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MALDI-ToF/MS as a Diagnostic Tool for the Confirmation of Sulfur Mustard Exposure†**

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Key words: laser desorption; mass spectrometry; chemical agents; diagnosis; sulfur mustard; protein adducts.

The continual threat of chemical and biological warfare has prompted the need for unambiguous analytical methods for the confirmation of agent exposure. In this paper, we have investigated the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) as a diagnostic tool for this purpose. Mass spectral studies of the interaction of sulfur mustard (bis-(2-chloroethyl) sulfide, HD) with hemoglobin and metallothioneine were conducted. *In vitro* experiments with purified proteins were performed, using both HD and chloroethylethyl sulfide (CEES), in an effort to determine the extent of alkylation and occurrence of HD cross-linking using the MALDI-ToF/MS technique.

In a typical experiment, 50 ml of 5 mM HD in acetonitrile was added to an equal volume of 0.5 mM hemoglobin in deionized water followed by vortexing and incubation at room temperature. After 24 h, the samples were analyzed by MALDI-ToF/MS. Mass spectral results indicated the presence of at least two distinct alkylation adducts for both HD and CEES experiments. These results demonstrate that MALDI-ToF/MS is a useful analytical technique to investigate the interaction of HD with biomolecules and may be employed potentially as a diagnostic tool for the confirmation of exposure to chemical warfare agents. Published in 2000 by John Wiley & Sons, Ltd.

INTRODUCTION

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) is a relatively new mass spectrometric technique that has been used in molecular weight determination as well as mixture analysis of biomolecules such as proteins and peptides,[1–5] nucleotides,[6–12] glycolipids,[13] carbohydrates[14] and oligosacharides.[15] This technique has been proven to be a comparatively advantageous analytical technique that allows for fast mass analysis time and requires little sample preparation of biomacromolecules. In addition, MALDI-ToF/MS exhibits high sensitivity (detection limits < subpicomolar), good mass accuracy (<0.05%), large detection range (above 100 kDa) and high resolution capabilities. Initially, a sample is prepared by mixing the saturated matrix solution (usually prepared with pure forms of α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid or 2,5-dihydroxy benzoic acid in 0.3% aqueous trifluoroacetic acid containing 30% acetonitrile) with the analyte solution at an experimentally determined ratio before 1–2 μl of the final mixture is placed on the sample probe. The 'spotted' sample is then allowed to dry either under vacuum or in air until the analyte and matrix molecules co-crystallize on the probe. Subsequently, the probe is placed into the MALDI-ToF mass spectrometer where the matrix is desorbed by an ultraviolet (UV) laser beam permitting the analyte to be ionized, through matrix ionization energy transfer, predominantly as intact quasimolecular ions. The generated ions are then accelerated down a ToF path where they are separated based on their relative mass-to-charge ratios (*m/z*). The ions are then detected at the end of the flight tube by the mass detector while a real-time mass spectrum is observed simultaneously on a computer monitor.

Studies have shown previously that MALDI-ToF/MS can be utilized as an effective and rapid means for determining and identifying site modifications of proteins by alkylating reagents.[16,17] Because it has been reported that chemical agents such as nerve agents and sulfur mustard (HD) form covalent linkages with various cellular macromolecules,[18] these covalent interactions can conceivably be monitored and/or specifically identified as potential biological markers of exposure rather easily using MALDI-ToF/MS. Our preliminary studies are focused on the identification of the HD adducts of hemoglobin (Hb) and metallothioneine (MT). Interpretation of the mass spectra was attempted to identify the structures of the HD–biopolymer adducts.

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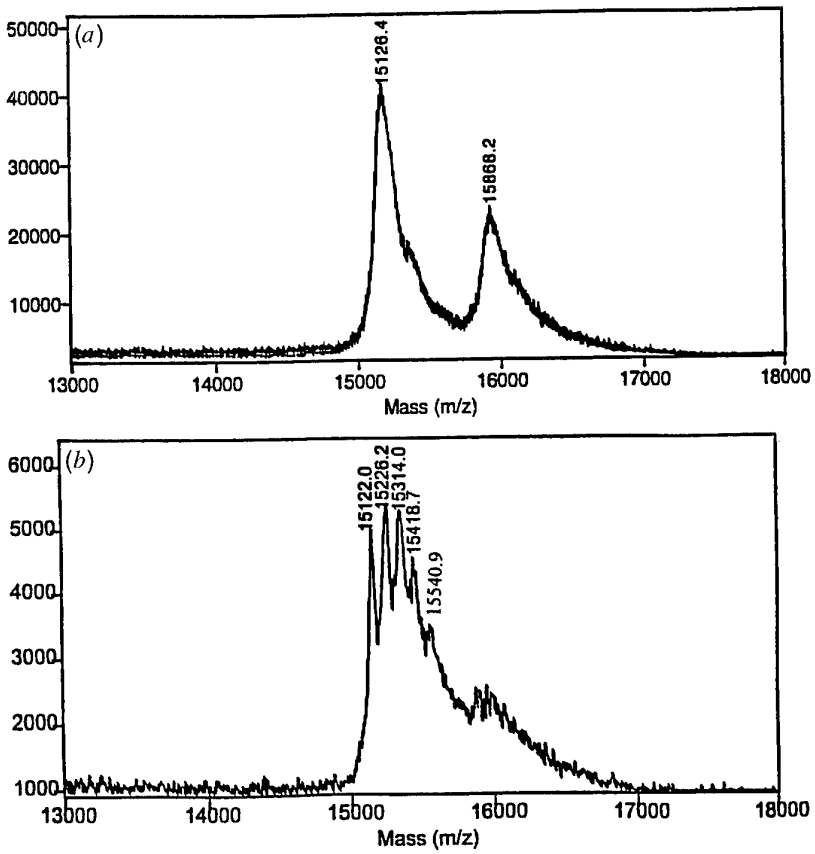

Figure 1. The MALDI mass spectra of Hb before (a) and after (b) sulfur mustard exposure.

EXPERIMENTAL

Instrumentation and sample preparation

The MALDI mass spectra were obtained in positive mode using a linear ToF instrument equipped with time-lag focusing (Voyager-DE Biospectrometry Workstation, PerSeptive Biosystems, Inc., Framingham, MA). Ionization was accomplished with a nitrogen laser operating at wavelength 337 nm with a 3-ns pulse width, and generated ions were accelerated at 20 and 25 kV. The matrix was prepared as a saturated solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in deionized water–acetonitrile (7:3) containing 0.3% trifluoroacetic acid (TFA). All protein samples, before and/or after exposures, were diluted to at least 5 μM concentration before MALDI/MS analysis. Approximately 1 μl of α-CHCA solution was placed on the MALDI sample probe, and 1 μl of the diluted MT sample(s) was immediately added on top of the matrix droplet. The resulting sample–matrix droplet was then allowed to dry in air until complete co-crystallization occurred. The Hb samples were prepared for MALDI mass analysis in the same manner except that the α-CHCA matrix was replaced with sinapinic acid matrix.

Table 1. Proposed Hb–HD alkylated products observed by MALDI-ToF/MS (data from Fig. 1b)

Proposed type of HD–Hb adduct	Total mass of HD moieties	Number of HD units adducted to Hb	Peaks observed (*m/z*)	Calculated mass (relative to *m/z* 15122.0, unreacted Hb)
MOH	105.2	1	15226.2	15227.2
M, MOH	193.4	2	15314.0	15315.4
M, 2MOH or 2M, MCl	298.6 or 300.0	3	15418.7	15420.6 or 15422.0
M, 2MOH, MCl or 2M, 2MCl	422.2 or 423.6	4	15540.9	15544.2 or 15545.6

M = $-CH_2CH_2SCH_2CH_2-$; MOH = $-CH_2CH_2SCH_2CH_2OH$; MCl = $-CH_2CH_2SCH_2CH_2Cl$.

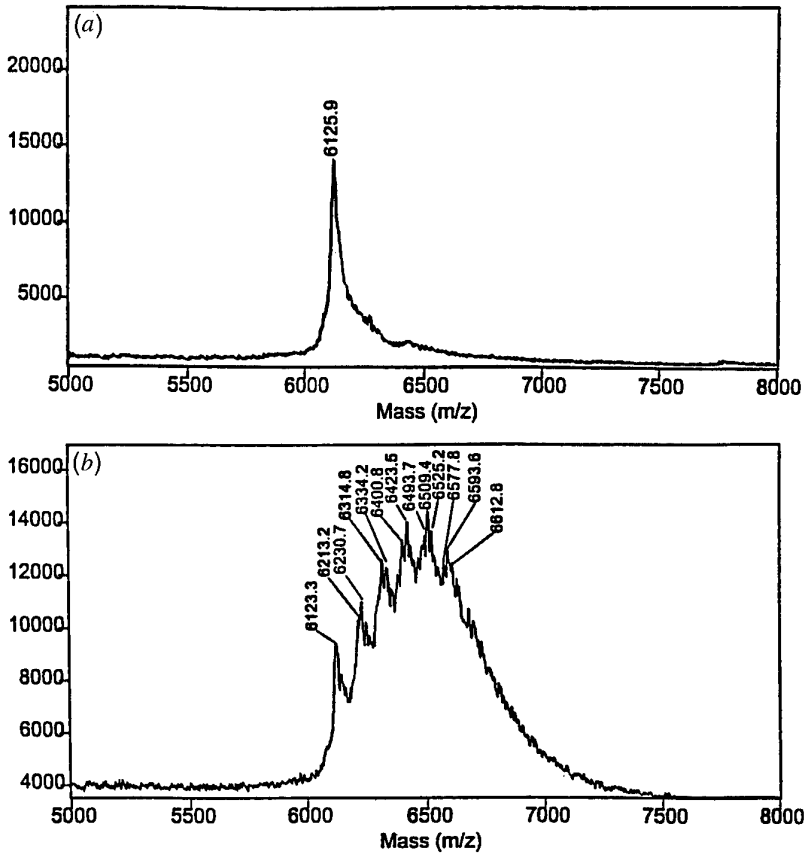

Figure 2. The MALDI mass pectra of MT before (a) and after (b) sulfur mustard exposure.

Chemicals

Rabbit liver MT, human Hb, chloroethylethylsulfide (CEES), α-CHCA, sinapinic acid, TFA and acetonitrile (HPLC grade) were obtained from Sigma Chemical Company (St Louis, MO). Sulfur mustard was obtained from the US Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD) in 97.5% purity.

Exposure of Hb to HD

Hemoglobin samples of 50 μL each of 3 mM, 0.5 mM and 0.025 mM were prepared in deionized water. Subsequently, 50 μl of 5 mM HD in acetonitrile was added to each sample, followed by vortexing. The three samples were allowed to incubate at room temperature for at least 24 h before they were subjected to MALDI/MS analysis.

Exposure of MT to HD

Aqueous MT samples of 0.5 mM (2 μl), 0.5 mM (4 μl) and 0.5 mM (8 μl) were each exposed to 2 μl of 4 mM HD prepared in acetonitrile. Samples were allowed to incubate for 24 h at room temperature before MALDI/MS analysis was performed.

Exposure of Hb to CEES

The CEES–Hb experiments were modeled after the HD–Hb experiments except that 10 mM CEES was substituted for HD.

Exposure of MT to CEES

Metallothioneine samples of 10 μl each of 0.50 mM, 0.20 mM and 0.10 mM were prepared in deionized water before mixing each sample with an equal volume of 5 mM CEES (prepared in acetonitrile). Additionally, two separate 2-μl samples of 0.5 mM MT were exposed to 2.5 mM and 0.50 mM CEES, respectively. Each exposed MT sample was allowed to incubate for 24 h at room temperature before analyzing the samples via MALDI/MS.

RESULTS AND DISCUSSION

Alkylated products were observed when Hb and MT were exposed to HD. Figure 1 depicts the MALDI-ToF/MS spectra of Hb before and after HD exposure. The peaks in Fig. 1a at *m/z* 15126.4 and *m/z* 15868.2 correspond to the singly protonated ($[M+H]^+$) α- and β-subunits of native human Hb (control), respectively. These experimental mass values closely compare to the calculated mass values of the α- and β-subunits (15127 and 15868 Da) of Hb. Figure 1b represents the HD-exposed Hb where the ion at *m/z* 15122.0 corresponds to the unalkylated Hb, and *m/z* 15226.2 corresponds to Hb with one adducted HD molecule with a hydroxyl functionality at the free end of the HD moiety. The additional peaks observed at higher mass shifts for all HD–Hb experiments are attributed to two or more alkylation events of the various possible types

Table 2. Proposed MT–HD alkylation products observed by MALDI-ToF/MS (data from Fig. 2b)

Proposed type of HD–MT adduct	Total mass of HD moieties	Number of HD units adducted to MT	Peaks observed (*m/z*)	Calculated mass (relative to *m/z* 6123.3, unreacted MT)
M	88.1	1	6213.2	6211.4
MOH	105.2	1	6230.7	6228.5
M, MOH	193.4	2	6314.8	6316.5
2MOH or M, MCl	210.4 or 211.8	2	6334.2	6333.7 or 6335.1
2M, MOH	281.6	3	6400.8	6404.9
M, 2MOH or 2M, MCl	298.6 or 300.0	3	6423.5	6421.9 or 6423.3
3M, MOH	369.8	4	6493.7	6493.1
2M, 2MOH or 3M, MCl	386.8 or 388.2	4	6509.4	6509.9 or 6511.5
M, 3MOH or 2M, MOH, MCl	403.8 or 405.2	4	6525.2	6527.1 or 6528.5
4M, MOH	457.6	5	6577.8	6580.9
3M, 2MOH or 4M, MCl	475.0 or 476.4	5	6593.6	6598.3 or 6599.7
2M, 2MOH, MCl or 3M, MOH, MCl	492.0 or 493.4	5	6612.8	6615.3 or 6616.7

M = $-CH_2CH_2SCH_2CH_2-$; MOH = $-CH_2CH_2SCH_2CH_2OH$; MCl = $-CH_2CH_2SCH_2CH_2Cl$.

of HD moieties adducted to Hb (Table 1). Commercial rabbit MT produced an $[M+H]^+$ ion at *m/z* 6125.9 (Fig. 2a), representing the major isoform of MT. Figure 2b of the HD-exposed MT displays several peaks that are attributed to HD alkylation products of MT and the unreacted MT. Table 2 lists the *m/z* values and the proposed structures of these HD–MT adducts.

Both MT and Hb were incubated with CEES to eliminate concerns of HD self-reaction. Based on the chemical structure of CEES ($ClCH_2CH_2SCH_2CH_3$), it was expected that the CEES molecule would add to MT and Hb in only one possible manner. The mass spectra of the alkylated products of the Hb–CEES and MT–CEES experiments are shown in Figs 3 and 4. The peaks that correspond to the CEES alkylated species of Hb and MT are well-defined single peaks that are separated from each other by ca. 89 ± 1.5 Da, reflecting the molecular weight addition of a single CEES molecule. As the concentration of HD or CEES was increased, while maintaining constant protein concentration, an increase in alkylation species was observed (data not shown).

CONCLUSION

Investigation of the interaction of HD with Hb and MT *in vitro* by way of MALDI-ToF/MS analysis is

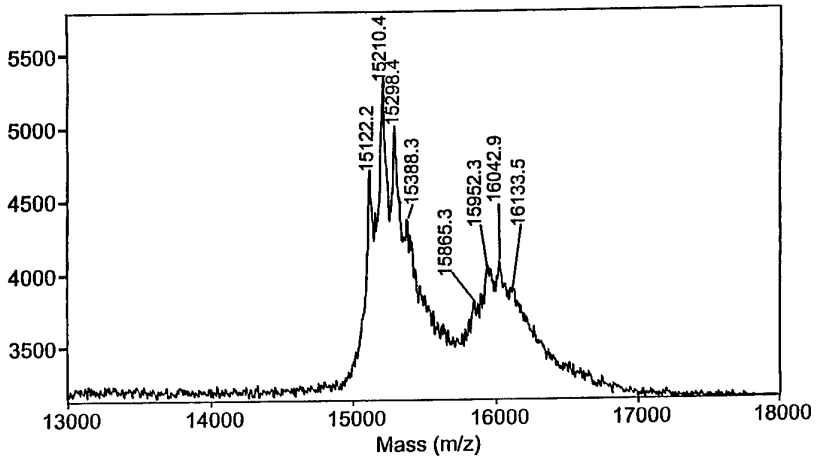

Figure 3. The MALDI mass spectrum of Hb after exposure to CEES: *m/z* 15122.2 represents the unreacted α-subunit of Hb (α-Hb); *m/z* 15210.4 (α-Hb + 1 CEES); *m/z* 15298.4 (α-Hb + 2 CEES); *m/z* 15388.3 (α-Hb + 3 CEES); *m/z* 15865.3 corresponds to the unreacted β-subunit of Hb (β-Hb); *m/z* 15952.3 (β-Hb + 1 CEES); *m/z* 16042.9 (β-Hb + 2 CEES); *m/z* 16133.5 (β-Hb + 3 CEES).

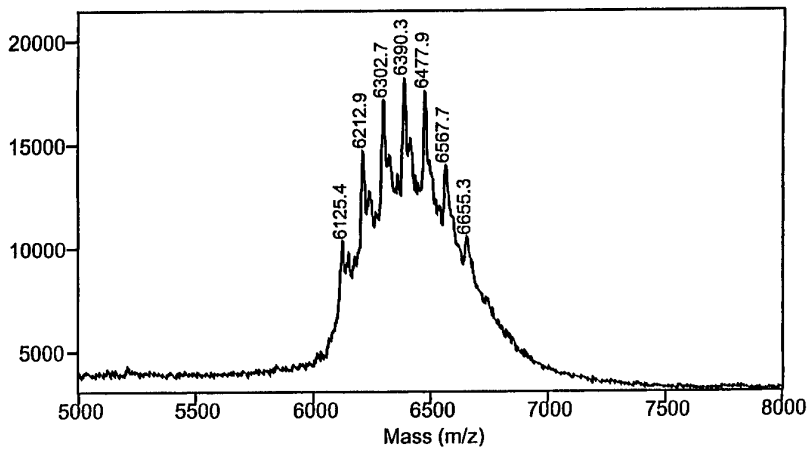

Figure 4. The MALDI mass spectrum of MT after exposure to CEES: *m/z* 6125.4 represents unreacted MT; *m/z* 6212.9 (MT + 1 CEES); *m/z* 6302.7 (MT + 1 CEES); 6390.3 (MT + 3 CEES); *m/z* 6477.9 (MT +4 CEES); *m/z* 6567.7 (MT + 5 CEES); *m/z* 6655.3 (MT + 6 CEES).

summarized and discussed. Multiple alkylated species were observed in the MALDI spectra as a result of the HD exposure to both Hb and MT. Based on the relative mass shifts of the alkylated species, we proposed the structures of the various HD–protein adducts generated from the experiments (see Table 1 and 2). We have demonstrated clearly that MALDI-ToF/MS can be utilized to study the *in vitro* interaction of HD with cellular macromolecules and, given the attractive characteristics of MALDI-ToF/MS, this technique may be employed potentially as a diagnostic tool for the confirmation of chemical agent exposure.

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