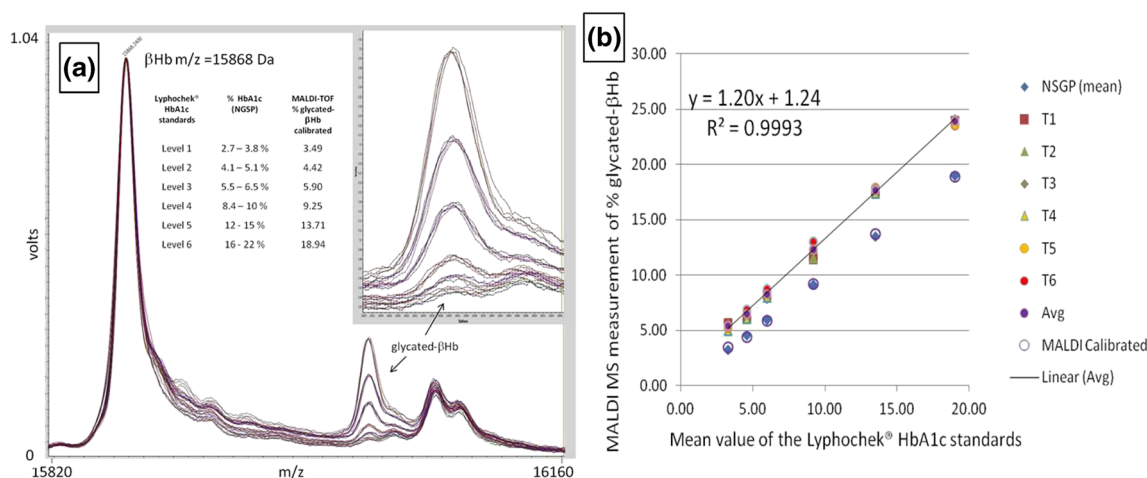
Figure 2.   An overlay (**a**) of all spectra obtained from the five technical replicates run on each of the six Lyphochek Hemoglobin A1c Linearity Set calibration standards from one of the six trial analyses (T1–T6) performed on these standards. The table inset in (**a**) shows the NGSP range for % HbA1c for each of the standards and the calibrated value for that the standard as determined by MALDI-TOF MS; (**b**) shows MALDI-TOF MS data obtained from six independent analyses of Lyphochek HbA1c standards (T1–T6). The MALDI-TOF MS data is plotted in reference to an idealized measurement (NGSP (mean) blue diamond) to demonstrate a perfect agreement between the mid-range of NGSP % HbA1c and the MALDI-TOF data. The curve generated from the average value (purple circle Avg) of the six experimental measurements is used to generate a calibration equation (y = 1.20× + 1.24) that expresses the relationship between the MALDI-TOF MS measurement of % glycated-βHb and % HbAlc in the standards. The calibration equation can be inverted x (x = (y-1.24)/1.2) and applied to the MALDI-TOF data and transform it into "MALDI calibrated" (O) data. As shown, for each concentration, the purple open O symbol surrounds the blue diamond
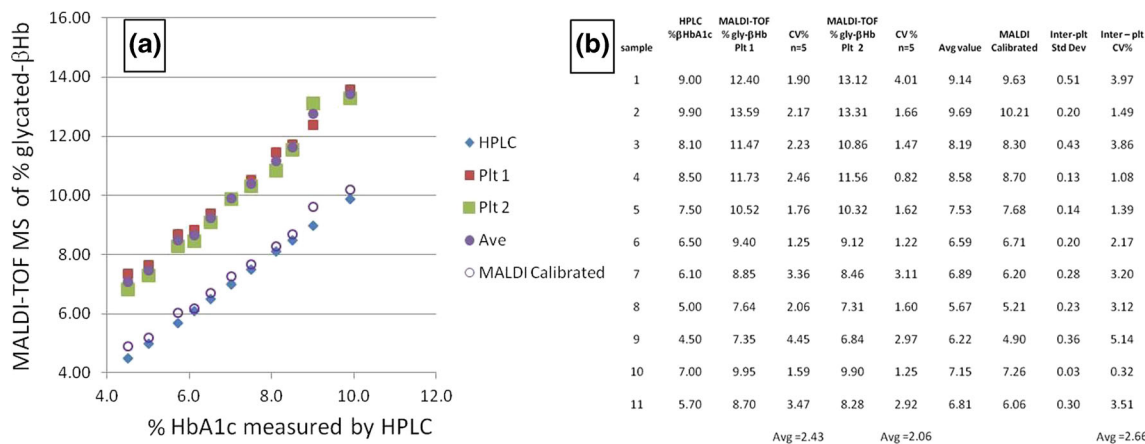
values of the Lyphochek HbA1c standards and the MALDI-TOF MS measurement of % glycated-βHb, and this calibration curve can be used to translate the MALDI-TOF MS measurements of % glycated-βHb from clinical samples (unknowns) into an equivalent measure of % HbA1c. For MALDI-TOF MS measurements, % glycated-βHb = (1.20 × % HbA1c) + 1.24 and, therefore, for MALDI-TOF MS measurement of % glycated-βHb the inverse of this equation can be used to translate the value into an equivalent value of % HbA1c [% HbA1c = (% glycated-βHb − 1.24)/1.20)]. This inverse calculation was performed on the average MALDI-TOF MS value for each of the six HbA1c standards and these data are plotted as "MALDI Calibrated" (O) in the figure.

Figure 3 shows results from two independent analyses of 11 whole blood samples specifically chosen to span the clinically relevant range of % HbA1c. The samples were diluted separately and spotted onto different plates. Prior to MALDI-TOF MS, these samples had been analyzed in a clinical laboratory using the validated HPLC method. As with the analysis of Lyphochek Standards in Figure 2b, a linear relationship between the HPLC data and the MALDI-TOF MS data is evident. The MALDI-TOF MS data from each plate are internally consistent but are once again elevated in comparison to the HPLC derived data. However, the same linear relationship established in analysis of the Lyphochek HbA1c standards (x = (y-1.24)/1.2) can be used to calibrated the data. The average value of the duplicate analyses was used for this calibration and the results are plotted as "MALDI calibrated" (O). These calibrated values demonstrate good agreement between the MALDI-TOF derived data and the HPLC derived data. Table 1 lists the MALDI-TOF derived % glycated-βHb and intra-

sample % CV as calculated from the five technical replicates of each sample. The average and calibrated values are also listed along with the inter-plate % CV for each sample. The average % CV for all measurements was 2.43% for plate 1 and 2.66% for plate 2.

Figure 4 shows the results from an analysis of a second set of 18 clinical blood samples. This second set of clinical samples is presented because they were prepared in a different laboratory, by a different person, ~ 4 mo apart in time, and analyzed on a different MALDI-TOF mass spectrometer than sample set 1 shown in Figure 3. For this second analysis, the six Lyphochek HbA1c samples were spotted and analyzed on the same MALDI targets as the samples. Samples were again prepared in duplicate on different plates and data for both analyses as well as the average value for each sample are plotted against the HPLC derived % HbA1c measurement. The Lyphochek HbA1c standards analyzed along with the samples are also plotted and used to create a calibration equation that worked out to be ($MS_{calibrated} = (MS_{measured} − 1.94)/1.15$) for this experiment. The calibration equation was applied to the MALDI-TOF data and the results are plotted as "MALDI calibrated" (O) and demonstrate good agreement with the HPLC derived % HbA1c values. The average % CV for five technical replicates of all sample were 2.12% and 1.47% for plates 1 and 2, respectively, with an inter-plate measurement CV of 2.51% (all data inlucued in auxiliary file).

Figure 5 shows plots of the MS derived % glycated-αHb plotted as a function of % glycated-βHb as determined for all samples in both sets of clinical samples. As seen from the plot, there is a clear correlation between the extent of glycation on αHb to that of the βHb. The difference in magnitude of the

sample	HPLC %βHbA1c	MALDI-TOF % gly-βHb Plt 1	CV% n=5	MALDI-TOF % gly-βHb Plt 2	CV% n=5	Avg value	MALDI Calibrated	Inter-plt Std Dev	Inter-plt CV%
1	9.00	12.40	1.90	13.12	4.01	9.14	9.63	0.51	3.97
2	9.90	13.59	2.17	13.31	1.66	9.69	10.21	0.20	1.49
3	8.10	11.47	2.23	10.86	1.47	8.19	8.30	0.43	3.86
4	8.50	11.73	2.46	11.56	0.82	8.58	8.70	0.13	1.08
5	7.50	10.52	1.76	10.32	1.62	7.53	7.68	0.14	1.39
6	6.50	9.40	1.25	9.12	1.22	6.59	6.71	0.20	2.17
7	6.10	8.85	3.36	8.46	3.11	6.89	6.20	0.28	3.20
8	5.00	7.64	2.06	7.31	1.60	5.67	5.21	0.23	3.12
9	4.50	7.35	4.45	6.84	2.97	6.22	4.90	0.36	5.14
10	7.00	9.95	1.59	9.90	1.25	7.15	7.26	0.03	0.32
11	5.70	8.70	3.47	8.28	2.92	6.81	6.06	0.30	3.51
			Avg =2.43		Avg =2.06				Avg =2.66

Figure 3. Plot of % glycated-βHb from the duplicate analyses of clinical sample set I (**a**) (plt 1 red square and plt 2 green square). Plot shows the MALDI- TOF MS measurements of the two sample plates along with average (purple circle Avg) of the two measurements and MALDI calibrated (O) results calculated using the calibration relationship established between MALDI-TOF MS measurments and the Lyphochek HbA1c standards demonstrated in (Figure 2) and these data show good agreement with HPLC measurement of HbA1c (blue diamond); (**b**) is a table with caluclated values for % glycated-βHb of the 11 samples on the both plates along with the % CVs calculated for intra-sample technical replicates (n = 5) and for the inter-plate duplicate measurement. The HPLC measurement of HbA1c and the calibrated MS value are also shown (all data contained in auxiliary data file)

glycation modification on the two chains can be determined by ratio of the precent ratios (% glycated-αHb/% glycated-βHb). For the samples analyzed in this study, the average for this ratio is 0.66 with a CV of 4.97% showing that βHb undergoes a higher degree of glycation and that the extent of each modification is dependent on blood glucose concentration.

In addition to the singly charged hemoglobin species, the doubly charged hemoglobin species are prominent peaks in the MALDI-TOF MS spectra of whole blood. Comparing the glycation measurements of βHb as calculated from the singly (βHb^{1+}) and doubly (βHb^{2+}) charged hemoglobin species for all samples analyzed in this study by ratio (% glycated-βHb^{2+}/% glycated-βHb^{1+}) led to a value of 0.96 (Std. Dev. = 0.05 and CV

of 5.55%), clearly demonstrating the expected correlation between these measurements (all data included in auxiliary file).

One can also monitor the relationships between other peaks and follow these trends as a means of assessing the "normalcy" of a given blood sample in regards to its consistency with a hemoglobin A profile. For example, monitoring the relationship between the main αHb and βHb chains using the same calculation (αHb/αHb + βHb) results in a ratio of 44.24% (Std. Dev. = 1.94 and CV of 4.38%) for the 48 samples including the standards (all data included in auxiliary file). The consistency of this relationship, as well as others, may be of diagnostic value for the detection of certain variant hemoglobin forms as demonstrated in Figure 6.
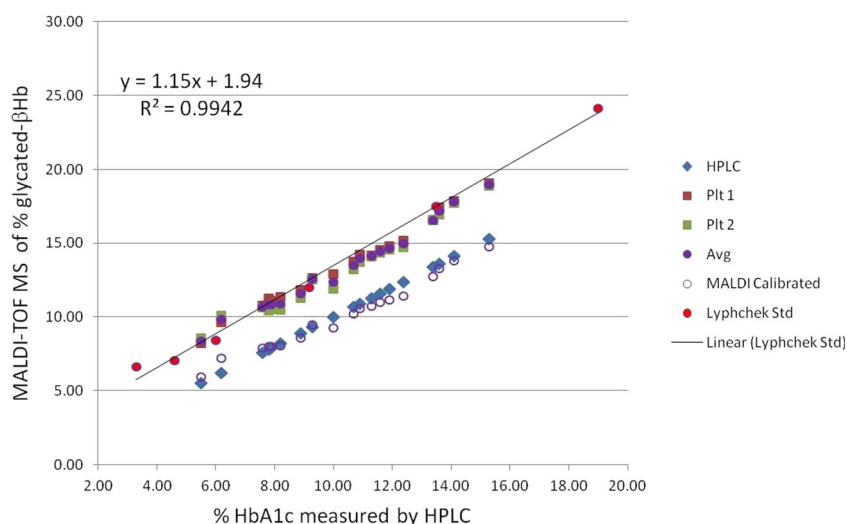
Figure 4. Plot of MALDI-TOF date for % glycated-βHb for clinical sample set II. Plot shows the MALDI-TOF MS measurements from duplicate analysis (plt 1 red square and plt 2 green square) for 18 clinical samples as well as average value (purple circle Avg) for each measurement. Lyphochek % HbA1c standards were analyzed along with standard are also plotted and the calibration equation generated from these standards is used to calibrate the MALDI-TOF Ms data (O MALDI Calibrated). Calibrated MALDI data show good agreement with HPLC (blue diamond) derived data

$Y = 0.57x + 0.93$
$R^2 = 0.9772$

♦ Clinical data set 1
■ Clinical data set 2
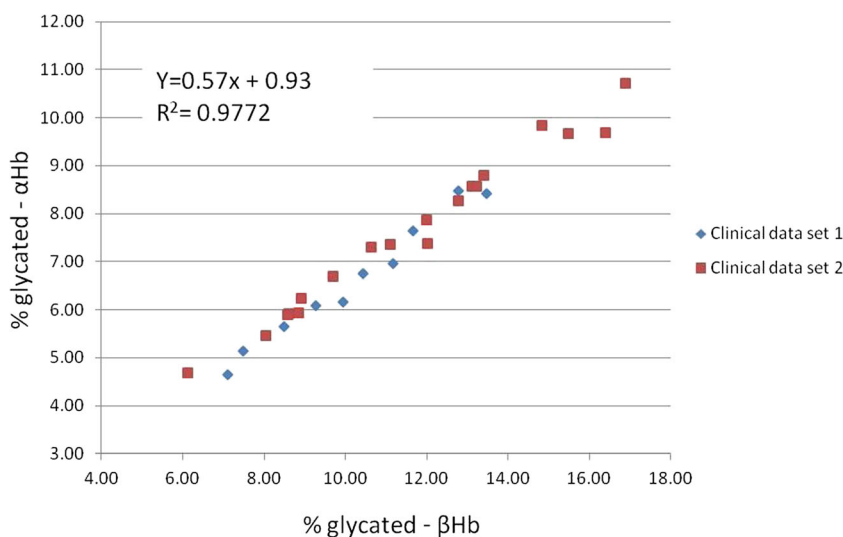
Figure 5. Plot of MALDI-TOF MS derived % glycated β-Hb versus % glycated-αHb for each sample in both of the clinical sample sets used in this study. Plot demonstrates that the extent of glycation on both hemoglobin subunits is correlated level and dependent on blood glucose concentration

Figure 6 shows an overlay of spectra from a hemoglobin A sample with that of variant hemoglobin S (HbS) [43]. The figure also demonstrates the various relationships that can be monitored by MALDI-TOF MS and used to assess the status of hemoglobin in any given sample. HbS is a common variant resulting from a single valine to glutamic acid substitution at position 6 of βHb. Figure 6 shows that this substitution reduces the mass of the Hb-subunit by 30 Da, and distorts the (αHb/αHb + βHb) ratio from 0.45 in the case of the hemoglobin A (green trace) sample to 0.39 in the case of hemoglobin S (blue trace) sample. By carefully monitoring the relationships among the prominent hemoglobin species as outlined in Figure 6, it may be possible to detect when a sample is "variant" and with further inspection, potentially identify the variant form. Additionally, based on the identity of the variant species, an alternative means for evaluating hemoglobin glycation may be

used, for example, determination by using the ratio obtained from % glycated-αHb as opposed to that obtained from % glycated-βHb.

Discussion

The current prevalence and predicted rise in the number of people afflicted with type-2 DM clearly marks the disease as a worldwide health concern. Regardless, with proper diagnosis, monitoring, and adaption and adherence to a prescribed lifestyle [44–46], type-2 DM can be prevented, or if present, reasonably managed. A key element to the effective and efficient management of type-2 DM is accurate, accessible, cost-effective diagnostic tests for monitoring blood glucose levels.

This study presents a protocol for an assay based on MALDI-TOF MS for the measurement and relative quantitation of glycated-βHb directly from whole blood and supports and validates the results of an earlier investigation into the potential for MALDI-TOF MS based quantitative assay for glycated-βHb. MALDI-TOF MS is well suited for this task because the glucose modification causes a 162 Da increase in the molecular mass of βHb subunit, a difference easily distinguished. MALDI-TOF MS measures both the βHb and glycated-βHb molecules in a single spectrum, and this allows the signal generated by each to be compared directly, and the extent of modification determined and reported as a ratio, in the same manner as current HPLC and CE methodologies: % glycated-βHb = 100 * (glycated-βHb/(βHb + glycated-βHb)). The ability to directly compare signals internal to the sample is a tremendous benefit to the assay as it negates the need for addition of an external standard for quantitation.

A quantitative response (analyte signal ∝ [analyte]) was determined for hemoglobin across a concentration range of ~1–5 μM. Based on factors such as health, age, sex, etc., hemoglobin concentration in blood can vary between individuals
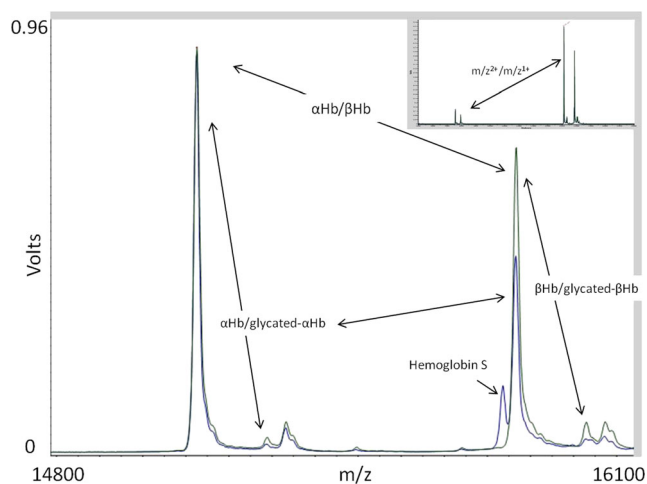
Figure 6. Overlay of "normal" hemoglobin A spectrum (green trace) with that of a sample containing the hemoglobin S variant (blue trace) demonstrating several of the hemoglobin relationships that can be monitored by MALDI-TOF MS

(~1.0–2.8 mM) [47]. In spite of this variation, an approximately 2000-fold dilution results in a sample preparation where the concentration of Hb is appropriate for MALDI-TOF MS analyses. Since the total spotted volume (i.e., matrix and sample) is only 1 μL, the equivalent of only 0.5 nL of blood is used for the analysis.

In whole blood samples, the high relative abundance of Hb in comparison to other blood proteins results in prominent MALDI-TOF MS signals for both the singly and doubly charged αHb and βHb species, allowing them to serve as internal mass calibrants for all spectra. The ability to calibrate the mass spectrometer on species internal to the sample simplifies the assay by eliminating the need to add external mass calibrants; also, calibrating directly on the analyte(s) of interest helps ensure exceptional accuracy in the mass determination of these molecules.

Precision in MALDI-TOF analyses is a consequence of thorough sample interrogation. A 1 μL sample deposition results in a ~2 mm diameter sample spot. Systematically rastering across a given spot at 100 μm intervals, using the conditions outlined the Experimental section, typically leads to a minimum of 200 individual spectra (individual data points) per sample. This corresponds to 20,000 laser shots summed per spectrum. The accumulation and averaging of data on this scale leads to statistical precision in measurement. To demonstrate the performance characteristic of the MALDI-TOF MS platform in regards to precision, all samples were run in 5× technical replication. The methodology outlined in our procedure resulted in a coefficient of variance of ~2.5% across entire analyses. HbA1c specifically designates glucose attachment to the N-terminal residue of βHb. As mentioned, glucose may also modify any one or more of the 11 lysine residues present on βHb. Although glycation of lysine is reported to be significantly lower than that of the N-terminal modification, our assay cannot distinguish between these isobaric monoglycated species. Regardless, the results presented in this study for % glycated-βHb obtained for the analysis of clinical patient samples are in good agreement with the results % HbA1c obtained from a clinical validated HPLC method. Therefore, any inaccuracy in the MALDI-TOF MS results due to the measurement of "mono" glycated-βHb as opposed to the exclusive measure of HbA1c does not appear to be significant. Also, in comparing the results derived by MALDI-TOF MS with those from HPLC it is important to remember that the MS data points represent the compilation of the analysis of five technical replicates and the accumulation of thousands of MS spectra, whereas the HPLC data points are the result of a single analysis.

Similar to the βHb subunit, the αHb subunit can also be glycated on its N-terminal valine residue or any of the 11 lysine residues in its primary sequence. The distribution of the modification is reported as being different between the two chains [33–35]; in particular, αHb Lys-61 is reported to undergo a greater degree of glycation than the N-terminal valine on αHb. This complication has no bearing on the present MALDI-TOF MS measurements that combine all "mono-glycated" species. Similar to βHb glycation, measurements of αHb glycation

demonstrate that the extent of the modification is proportional to blood glucose concentration; also, within a given sample, comparison of the results for % glycated-αHb with those of % glycated-βHb yields a consistent proportionality of % glycated-αHb/% glycated-βHb of ~0.66. The reasons behind variability in the degree of glycation amongst the numerous sites on both the αHb and βHb have been speculated to be due, in-part, to steric hindrance, primary structure, acidity, and tertiary structure of the particular site involved; regardless, present emphasis is placed on the consistency in the extent of total glycation that occurs on the two chains within a given sample. We hypothesize that the routine measurement of % glycated-αHb, along with that of % glycated-βHb, can be used to establish the relationship between the two phenomena with a defined degree of statistical certainty for individuals who express hemoglobin A (i.e., normal or non-variant hemoglobin; 95% of the population). Once sufficiently characterized, we hypothesize that this relationship can be used as an additional piece of information to either validate or challenge either of the calculated values as a matter of routine in the course of running the assay. All of the information needed for % glycated-αHb determination is included in every MALDI-TOF spectrum, and determination of % glycated-αHb is achieved with the same ease and has the same analysis-inherent characteristics as those discussed for % glycated-βHb measurements. Therefore, for 95% of the population, the correlation between the % glycated-αHb and % glycated-βHb measurements should agree within the established statistical confidence intervals. Any discrepancy between these two independent values may be flagged as it may indicate either a mutated hemoglobin chain or an error in the assay of that particular sample.

In addition to the singly charged hemoglobin species, the doubly charged hemoglobin species are prominent peaks in the MALDI-TOF MS spectra of whole blood. The only difference between singly and doubly charged molecules of a given species is that the doubly charged molecule has obtained an additional proton during the course of ionization. Statistically, this occurrence should be proportional and precise for a given molecule in a given sample. Therefore, evaluation of the % glycated-βHb as determined by analysis of βHb^{2+} and glycated-βHb^{2+} should yield values equivalent to those obtained by the analysis of the singly charged species. In this study, the ratio of the percent glycation determined from these differently charged βHb ions (% glycated-βHb^{2+}/% glycated-βHb^{1+}) was 0.96 with a CV of 5.55%. Here again, we hypothesize that the routine measurement of % glycated-βHb as derived from these two sets of ions can serve as an additional piece of information to either validate or challenge a given % glycated-βHb measurement.

Our results also demonstrate a consistency in the signal intensities of unmodified αHb and βHb with calculated ratio for αHb/(αHb + βHb) of 44.24% and CV of 4.38% across all samples. Analysis of a small subset of samples containing the HbS modification showed significant distortion in this ratio and demonstrates the potential usefulness of monitoring the

relationship between αHb and βHb for detecting HbS, and potentially other variant hemoglobin forms.

Our study has focused almost exclusively on the analysis of hemoglobin A. Although hemoglobin A is expressed in ~95% of the population, there are more than 1200 know variant hemoglobin forms [48, 49]. There is great interest and need for the accurate detection and assignment of hemoglobin variants chains, not only for the blood glucose assessment but for the potential diagnosis of other medical disorders (sickle cell anemia, thalassemia) [50, 51]. Although MALDI-TOF MS may not be suitable for distinguishing all variants, as cases exist where the mass difference between forms is only ±1 Da (i.e., HbC and HbE), there are many other hemoglobin variants that are expected to be readily detectable.

Currently, spectra are acquired by rastering across samples at 100 μm intervals at a scan rate of 1 mm/s. Accordingly, the analysis of a single spot takes ~40 s and a complete sample spotted in 5× replication takes ~4 min. The speed of this current protocol is fast in comparison to other methods (~3 min for one measurement by HPLC) [52] and it comes with intra-sample replication, corroborative information to validate the primary measurement, and the capability for re-analysis of the same sample if necessary. Regardless, by further exploiting the capabilities of the mass spectrometer's maximum 5 kHz laser and 10 mm/s scan speed, there is potential to decrease the analysis time by an order of magnitude. In addition to an increase in acquisition speed, we also believe that an assay for % glycated-βHb based on MALDI-TOF MS will come at a reduced cost of analysis per sample in comparison with current methods. Although the initial instrument cost of a MALDI-TOF mass spectrometer may exceed that of HPLC or CE unit, the low cost of sample preparation, low cost of instrument operation and maintenance, and the potential for a much higher through-put analytical platform should make MALDI-TOF MS an economically attractive option for performing % glycated-βHb analyses.

Conclusion

MALDI-TOF MS analysis and quantitation of glycated-βHb is both feasible and practical and contains some distinct advantages over currently practiced methods. The approach is accurate, precise, sensitive, rapid, and requires minimum sample workup. The analysis is calibrated with commercially available reference materials and is demonstrated to be portable between different laboratories and different mass spectrometers. Both quantification and mass calibration are performed by using signals internal to each sample, thereby eliminating the need for addition of external reference materials. Additional and independent measurements are made simultaneously and these measurements serve to strengthen the confidence of the primary % glycated-βHb measurement and potentially aid in Hb variant-form determination.

Our future goal is to analyze a larger cohort of clinical samples (~1000s). The results from this large study will serve to better quantify and assign statistical significance to the relationships described herein and to assess the ability of the assay to correctly detect and potentially assign different variant forms of hemoglobin.

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