Quantitation of Glycated Hemoglobin by MALDI Mass Spectrometry

Stephen J. Hattan[1], Kenneth C. Parker[1], Marvin L. Vestal[1], Jang Y. Yang[2], David A. Herold[2,3], Mark W.Duncan[4]

[1]SimulTof Systems, 60 Union Avenue, Suite 1-R, Sudbury, MA 01776
[2]Department of Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-9113
[3]VAMC-San Diego, 3350 La Jolla Village Drive, San Diego, CA 92161
[4]University of Colorado School of Medicine, Division of Endocrinology, Metabolism & Diabetes
MS 8106, 12801 E. 17th Avenue, Room 7103, Aurora, CO 80045

Hemoglobin A1c (HbA1c) is a stable product resulting from glucose attachment to the N-terminus of the β-chain of hemoglobin. Quantification of hemoglobin A1c is clinically significant because it reflects the average, standing blood glucose concentration, and is therefore a statistically relevant diagnostic for diabetes. A healthy level of HbA_{1c} in the blood is 4%-5% of the total hemoglobin, as compared to 10%-15% in the case of an untreated diabetic. Currently, the most widely accepted method for measuring hemoglobin A1c is based on quantification by absorbance following separation by liquid chromatography. Although effective, this methodology ignores other glycosylated hemoglobin species, and can fail if patients express variant forms of hemoglobin. Moreover, it requires samples to be injected and separated sequentially.

In this paper we demonstrate a new method for quantification of hemoglobin glycosylation levels based on MALDI mass spectrometry. The method we present has several advantages over existing LC based methods. It offers direct detection of unmodified hemoglobin alpha and beta chains, and their glycosylated forms, in a single mass spectrum based on molecular mass. Additionally, spectral information may be used to determine if variant hemoglobin forms are present. Sample preparation requires mere dilution of whole blood samples into the appropriate formulation of MALDI matrix. Multiple sample preparations from an individual patient, or from different patients, can be loaded together into the mass spectrometer, and spectra can be collected within minutes.

Covalent attachment of glucose (glycation) at the N-terminal valine residue of Hb changes the overall charge on the molecule. Based on this charge difference, ion exchange chromatography can be used to separate HbA1c from Hb, while the absorbance of heme is used for quantification. Thus, the percentage of HbA1c is calculated from the areas of the peaks

known to correspond to unmodified hemoglobin and HbA1c. Hemoglobin molecules modified elsewhere and any hemoglobin genetic variants are detectable by absorbance, but do not elute in the expected locations, and sometimes compromise correct quantification of the Hb and HbA1c peaks.

As an alternative strategy, MALDI mass spectrometry can be used to quantify glycosylation, because covalent attachment of glucose also *changes the mass* of the molecule by adding an additional 162 Da for every glucose attachment. From the mass spectra, the integrated peak area (ion count) for the two species can be accurately quantified to obtain an HbA1c ratio (HbA1c / (Hb + HbA1c)) in a manner similar to LC methods. However, in addition to direct detection of HbA1c, MS methodology also offers information regarding comparative α-chain glycosylation. We have found that this second intensity ratio tracks closely with β-chain glycosylation if no variants are present, and can therefore be used to rule out variants or spectra of low quality. Also, doubly charged and triply charged hemoglobin molecules are commonly detected, and additional corroborative quantitative information about glycosylation can be collected from them. If the alpha and beta glycosylation ratios are inconsistent, then variant hemoglobin forms can be sought, for example, the most commonly encountered hemoglobin S variant. If the quality of the spectra is poor, based on inconsistent alpha, beta, doubly charged alpha, and doubly charge beta glycosylation ratios, more spectra can be collected from the same sample within minutes, or if necessary, from duplicate samples.

In this presentation we will give a brief overview of hemoglobin chemistry and the disease of diabetes. We will discuss the pros and cons of the current methodologies used for making HbA1c blood level estimates and outline the utility and advantages of an HbA1c assay based on MALDI MS. We will discuss the logic and present the experimentation used in developing our current protocol and present a statistically based argument regarding its merits. We will present comparative data, contrasting HbA1c estimates obtained from samples analyzed by both LC and MS methods. Lastly, we will highlight the additional, clinically-relevant information that our MS based analysis can capture, including the detection of Hb variant forms, and auxiliary information regarding α-chain glycosylation that may be used to make a more accurate determination of health or disease in a patient.

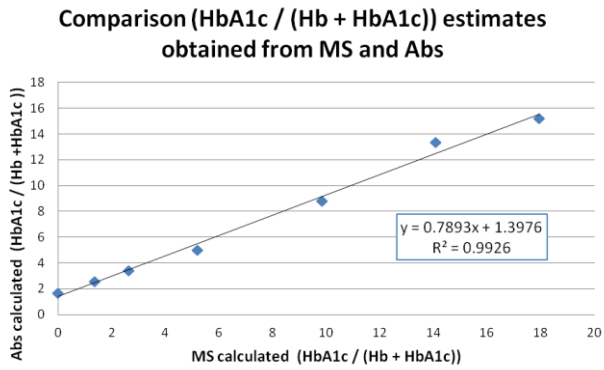
Methods:

Hb / HbA1c Standard Curve preparation

Lyphochek® Hemoglobin A1c Linearity Set was purchased from BioRad (Hercules, CA). The level 6 standard (16-22% HbA1c) was separated following A1c analytical protocol using a Mono S cation-exchange column (GE Healthcare, Uppsala, Sweden). Lyophilized hemoglobin standard was purchased from Lee Biosolutions (St. Louis, MN). Lyophilized hemoglobin was weighed and diluted to create a range of protein concentrations; triplicate absorbance measurements at each concentration were averaged and used to construct an absorbance based calibration curve. Hb and HbA1c separated from the BioRad A1c calibration standard were collected and reanalyzed by absorbance against our calibration curve for protein concentration determination. Isolated Hb and HbA1c were then remixed in proportion to mimic and span clinically relevant HbA1c blood levels (0 – 20 %). Constructed standards were then analyzed by MALDI MS (according to protocol outlined below) to create an MS calibration curve. Figure 1 shows the results obtained by MS plotted against those obtained by absorbance with the 7 data points used in curve construction. Table 1 shows the average (HbA1c / (Hb + HbA1c)) ratio calculated at each calibration point and coefficient of variance calculated from amongst the 5 replicates at each concentration. Figure 2 shows an overlay of averaged MALDI spectra collected for each calibration point with an expansion of the HbA1c peak.

Patient Samples

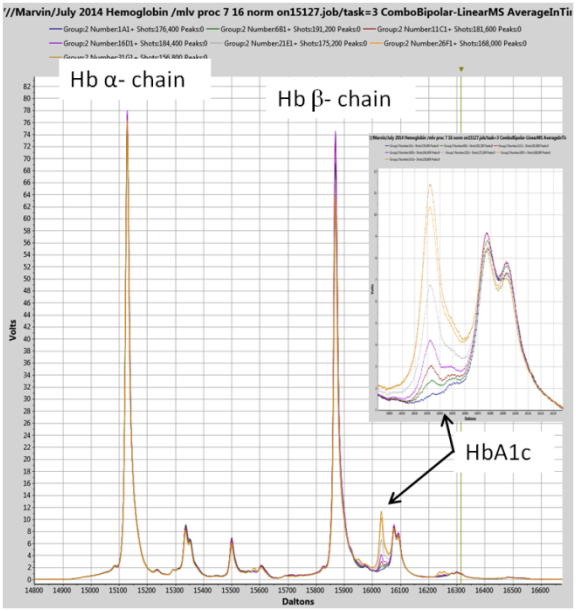
Whole blood is collected in heparinized tubes following normal phlebotomic protocol. Collected samples are then further diluted 1:1000 in DI H_2O, vortexed and centrifuged (3000 rpm) then mixed 1:1 with 10 mg / mL Sinapinic Acid (50% ACN, 0.1% TFA). One µL of this preparation is spotted onto a stainless steel MALDI target. For the purposes of assay evaluation and development, all samples are currently analyzed in 5X replicate. Table 2 shows the results from the analysis of single sample analyzed in 5X replicate 16 consecutive times (16 trials of 5X each). Table 2 displays the average (HbA1c / (Hb + HbA1c)) ratio calculated for each of 16 trials based on the average measurement of 5 replicates and coefficient of variance calculated amongst the 5 replicates at each trial point.



HbA1c / (Hb + HbA1c)	CV 5 reps
0	2.80
1.36	1.65
2.64	1.66
5.79	0.26
9.83	1.11
14.08	1.41
17.94	0.96

Figure 1: HbA1c concentration study to evaluate MS based assay's ability to measure (HbA1c / (Hb + HbA1c)) over the clinically relevant range.

Table 1: Constructed (HbA1c / (Hb + HbA1c)) concentrations and coefficient of variance calculations for the 5 replicates measured at each concentration



	A1c	CV
T 1	13.52	1.39
T 2	13.48	0.71
T 3	13.20	0.62
T 4	13.07	0.99
T 5	13.07	0.96
T 6	13.11	0.90
T 7	12.98	0.67
T 8	12.99	1.19
T 9	13.05	0.96
T 10	12.99	0.99
T 11	13.08	0.92
T 12	13.01	0.66
T 13	13.16	1.00
T 14	13.15	0.84
T 15	13.22	0.70
T 16	13.17	0.85
Average	13.14	0.90
Std Dev	0.16	
Rel Std Dev	1.22	

Figure 2: Overlay of averaged spectra for each of 7 data points in the HbA1c concentration study. Expansion highlights the HbA1c peak. Figure 2 serves to show the utility of an HbA1c assay based on MALDI MS.

Table 2: Results for a 16 trial analysis of a single sample analyzed in 5X replicates. The table demonstrates the reproducibility of the MS assay.