The Solid State Oxidation of Methionine Containing Peptide: A Preliminary Study Using Time of Flight Secondary Ion Mass Spectrometry

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Purpose. A surface sensitive mass spectrometric technique: Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) was introduced to study the solid state instability of a methionine containing peptide caused by the oxidation of the methionine residue.

Methods. The oxidation of a neuropeptide Methinonine-Enkephalin (ME) in air and under UV acceleration was studied by ToF-SIMS.

Results. The apparent oxidation rate is defined by the peak ratio of oxidized molecular ion over unoxidized molecular ion. ME is oxidized at a faster rate to its sulfoxide derivative in the UV accelerated oxidation environment than in lab air. The calibration curve for evaluating the ionization probability ratio of the oxidized deprotonated molecular ion divided by the unoxidized deprotonated molecular ion was obtained. This could be used to extract the real oxidation rate of ME in the solid state.

Conclusions. The preliminary results showed that ToF-SIMS with simple sample handling, fast data acquisition, together with excellent surface sensitivity and detection limit could be an applicable and convenient tool to study peptide reactions in the solid state such as oxidation and deamidation process.

KEY WORDS: methionine-enkephalin; oxidation; solid state; secondary ion mass spectrometry; peptide stability; methionine.

INTRODUCTION

The instability of methionine containing peptides and proteins caused by oxidation of the methionine (met) residue to the corresponding sulfoxide has been a particular problem in the pharmaceutical industry (1). It has been reported that oxidation of the methionine residue can occur in isolation, synthesis, or during the formulation and storage (1). In recent years, oxidation in solution, especially in the presence of hydrogen peroxide and other oxidants, has been extensively studied for pharmaceutical and biochemical applications (2–4). However, less attention has been paid to the study of solid state oxidation (5,6). As more and more peptide and protein based drugs are formulated as lyophilized or freeze-dried products (5), it becomes more important to investigate solid-state stability of the methionine residue at the molecular level.

In this paper, we demonstrate the application of a surface sensitive mass spectrometric technique to study the oxidation of a methionine containing peptide. This study is aimed at developing a model to evaluate the factors influencing the oxidation of peptide and protein powders during the formulation and storage process.

The technique we use in this work is Time of Flight-Secondary Ion Mass Spectrometry (ToF-SIMS). ToF-SIMS is a mass spectrometric technique providing molecular structure information like conventional mass spectrometric methods, but the samples that are examined by ToF-SIMS measurements are solid. The unique characteristic of ToF-SIMS is its extremely high surface sensitivity, which allows detection of top-most layers. Low detection limits (typically parts per billion) makes ToF-SIMS very powerful for diagnosing organic impurities as well as inorganic dopant levels in the semiconductor industry (7).

In the past ten years, our group has been actively working on the development of quantitative methods for SIMS studies on model systems such as Langmuir Blodgett (LB) thin films (8). The ion formation mechanism in SIMS as well as kinetics of polymerization reactions have been studied using LB films. Recently, we have developed quantitative SIMS methodology to study the kinetics of hydrolytic degradation of polymer drug delivery materials (9).

In present paper, we report preliminary data for quantifying the oxidation of methionine-enkephalin(ME) peptide powder using ToF-SIMS. Methinonine-enkephalin is a neuropeptide and its oxidation in solution has been studied (10,11). It has been found that some of the biological properties changed when the methionine residue was oxidized to sulfoxide and sulfone (12). In this surface oxidation study, the solid state stability of ME was investigated in two oxidation conditions: (1) laboratory air oxidation and (2) UV accelerated oxidation. Comparing the apparent oxidation rates of ME in these two conditions, the UV accelerated environment did enhance the oxidation of methionine to corresponding sulfoxide.

MATERIALS AND METHODS

Materials

Methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) acetate salt was purchased from Sigma Chemical Co.(St. Louis, MO), and used as received (purity $\sim$ 99%). Methionine-Enkephalin sulfoxide(H-Tyr-Gly-Gly-Phe-Met(O)-OH) was purchased from Bachem Bioscience Inc. (King of Prussia, PA), and used without any further purification (purity $>$ 99%). Silver foil used as a substrate (99.998%, 0.25 mm thick) was purchased from Alfa AESAR (a Johnson Matthey company, Ward Hill, MA). Silver foil was etched in 20% nitric acid to clean and oxidize its surface then rinsed copiously with triply distilled water.

Sample Preparation

Peptides were dispersed to the etched silver substrate with an area of 1 cm^2 by a solution casting method. Peptide solution was deposited on the silver substrate using a microsyringe and then the solvent was evaporated in the vacuum. The standard samples used for the calibration were prepared by mixing ME peptide and its oxidized derivative Methionine-Enkephalin sulfoxide (MEO) with certain molar ratios and the solution of these

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mixture were also deposited on the silver substrate using a solution casting method. The solvent for ME peptide solution is methanol, the solvent for the ME and MEO mixture is a water and methanol mixture with a volume ratio of 1:3 (H_2O:CH_3OH).

UV Oxidation Set-up

The UV accelerated oxidation set-up is shown in Fig. 2. The UV lamp is a GE germicidal lamp with electric power of 15 watts. The dimensions of the chamber are 23″ × 10.5″ × 9.5″ (length × height × width). A silver substrate was mounted with double-sided tape to the sample holder which was placed 5 cm away from the UV lamp. The UV density was measured by a photodetector (International Light Inc., Newburyport, MA). In the configuration which is shown in Fig. 2, the peptides were exposed to the UV light with the density of 4.8×10^{-5} Watt/cm^2. We analyzed for ozone in the air in this chamber and ozone could not be detected using Draeger ozone detector (Draeger Safety, Inc.) with a detection limit of 0.05 ppm.

Instrumentation

The ToF-SIMS instrument used in this work is a Physical Electronics (PHI) 7200 ToF-SIMS spectrometer (Fig. 1). Solid samples are placed in the examination chamber under vacuum of ca 10^{-8}–10^{-10} Torr. In the ToF-SIMS experiment, a surface of the sample is bombarded by a primary ion beam (in this case Cs^+) with a kinetic energy of 1–8 Kev. According to the SIMS collision cascade ionization theory (13), the kinetic energy from the Cs^+ is dissipated to the adjacent atom or molecule in the form of a collision cascade. When the energy from

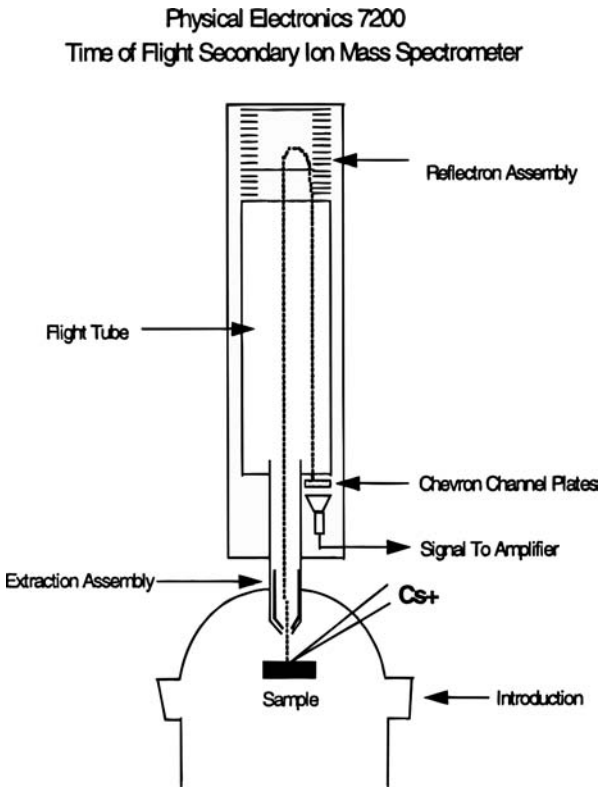

Fig. 1. Schematic of PHI 7200 ToF-SIMS Spectrometer.

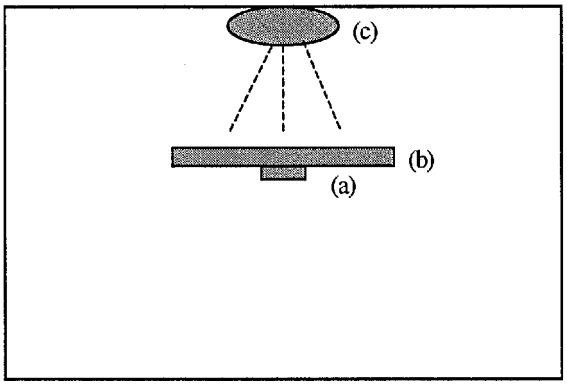

Fig. 2. Schematic of UV accelerated environment. (a): sample; (b): sample holder; (c): UV lamp.

Cs^+ transfers to the atom or molecule on the topmost layer at surface, these atoms or molecules could be desorbed either as a neutral atom/molecule or negatively charged ion or positively charged ions from the surface. These charged ions are called secondary ions and can be extracted by the high voltage electric field (extraction assembly in Fig. 1) to the flight tube and analyzed by the Time of Fight analyzer. The flight time of each ion in the field free flight tube is eventually converted to the mass to charge ratio, yielding secondary ion mass spectra (13).

ToF-SIMS Analysis

For all spectra, the negative ion mode was chosen for this study. Low damage or "static" conditions (i.e., dosage is less than 10^{12} ions/cm^2) were used in all acquisition with low ion current. The acquisition time for each spectrum was 0.67 mins. There was no charging during the acquisition, so no charge neutralization was used. Data reduction was performed using Physical Electronics TOFPak software (Version 2.0)

RESULTS AND DISCUSSION

In the SIMS spectra of these peptide samples, many signals were detected representing peptide molecular ions and peptide fragment ions. In this paper, we chose to focus on using the deprotonated molecular ion to qualitatively and quantitatively characterize ME and MEO in the negative ion spectra (Fig. 3a). The deprotonated molecular ion is formed by the intact molecule losing one proton, which is designated as $(M\text{-}H)^-$. Since the molecular weights of ME and MEO are 573 and 589 respectively, the $(M\text{-}H)^-$ of ME is at $573-1 = 572$ (m/z) represented by ME-H; and the $(M\text{-}H)^-$ of MEO is at $589-1 = 588$ (m/z) represented by MEO-H (See Fig. 3a). The peak areas of ME-H and MEO-H were integrated using TOFPak software and used for quantification. In the ToF-SIMS spectrum, isotopic peaks were also clearly detected (see Fig. 3a). Based on a theoretical calculation, the isotopic distribution of the ME-H is quite similar to that of MEO-H, so for both ME-H and MEO-H only the major isotopic peak was analyzed and used for the quantification in this study (14). For each sample, four different spots were measured by ToF-SIMS. All the data were statistically analyzed using Origin 4.0 (MICROCAL™ Software, Inc.).

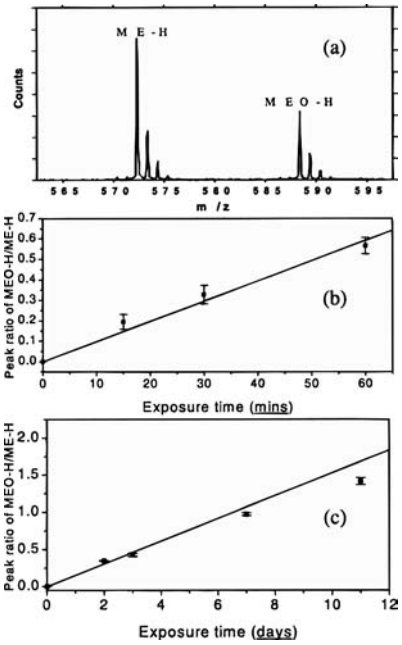

Fig. 3. SIMS characterization of ME oxidation at surface. (a) Negative SIMS spectrum for ME after 60 mins exposure to UV environment; (b) SIMS peak ratio as a function of exposure time of ME to UV environment; (c) SIMS peak ratio as a function of exposure time of ME to laboratory air.

Apparent Oxidation Rate

We first related the peak area ratio of MEO-H/ME-H with the surface oxidation reaction time (exposure time), which is shown in Fig. 3b for UV oxidation and is shown in Fig. 3c for air oxidation. Figure 3b shows that ME is gradually oxidized to MEO after it is exposed to the UV accelerated oxidation environment for one hour. The peak ratio of MEO-H/ME-H ranged from about 0.2 for 15 mins exposure to 0.55 for 60 mins UV exposure. In Fig. 3c, the peak ratio of MEO-H/ME-H changed from 0.35 for 2 days exposure to 1.4 for 11 days exposure to lab air. We fitted the data in Fig. 3b and Fig. 3c with linear functions using linear least square techniques in Origin. The equations from the fittings and correlation coefficients are: $y = 0.0099x$ and $R^2 = 0.99$ for Fig. 3b, and $y = 0.15x$ and $R^2 = 0.98$ for Fig. 3c. Based on the results of the linear fits of experimental data in Fig. 3b and Fig. 3c, we defined the apparent oxidation rate in terms of peak ratio in Eq. (1):

$$I_{MEO\text{-}H}/I_{ME\text{-}H} = K_{apparent} * t \quad (1)$$

Where $I_{MEO\text{-}H}$ and $I_{ME\text{-}H}$ are peak area for MEO-H and ME-H peak respectively; t is exposure time; $K_{apparent}$ is defined as "apparent oxidation rate" with a unit of 1/min for UV oxidation (Fig. 3b) or 1/day for air oxidation (Fig. 3c). These apparent oxidation rates are the slopes of the linear plots in Fig. 3b and Fig. 3c, which are 14/day (0.01/min) and 0.15/day respectively. Comparing the apparent oxidation rates, exposing ME to UV accelerated oxidation environment did accelerate the oxidation of the methionine residue to sulfoxide by a factor of 100 compared to the lab air oxidation.

SIMS Calibration Curve for ME and MEO

In the above discussion, we used the peak ratio to calculate the "apparent oxidation rate" defined in equation (1). In order to obtain a real oxidation rate, the SIMS peak ratio of MEO-H/ME-H ($I_{MEO\text{-}H}/I_{ME\text{-}H}$) should be converted to the molar ratio of MEO/ME. The relationship between the peak ratio and molar ratio was built by a calibration curve (Fig. 4). In this calibration curve, four data points were taken corresponding to molar ratio of MEO/ME of 0, 1, 5, 10. The fitting result is $y = 0.15x$ and $R^2 = 0.99$. The slope of the line was used to obtain the ionization probability ratio which is defined in Eq. (2):

$$I_{MEO\text{-}H}/I_{ME\text{-}H} = \alpha_{MEO\text{-}H/ME\text{-}H} * C_{MEO}/C_{ME} \quad (2)$$

Where C_{MEO} and C_{ME} are the molar concentration for MEO and ME molecule and C_{MEO}/C_{ME} represents molar ratio of MEO/ME; $\alpha_{MEO\text{-}H/ME\text{-}H}$ is defined as ionization probability ratio of MEO-H/ME-H. So $\alpha_{MEO\text{-}H/ME\text{-}H}$ is assumed to be 0.15 and this number allows the conversion from peak ratio to molar ratio, for example if peak ratio of MEO-H/ME-H is 2, the molar ratio of MEO/ME is 2/0.15 = 13:1

Estimation of Absolute Kinetic Information from SIMS Data

From SIMS data, we can directly obtain the relationship between peak ratio and exposure time which given Eq. (1) in this study. Furthermore, the peak ratio ($I_{MEO\text{-}H}/I_{ME\text{-}H}$) in equation (1) can be replaced with the molar ratio (C_{MEO}/C_{ME}) in equation (2) and this yields Eq. (3):

$$C_{MEO}/C_{ME} = K_{apparent} * t \div \alpha_{MEO\text{-}H/ME\text{-}H} \quad (3)$$

We assumed during the oxidation reaction that the total number of the molecules are kept constant ($C_{ME} + C_{MEO} = C_0$, where C_0 is the initial molar concentration of ME before the oxidation occurs). Under this assumption, the derivative of equation (3) with respect to time turns to be:

$$\delta C_{MEO}/\delta t = \{K_{apparent}/\alpha_{MEO\text{-}H/ME\text{-}H} \div [1 + (K_{apparent}/\alpha_{MEO\text{-}H/ME\text{-}H})*t]\} * C_{ME} \quad (4)$$

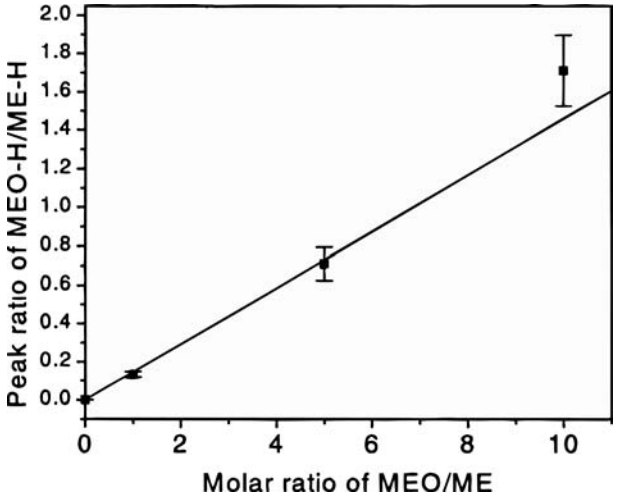

Fig. 4. SIMS calibration curve for ME and MEO.

Equation (4) shows that the reaction rate in terms of the formation of oxidized molecule MEO is first order with respect to ME for both lab air oxidation in early stage (about 7 days of air exposure based on Fig. 3c) and for the short time UV accelerated oxidation (about one hour exposure based on Fig. 3b) in this study.

CONCLUSION

The preliminary data in this paper shows that ToF-SIMS can be an applicable and convenient tool for studying the oxidation of the methionine residue of short peptides in the solid state. Detailed ToF-SIMS kinetic studies of the oxidation of other methionine containing peptides are being carried out in our group. The factors being investigated include UV light density as well as moisture and oxygen content. These have been found to influence the stability of methionine in solid state (6) and will be evaluated by determining the oxidation rate constant.

Due to the need to determine the ionization probability, a small peptide molecule is easier to be quantitatively analyzed than a large protein molecule in a SIMS measurement. But studying a short peptide might also be helpful to understand the oxidation kinetics of a protein if we assume that the peptide studied can have the same structure as the equivalent peptide sequence in a large protein molecule, including its conformation. A second point to note is that the mechanism for methionine residue oxidation which occurs at the surface may be different from that in solution, thus surface oxidation might provide a way to oxidize those methionine residues which are difficult to be oxidized in solution due to the poor accessibility to the oxidant in the liquid phase (15). So it is also important to investigate the surface oxidation of the thioether group to sulfoxide from the synthetic point of view.

Finally, we also want to emphasize one of the great priorities for using ToF-SIMS to study the oxidation of peptide or other chemical processes such as deamidation, hydrolysis etc. is: the time from sample preparation to data acquisition to finally extracting the oxidation rate will be much shorter than with other techniques. We also hope peptide stability studies would be another important application of SIMS in organic molecular analysis in the future.

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