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Characterization of Hemoglobin Variants by MALDI-TOF MS Using a Polyurethane Membrane as the Sample Support

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A new method for the sampling and off-site analysis of hemoglobin variants by mass spectrometry is reported. This technique uses a nonporous polyurethane membrane as the collection device and transportation medium of a blood sample for analysis. The same membrane is then used as the MALDI-TOF MS sample support for mass spectrometric analysis. Minimal invasive sample collection is afforded by collecting less than 1 μ L of blood using a common lancet device. MALDI-TOF MS is performed directly on the membrane, after washing off the interfering plasma components, followed by the addition of matrix. This reduces the time of analysis and prevents sample loss. Enzymatic digestion can be performed directly on the membrane, using in this case trypsin, allowing for further characterization of the sample. The method is much less invasive compared to drawing blood with a syringe. The sample may be transported to the laboratory by regular mail, and thus the method can serve remote locations. We demonstrate the procedure by characterizing the Hb Shepherds Bush hemoglobin variant, b74-(E18)Gly $\rightarrow$ Asp.

Hemoglobin disorders arise due to structural changes in the chains of hemoglobin and are responsible for a number of disease states. Essentially, four types of structural modifications may occur in abnormal hemoglobin: amino acid substitution, deletion of an amino acid, elongation of a chain, and fusion or hybridization of the DNA during meiosis. The most common modification is replacement of one amino acid with another. Several hundred variants have been described and approximately two-thirds affect the β chain.¹ The modifications may remain asymptomatic or may manifest themselves clinically. With the implementation of newborn screening programs, the health risks associated with the late detection of an abnormality could be minimized, but because

of the large number of possible variants, the need exists for a rapid and cost-effective method of analysis.

The standard methods used for the characterization of hemoglobin variants are based upon traditional electrophoresis techniques and are time-consuming. This limits the number of samples that can be analyzed in a given time period.^{1,2} These methods involve isolation of hemolysate from red blood cells followed by analysis by electrophoresis to establish normality. Further characterization may be performed by proteolytic digestion of the chains followed by electrophoretic or high-performance liquid chromatographic (HPLC) separation of the fragments. The resulting peptide maps must be interpreted by a skilled technician in order to diagnose the existence of a variant. More complete characterization requires sequencing of the globin chains.

Application of mass spectrometry to the analysis of hemoglobin variants constitutes a new, rapid, and accurate means for the detection and characterization of changes occurring in the globin chains.³ The role of mass spectrometry (MS) when applied to the identification of hemoglobin variants has recently been discussed in detail by Shackleton.³ A number of approaches have been investigated, including analysis of the intact globin by electrospray ionization (ESI)⁴⁻⁸ and matrix-assisted laser desorption/ionization (MALDI)⁹⁻¹² and characterization of proteolytic

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(1) Alphabetical Hemoglobin Variants List *Hemoglobin* **1994**, 18, 163–183.

fragments by liquid secondary ion mass spectrometry (LSIMS)¹³ and ESI.¹⁴ Most of these approaches involve the application of several sample pretreatment steps prior to mass spectrometric analysis, including sample purification to a varying extent. These steps are necessary in order to remove some of the components present in the blood matrix, such as plasma proteins and salts, which could cause interference during acquisition of mass spectra.

Characterization of hemoglobin specifically by MALDI has been the subject of a few reports, and some degree of sample purification was most often required.^{9–12} For instance, MALDI was used to examine the extent of glycation of hemoglobin as a possible indicator for diabetes.^{10,11} Recently, Houston and Reilly reported on the characterization of sickle cell hemoglobin.⁹ In this example, good-quality MALDI spectra of human hemoglobin were obtained by simply diluting whole blood prior to analysis.

In general, MALDI is relatively tolerant of impurities, i.e., salts and buffers, which would hamper other ionization processes such as ESI. Delayed extraction, combined with reflecting time-of-flight (TOF) mass analysis, provides high resolution and allows accurate mass measurements in the ppm range for sample components of fairly complex mixtures.¹⁵ MALDI-TOF MS^{16–19} has therefore been used by many research groups as a convenient and rapid means for the characterization of proteins and peptides derived from biological samples. Despite MALDI being tolerant of impurities, biologically derived samples must still be isolated and purified prior to analysis to obtain the best results. High contaminant concentrations strongly disrupt crystallization and quench MALDI signals. Lower concentrations result in adduct formation on the analyte molecular ions and thus produce broadened and poorly resolved peaks. Several methods of sample purification prior to MALDI-TOF MS analysis have been developed and include dialysis and chromatography. Both methods have limitations, such as sample loss and time-consuming sample preparation.

Carrying out the sample purification, and other treatment processes including proteolytic digestion, on the surface of the MALDI probe avoids many sources of sample loss. Different approaches have been studied, including the use of membranes and films placed on the surface of MALDI probes and of chemically modified probes supporting active surfaces. Examples of the latter have been reported by Brockman and Orlando and include a C₁₈ reversed-phase surface²⁰ as well as an antibody/antigen surface.²¹ MALDI probes with covalently bound trypsin and other enzymes have been extensively developed by Nelson and co-workers, who demonstrated the on-probe digestion of

bovine hemoglobin using a trypsin-active probe.^{22,23} According to this study,²³ detergents were used to facilitate the denaturation of hemoglobin on the probe surface, which resulted in an increased extent of digestion compared to digests performed without the detergent. However, modification of the probe surface as described above is time-consuming, and the probes have a limited lifetime. Also, there exists the possibility of sample carry-over if the probe is reused. In addition, samples must still be transported to the MALDI-TOF MS laboratory by conventional means, i.e., in solution and on ice.

The use of membranes as sample supports has recently been adopted as a means of sample purification and sample introduction into the mass spectrometer.^{24–28} Membrane supports investigated include nylon,^{24,27} polyethylene,^{25–27} polypropylene,²⁶ nitrocellulose,²⁶ poly(vinylidene difluoride) (PVDF),²⁷ regenerated cellulose,²⁷ and polyurethane (PU).²⁸ Deposition of aqueous protein solutions onto membrane supports has been shown to enhance MALDI signals, especially for samples containing buffer components in higher concentrations than can generally be tolerated. If the analyte of interest is selectively adsorbed onto the modified probe, interfering substances can be washed off, while the analyte is retained. Purification by on-probe washing and performance of on-probe enzymatic digestion result in minimal sample loss, since proteins and peptides are bound fairly strongly to the membranes by hydrophobic interactions. Membranes are also very convenient for transporting dry protein samples from the preparation laboratory to the MALDI-TOF MS laboratory and allow for easy introduction of the samples into the mass spectrometer.

It has been demonstrated that it is not necessary to use a liquid sample in order to apply mass spectrometry to the analysis of whole blood. Methods have been developed using samples of dried blood spots obtained from newborn screening cards.²⁹ Chace et al. developed a method for the diagnosis of phenylketonuria and maple syrup urine disease.^{30,31} The procedure involved extracting the blood proteins from the screening card, followed by hydrolysis, chemical derivatization, and analysis by tandem mass spectrometry (MS/MS). Wada et al. applied LSIMS to the characterization of γ -globins as part of a newborn screening program.¹³ After reconstituting the dried blood from the screening cards, initial screening was performed using electrophoresis. The samples were then separated by HPLC to yield purified γ -globins. These were subjected to tryptic digestion and the fragments were

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characterized by LSIMS. These studies demonstrated that MS/MS and LSIMS can yield rapid, accurate, and informative results when dried blood is used as the analyte. However, both experiments required a relatively large sample size and considerable extent of workup prior to the analysis by MS.

In this paper, we demonstrate the application of a PU membrane as a sample support for the mass spectrometric characterization of the Hb Shepherds Bush hemoglobin variant b74(E18)Gly→Asp.^{32–35} Sampling is performed outside of the laboratory with a common lancet device, and less than 1 μ L of whole blood is collected directly on the PU membrane. This method does not require a large volume of liquid sample for analysis. Once dried, the sample may be sent to the mass spectrometry laboratory by regular mail, i.e., no particular precautions are required for transportation.²⁹ It is not necessary to transport the sample on ice, since the sample has been dried on the membrane. On-membrane washing protocols are used to process the sample with minimal extent of workup prior to analysis by mass spectrometry. On-membrane proteolytic digestion allows for further characterization of the globin chains. The choice of PU instead of other sample supports is based on an earlier study, where it was discussed in detail.²⁸

EXPERIMENTAL SECTION

Reagents and Materials. Regular hemoglobin was obtained from whole blood provided by some of the authors. The Shepherds Bush variant hemoglobin was provided by M. Smith. Solutions of horse heart myoglobin (16 951 Da) and bovine insulin (5733 Da) (Sigma Chemicals, St. Louis, MO) were prepared in water (10^{-6} – 10^{-4} M) and used without further purification. Deionized, filtered water was obtained from a Barnstead Nano-Pure water filtration system supplied by a reverse-osmosis feedstock. Analytical grade acetic acid, HPLC-grade acetonitrile, and electronic-grade methanol were purchased from Mallinckrodt (Paris, KY). Sinapinic acid, used as the MALDI matrix (saturated in 50:50 water/acetonitrile), was obtained from Sigma. The nonporous ether-type PU membrane, 50 μ m in thickness (XPR625-FS), was supplied by Stevens Elastomerics (Northampton, MA). The PU membrane was washed with water and methanol prior to use.

Collection of Whole Blood on PU Membranes. The sampling protocol is similar to that used for blood collection onto newborn screening cards.³¹ Prior to sample collection, the PU membrane was cleaned in methanol and allowed to dry. Under aseptic conditions, using a common lancet device, approximately 0.5–1 μ L of blood was drawn, which was sufficient for preparing two samples. The drop of blood was placed onto the PU membrane by lightly touching the membrane against the subject's finger. To avoid possible contamination, the contact time between the finger and the PU membrane was minimized. The samples were allowed to dry under ambient conditions. To some samples, 2 μ L of methanol was added prior to drying in order to disrupt coagulation, enhance cell lysis, and enhance protein binding onto

the membrane. The Shepherds Bush variant samples were shipped to our MALDI-TOF laboratory via courier. No particular precautions were taken when the samples were transported; i.e., they were not shipped on ice.

Sample preparation for mass spectrometry was performed according to the protocols previously developed and used with PU membranes.²⁸ Matrix preparation for MALDI was based on the dried drop method.³⁶ In the case of standards used for calibration, 2 μ L of standard solution was placed on the membrane and allowed to dry slowly. Methanol (2 μ L) was added to the dried blood samples or the dried calibrant samples and allowed to dry. The samples were then rinsed at least twice with 20- μ L aliquots of water. The water was removed each time with a pipet after 1 min, and the samples were allowed to dry. The matrix solution (2 μ L) was then added, and the mixture was allowed to crystallize slowly. After drying of the matrix, the membrane was placed onto a 1-cm-diameter metal disk which had been coated with a thin layer of adhesive (Spraymount, 3M). The excess membrane was trimmed from the disk and the disk placed into the MALDI probe. The probe assembly and sample preparation are described in more detail in an earlier publication.²⁸

On-Membrane Tryptic Digestion. Tryptic digestions of blood samples were performed directly on the PU membrane. Prior to any washing steps, trypsin, (2–10 μ L, 0.01 mg/mL in 20 mM Tris-HCl, pH 7.5) was placed directly onto the protein spots on the PU membrane. Digestion was allowed to proceed for periods of time ranging from 2 to 60 min. Digestions were stopped by adding 1 μ L of a 1% solution of acetic acid. The samples were then processed as outlined above with the washing protocol.

MALDI-TOF MS. MALDI-TOF MS was performed in the linear mode on a reflecting TOF instrument constructed in our laboratory,³⁷ using sinapinic acid as the matrix (saturated in 50:50 H₂O/ACN),³⁸ and an accelerating potential of 25 kV. To avoid saturation of the detector by low-mass matrix ions, the electron converter plate was pulsed on $\sim$ 19 000 ns after each laser shot. Delayed extraction experiments were performed on the same instrument with a delay time of 700 ms, a pulse height of 3 kV, and an accelerating potential of 20 kV. Spectra were obtained using a nitrogen laser (337 nm) with the fluence adjusted slightly above threshold. Each spectrum presented here results from the summation of 40–50 consecutive shots. External and internal calibrations were performed. External calibrations for measurements using the PU membrane were performed with standards prepared on similar membrane targets.

Safety Considerations. Standard precautions regarding the collection of blood and handling of biological fluids should be followed. Gloves should be worn during collection and manipulation of the samples.

RESULTS AND DISCUSSION

MALDI-TOF MS of Normal and Shepherds Bush Hemoglobin. Hb Shepherds Bush is a mutation in the β chain of hemoglobin, b74(E18)Gly→Asp, which is presumed to result from a mutation in the DNA sequence at codon 74: GGC → GAC.^{32–34}

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Figure 1. MALDI-TOF mass spectra of the α and β chains of (a) normal hemoglobin and (b) hemoglobin containing the Shepherd's Bush variant. Approximately 1 μ L of whole blood was collected on the PU membrane and allowed to dry. The samples were then subjected to the washing protocol. Each spectrum is the sum of 40–50 shots. Delayed extraction was used with external calibration. Peaks correspond to the α and β chains of hemoglobin, in the +1 and +2 charged states. The variant b74(E18)Gly→Asp is indicated by a mass shift of 58 Da.

Figure 2. Expanded MALDI-TOF mass spectra of the α and β chains of hemoglobin containing the Shepherd's Bush variant. Conditions were the same as in Figure 1. Peaks correspond to the α and β chains of hemoglobin, in the (a) +2 and (b) +1 charged states. The variant b74(E18)Gly→Asp is indicated by a mass shift of 58 Da.

This variant results in an increased oxygen affinity and expresses itself as mild hemolytic anemia in the patient. The variant hemoglobin makes up for $\sim 24\%$ of the total hemoglobin and exhibits thermal instability.³² The Shepherd's Bush mutation is relatively rare but serves as a good example to demonstrate the feasibility and advantages of our MALDI analysis protocol. The MALDI-TOF MS spectra of the α and β chains of hemoglobin are shown in Figure 1. Here, a comparison is made between the normal chains and those from the Shepherd's Bush variant. An expansion of the mass spectrum of the Shepherd's Bush variant is shown in Figure 2. The measured masses of the normal globin chains were α $[M + H]^+ = 11\,528.98$ Da and β $[M + H]^+ = 15\,870.38$ Da ($n = 3$), in agreement with the calculated average masses derived from the sequence ($[M + H]^+ = 15\,127.3$ and

15 868.2 Da, respectively). Mass accuracy was $\sim 0.01\%$ using internal calibration and the resolution was ~ 800 (fwhm). The mass obtained for the variant was $[M + H]^+ = 15\,928.83$ Da, an increment of 58.45 Da (58.04 Da theoretical) relative to the normal β chain, which was clearly observable in the mass spectrum. The peak area ratio observed for the normal α and β chains was similar to that reported by others, with the α chain being predominant.⁹ However, the peak area for the shepherd's bush chain was observed to range from about 25 to 100% of the normal β chain peak area. The upper value is larger than the reported value of 24% based on electrophoretic measurements.³² This discrepancy may be attributed to nonuniform MALDI ionization phenomena.^{39,40}

Preparation of samples on PU membranes and introduction of the samples into the mass spectrometer were relatively simple. Both steps were facilitated by the probe design. Samples could be prepared on the PU membranes and affixed to the metallic disks, allowing for analysis at a later date in the MALDI-TOF MS laboratory. The dual-part probe design enabled the introduction of samples at a rate of one every few minutes. The longest delay was due to the evacuation of the mass spectrometer.

Collection of samples of whole blood on the membrane with the lancet device constitutes a relatively noninvasive means of obtaining a sample, in comparison to the use of a syringe. The sampling protocol is easy to follow and may be performed by an untrained individual. The presence of a health care practitioner is thus not necessary to draw a liquid sample. Samples were shipped to the laboratory by courier and no precautions were taken with regards to transportation. Samples were analyzed up to seven days after acquisition with no observable change in the quality of the mass spectra. For prolonged storage, the sample-loaded membranes were placed in the freezer at $\sim -20^\circ\text{C}$.

When the blood was collected on PU membranes, smaller sample amounts provided for better results than larger amounts. More than 1–2 μ L of whole blood resulted in excessive coagulation within the sample itself. This produced a scab, which did not strongly bind to the membrane and which in one case actually detached itself from the membrane during transportation. In addition, larger samples of whole blood proved to be more difficult to resolubilize for cocrystallization with the matrix or for on-membrane digestion. In most cases, not all of the blood sample was resolubilized. Excess material was removed in the washing procedure. However, this did not decrease the intensity of the MALDI-TOF MS signals, presumably due to the high sensitivity of the MALDI method. A 1- μ L sample of whole blood contains ~ 2.5 pmol of hemoglobin, more than that required for MALDI-TOF MS.

The formation of scabs led us to investigate the use of modifiers that would disrupt coagulation of the sample on the membrane. Our initial work on PU membranes²⁸ showed that the addition of methanol to samples deposited on the membrane caused swelling of the PU and enhanced protein sorption. Proteins prepared on PU without the addition of methanol were desorbed from the membrane more easily when washed with water. Methanol possibly disrupts the intermolecular forces

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holding the polymer chains together, thus allowing an increase of the effective surface area available for protein sorption. At the same time, it facilitates the partitioning of proteins and peptides from more polar components, such as salts. The effectiveness of methanol in disrupting the coagulation process was thus investigated. The addition of methanol to freshly collected liquid samples resulted in a decrease in the extent of coagulation compared with samples prepared without methanol. The MALDI-TOF MS spectra were slightly improved compared to those obtained for the samples prepared without the addition of methanol. This improvement was attributed to cell lysis rather than a decrease in coagulation. We are currently investigating other chemical modifiers, such as heparin, which would disrupt coagulation and thus favor protein/membrane interactions.

As discussed previously by Houston and Reilly,⁹ a mass resolution of 1000 (fwhm) will allow the detection of a variant with a mass difference of ~ 16 Da at $m/z = 15867$ from that of the normal component. This is insufficient to resolve all possible variants occurring in the hemoglobin chains. One possible means of improving the identification of variants is to use ESI-MS in combination with a separation technique such as HPLC.^{3,5} This improves the resolution to ~ 6 Da when deconvolution is used. Another method is to perform a proteolytic digestion of the α and β chains of hemoglobin in order to produce smaller fragments. For example, with a mass resolution of 1000 (fwhm) and a molecular mass of a proteolytic fragment of 5000 Da, it would be possible to resolve two peaks with a mass difference of 5 Da. This method would extend the number of variants that could be characterized using a MALDI-TOF instrument with moderate resolution. Recently, Krutchinsky et al. introduced an orthogonal injection MALDI-TOF mass spectrometer with a resolution of ~ 5000 (fwhm) at 6000 Da and a mass accuracy of ~ 30 ppm.⁴¹ With this resolution and accuracy it would be possible to identify mass differences of ~ 3 Da for the globin chains. Tryptic fragments may be characterized with isotopic resolution, thus ensuring accurate peptide mass mapping. We are currently investigating the use of this instrument for hemoglobin characterization using PU membranes as sample supports.⁴²

On-Membrane Tryptic Digestion of Hemoglobin. Proteolytic digestion allows for peptide mapping and may be used to pinpoint the site of the mutation in the peptide chain. Peptide maps can be used to identify a protein but, in this case, can also be used to generate patterns that are indicative of a specific variant.³ Not only will there be a mass shift in one of the peptides as a result of the variant, but the extent of proteolysis and the cleavage sites may change due to the relative instabilities of variant chains compared with those of normal hemoglobin, which are generally more stable.^{32,33}

Our membrane methodology was applied by performing tryptic digests of the normal and Shepherds Bush hemoglobin directly on the membrane. Digests were performed on the membrane for periods of time varying from 2 to 60 min. Good-quality MALDI spectra were obtained once buffer components were removed using the washing procedure. Figure 3 shows the mass spectra

Figure 3. MALDI-TOF mass spectra of the tryptic fragments of the α and β chains of normal hemoglobin. Digestion was performed directly on the membrane for (a) 2, (b) 5, (c) 10, and (d) 60 min. Each spectrum is the sum of 40–50 shots. Spectra were acquired in linear mode with internal calibration based on known peaks.

Figure 4. MALDI-TOF mass spectra of tryptic fragments of the α and β chains of (a) normal hemoglobin and (b) hemoglobin containing the Shepherds Bush variant. Each spectrum is the sum of 40–50 shots. Delayed extraction was used with internal calibration based on known peaks. Each sample was digested directly on the membrane, with trypsin, for 10 min. Peak assignments are in Table 1. Asterisked peaks indicate fragments derived from the variant b74-(E18)Gly→Asp.

obtained for the 2-, 5-, 10-, and 60-min digests of normal hemoglobin. During digestion, the initially abundant high-mass ions were gradually replaced with lower mass ions. Also, the

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Table 1. Fragments of the α and β Chains of Normal and Shepherds Bush Variant Hemoglobins^a

tryptic		calcd mass [M + H] ⁺	normal hemoglobin		Shepherds Bush Variant	
fragment	sequence ^b		mass [M + H] ⁺	% error	mass [M + H] ⁺	% error
b4	31–40	1275.55	1275.54	0.001		
b13	121–132	1379.56			1383.56	0.289
b12	105–120	1721.13	1720.52	0.035	1719.64	0.087
a6	41–56	1835.03	1834.27	0.041		
b5	41–59	2060.30	2059.99	0.015	2059.37	0.045
b2–3	9–30	2229.50	2229.69	0.009		
b10–11	83–104	2530.83	2531.07	0.009		
a5–6	32–56	2888.32	2888.64	0.011		
a12	100–127	2969.52	2968.77	0.025		
a9	62–90	2998.35	2997.17	0.039		
b12–13	105–132	3081.66	3082.02	0.012		
b1–3	1–30	3163.56	3162.45	0.035		
a9–10	62–92	3267.70	3267.39	0.009		
a5–7	32–60	3267.74	3267.39	0.011		
b4–5	31–59	3316.81	3316.63	0.005	3314.55	0.068
b2–4	9–40	3486.01	3483.72	0.066		
a7–9	57–90	3505.94			3500.99	0.141
a11–12	93–127	3769.45	3767.42	0.054		
a10–11	91–127	4038.80	4039.56	0.019		
a12–13	100–139	4203.98	4205.26	0.030	4202.02	0.047
b10–12	83–120	4232.93	4230.62	0.055		
a1–5	1–40	4250.87	4251.40	0.012		
a4–6	17–56	4399.94	4400.48	0.012		
b1–4	1–40	4420.07			4417.83	0.051
a12–14	100–141	4523.35	4524.37	0.023	4520.77	0.057
b5–9	*41–82	4519.14			4520.77	0.036
b3–5	18–59	4613.21	4613.72	0.011	4611.76	0.031
a11–13	93–139	5003.91	5003.91	0.000	5001.77	0.043
a10–13	91–139	5273.26	5273.60	0.006	5271.72	0.029
a6–9	41–90	5321.95	5323.34	0.026	5320.6	0.025
b11–14	96–144	5321.23	5323.34	0.040	5320.6	0.012
b2–5	9–59	5527.29	5527.58	0.005	5526.11	0.021
a6–10	41–92	5591.30	5593.17	0.033	5591.92	0.011
a10–14	91–141	5592.62	5593.17	0.010	5591.92	0.013
b10–13	83–132	5593.46	5593.17	0.005	5591.92	0.028
a2–7	8–60	5735.47			5733.28	0.038
b2–6	9–61	5754.59			5753.29	0.023
b9–12	*67–120	5942.85			5939.32	0.059
b1–5	1–59	6461.35	6461.53	0.003	6461.24	0.002
b1–6	1–61	6688.65	6687.57	0.016	6686.94	0.026
b10–15	83–146	7025.12	7023.64	0.021	7024.54	0.008
b9–13	67–132	7245.35	7245.85	0.007		
b9–13	*67–132	7303.38			7302.55	0.011
b8–13	66–132	7373.52	7374.04	0.007	7371.98	0.021
b8–13	*66–132	7431.55			7431.32	0.003
a9–13	62–139	8252.58	8251.99	0.007	8252.92	0.004
a8–13	61–139	8380.76	8380.84	0.001	8379.47	0.015
a9–14	62–141	8571.95	8571.95	0.000	8571.42	0.006
b9–15	67–146	8677.01	8677.13	0.001		
a8–14	61–141	8700.12	8699.75	0.004	8699.48	0.007
b9–15	*67–146	8735.04			8734.65	0.004
b8–15	66–146	8805.19	8803.01	0.025		
b8–15	*66–146	8863.21			8862.21	0.011
b1–9	*1–82	8920.19			8919.16	0.012

^a Peak assignments correspond to those observed for the 10-min digests referred to in Figure 4. ^b * labeled fragments are from the Shepherds Bush variant β -globin.

protein underwent significant digestion after only 2 min. This may indicate that the protein becomes denatured upon sorption onto the membrane, thus facilitating rapid digestion. The digestion times were varied in order to optimize the procedure. Ideally, the best situation would be a relatively short digestion time which would allow formation of enough fragments to identify the variant. A 10-min digestion was chosen as it produced an abundance of fragments within the mass range of 1000–9000 Da with good reproducibility.

Figure 4 shows the MALDI-TOF spectra obtained for 10-min tryptic digests of (a) normal hemoglobin and (b) the Shepherds Bush variant. Most segments of the protein were mapped against calculated fragments by mass, as shown in Table 1. Digestion of the variant produced seven observable fragments which contained the mass shift corresponding to the Gly-Asp mutation. These are indicated with an asterisk in Table 1. Significant digestion was observed after 10 min, allowing for the characterization of the entire β variant. Three ambiguous assignments appear in Table

Figure 5. Peptide map of tryptic fragments (10-min digest) of the (a) α and (b) β chains of normal hemoglobin, and the (c) α and (d) β chains of a sample containing Shepherds Bush variant hemoglobin.

1, in pairs at 3267, 5321, and 5593 Da. It was not possible to resolve these pairs of peptides using MALDI, due to the small mass differences. Further characterization would thus require digestion with a different enzyme or the use of sequencing methods. The 10-min digestion of normal hemoglobin and of the variant produced a number of different fragments, as indicated in Table 1 and on the peptide map shown in Figure 5.

The peaks associated with β -chain fragments containing the variant were much more intense in comparison to analogous peaks from the normal β chain appearing in the same mass spectrum. In some cases, the peaks corresponding to the normal fragments were not observed. For example, in the mass spectrum of Figure 4b, there is no peak observed at 7245.85 Da (normal); only the variant peak at 7302.55 is observed. This is surprising, as the variant makes up for only 24% of the heterozygote. It is unlikely that the fragments containing the variant would produce a more intense signal based only on MALDI considerations, as the Gly-Asp shift should not significantly enhance the proton affinity of the fragment. A possible but unlikely explanation is that the fragments containing the variant are retained more strongly on the PU membrane during the washing process. A more reasonable possibility is that the variant hemoglobin is likely to undergo more extensive proteolytic digestion because of its decreased stability due to the amino acid substitution.^{32,33} Replacement of Gly with Asp results in an increased charge within the heme pocket of the globin. This weakens the hydrophobic forces in the center and destabilizes the globin, allowing easier penetration of water. The digestion results suggest that the variant, due to its reduced stability relative to that of normal hemoglobin, undergoes slightly different denaturation and digestion.^{32,33}

Mapping of the proteolytic fragments may be used for positive identification of the variant in addition to simply measuring the mass differences of the globin chains. By performing the digest, it was possible to further characterize the Shepherds Bush variant. The mass difference observed in the β chain, following the tryptic

digest, could be assigned to the sequence 67–82. With a second digestion using a different enzyme, or with the use of postsource decay methods, it may be possible to sequence the chains and accurately pinpoint the substitution site.

Our method allows for the rapid generation of a peptide map, which in turn allows for rapid identification with the help of computerized searches. In combination with automated laboratory methods of analysis⁴³, this technology would open the possibility to routinely characterize samples from various locations and would favor accuracy and cost-effectiveness.

CONCLUSION

Our preliminary results on the characterization/differentiation of normal and variant hemoglobin are favorable. MALDI mass spectra obtained on both the normal and Shepherds Bush hemoglobin samples are of good quality and informative. Sampling is easy to effect and may be performed by an inexperienced user. Sample handling and shipment is facilitated by using the membrane; i.e., a liquid sample is not required. Analysis is rapid, and minimal time is required for hands-on preparation in the laboratory. Samples may be collected in any setting outside of the mass spectrometry laboratory, with a lancet device and a small piece of PU membrane ($1 \times 1 \text{ cm}^2$). Less than $1 \mu\text{L}$ of whole blood is sufficient to produce good-quality MALDI-TOF MS spectra, and there is no need for a large volume of liquid sample. The analysis is sensitive because loss is minimized during digestion and during the washing steps as both processes are conducted on the membrane. The use of PU membranes with MALDI adds no significant cost to the analysis and thus may be applied on a routine basis. On-membrane digestion with database mapping should facilitate identification of abnormal amino acid sites in the sequence of the α and β chains. MALDI-TOF MS

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spectra of the intact chains may give molecular weight information on the chain, which may constitute insufficient information for complete identification of a variant. We are currently refining our methodology and are investigating the possibility of analyzing whole blood for thalassemias.

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