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Real-time reaction monitoring by probe electrospray ionization mass spectrometry

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Probe electrospray ionization (PESI) is a modified version of the electrospray ionization (ESI), where the capillary for sampling and spraying is replaced by a solid needle. High tolerance to salts and direct ambient sampling are major advantages of PESI compared with conventional ESI. In this study, PESI-MS was used to monitor some biological and chemical reactions in real-time, such as acid-induced protein denaturation, hydrogen/deuterium exchange (HDX) of peptides, and Schiff base formation. By using PESI-MS, time-resolved mass spectra and ion chromatograms can be obtained reproducibly. Real-time PESI-MS monitoring can give direct and detailed information on each chemical species taking part in reactions, and this is valuable for a better understanding of the whole reaction process and for the optimization of reaction parameters. PESI-MS can be considered as a potential tool for real-time reaction monitoring due to its simplicity in instrumental setup, direct sampling with minimum sample preparation and low sample consumption. Copyright © 2010 John Wiley & Sons, Ltd.

After the combination of electrospray ionization (ESI) and mass spectrometry (MS) was first introduced in 1984 by Yamashita and Fenn,[1,2] it has been widely recognized as an irreplaceable and unique means for rapid characterization and analysis in chemistry, biochemistry, pharmacy and related sciences. After more than 20 years of development, ESI is widely accepted as a versatile tool which can gently transfer a broad range of chemical or biological molecules from the condensed phase into the gas phase, particularly in multiply charged states. In general, ESI employs capillaries for continuous sample delivery, which means that the procedures of dissolution, extraction and purification of samples should be conducted before ESI-MS analysis. This requirement may restrict the use of ESI-MS for direct analysis and real-time monitoring where a quick response is important. It was recently recognized that charged droplets of the electrosprayed plume could react with neutral molecules and lead to secondary ionization. Thus, many ESI-based desorption/ionization methods have been proposed in recent years, of which desorption electrospray ionization (DESI)[3] is one prominent example. In DESI, a pneumatically assisted charged jet is sprayed directly onto the sample surface, and secondary charged droplets/ions are generated and scattered off the sample surface during the process of surface collision and charge transfer.[3,4] A similar approach related to DESI, where the solution is sprayed with a high-speed nebulizing gas but no voltage is applied, is called desorption sonic spray ionization (DeSSI),[5] recently renamed easy ambient sonic-spray ionization (EASI).[6] In 2006, Cooks' group introduced extractive electrospray ionization (EESI),[7] which shared some basic design features with a previously published fused-droplet electrospray ionization (FD-ESI)[8] technique, where two sprayed plumes, one for nebulization of the sample solution and the other for charged droplets, were cross-dispersed in continuous liquid-liquid extraction with collision, interaction and ionization. Unlike those methods mentioned above, there are some two-step approaches where the desorption and ionization stages are separated. The combination of laser ablation and ESI led to the appearance of the family of laser-assisted desorption/electrospray ionization techniques including electrospray-assisted laser desorption ionization (ELDI),[9] matrix-assisted laser desorption electrospray ionization (MALDESI),[10] and laser ablation electrospray ionization (LAESI),[11] or, alternatively, infrared laser assisted desorption electrospray ionization (IR-LADESI).[12]

Real-time monitoring of chemical or biological reactions is of great importance and interest because better understanding of mechanisms as well as optimization for high production efficiency can be achieved by knowledge of the kinetics of these reactions. With its advantageous properties including high sensitivity, high accuracy and fast detection of stoichiometric ratio of reactants, MS is becoming more widely used in this field.[13] The first application of ESI-MS to real-time reaction monitoring was demonstrated by Lee *et al*. in 1989.[14] However, capillary-based ESI-MS for real-time monitoring of

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reactions causes a delay in the analysis due to the complexity of the sample pretreatment procedures.[15,16] Coupling of the rapid mixing of reactants to ESI-MS could reduce the delay in the analysis from several minutes to a few microseconds.[15] This method was found, however, not to be applicable to process scale reactions.[16] Furthermore, ESI-MS is also unsuitable for high-salt-containing reactions, which bring a high risk of clogging of the ESI capillaries. Recently, the use of extractive electrospray ionization mass spectrometry (EESI-MS) for real-time monitoring of organic reactions was reported by Zenobi and co-workers.[16] Initially, nitrogen gas was introduced through a three-necked flask, the container of the chemical reaction, to bring volatile compounds from the reaction system. The carrier nitrogen gas was then cross-dispersed with an electrospray plume jet for ionization. This method had only a short time delay. Although this method can be applicable to organic reactions, reactions occurring in aqueous solution containing nonvolatile compounds such as carbohydrates and peptides may need special treatments for nebulization.[8] Thus, a new ESI-based method is needed with the high sensitivity and high production efficiency of ions provided by ESI, for real-time reaction monitoring in both aqueous and organic solutions with minimal or no sample pretreatments.

Recently, a modified version of the ESI source, probe electrospray ionization (PESI), was developed in our laboratory,[17] where the philosophy of using a capillary for sampling was totally abandoned. A solid needle was utilized to load a small amount of sample solution repeatedly in a perpendicular direction by penetrating the tip into the surface of a sample solution, or even of real-world samples such as biological tissues[18–22] and foods.[20,24] When the needle reached the upper position, an electrospray could be generated by applying a direct current high voltage to the needle.[23] PESI has been proven to be suitable for various biological samples and a high tolerance to salts is one of its advantages compared with the conventional ESI,[17] where the clogging of capillaries is a serious problem. Herein, some basic biological/chemical reactions such as acid-induced protein denaturation, hydrogen/deuterium exchange of peptides, and Schiff base formation have been chosen to demonstrate the application of PESI-MS to the real-time monitoring of reactions. In this study, the strategy of diffusive mixing of reactants was combined with PESI-MS measurements to decrease reaction rates. By means of the slow diffusive mixing of reactant solutions, the reaction time could be extended greatly, from microseconds to seconds, or even minutes.

EXPERIMENTAL

The side view of the configuration of PESI setup is shown in Fig. 1. Stainless steel disposable acupuncture needles were purchased from Seirin Co., Ltd. (Shizuoka, Japan). The diameter of the needle is 140 μm with a submicron-diameter tip.[23] As shown, an acupuncture needle was vertically aligned in the orthogonal direction to the axis of the ion sampling orifice. The needle could be driven up and down along the vertical axis by a linear actuator system (ARIOS, Akishima, Tokyo, Japan). When the needle was at the highest

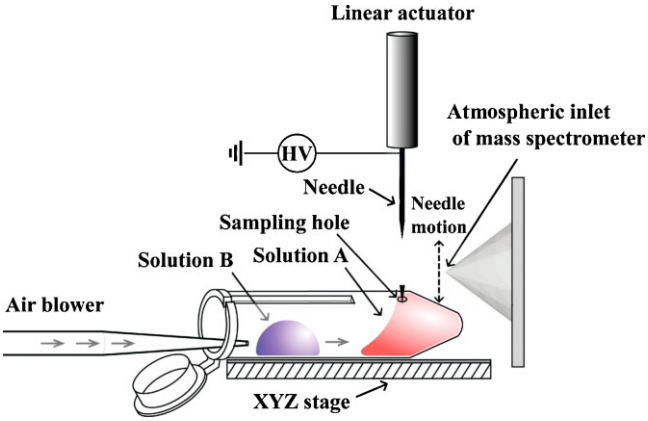

Figure 1. Schematic depiction of the PESI-MS setup combined with diffusive mixing strategy for real-time monitoring.

position, the vertical and horizontal distances between the tip of needle and the apex of the ion sampling orifice were 2 and 3 mm, respectively. The frequency of the needle motion was set as 3 Hz with an 8 mm moving stroke.

A 15-W electric soldering iron (Taiyo Electric Inc., Tokyo, Japan) was used to melt a small hole in a 1.5 mL polypropylene microcentrifuge tube (Simport Plastics Ltd., Belloeil, Quebec, Canada). A rectangular piece (about 2 mm × 20 mm) of the side wall of this tube was then carefully removed. After addition of a certain volume (typically 200 μL) of solution A in the bottom, containing one reactant, this microcentrifuge tube was carefully fixed on an XYZ sampling stage below the ion sampling orifice with the small sampling hole upward. The XYZ stage was adjusted manually to a position where the tip of the needle could touch solution A through the sampling hole, when the needle was driven to the lowest position. A typical penetration depth for sampling was achieved by controlling the sample stage moving the sample up for about 0.5 mm. A small amount of sample solution could then be loaded onto the tip of the needle and electrosprayed when a high voltage of 2.5–3.0 kV was applied to the needle.[23] The strongest ion signals were observed when the needle reached the highest position.[23] Periodic sample loading and electrospraying processes were achieved by the linear actuator system mentioned above. A certain volume (typically 50 μL) of solution B, containing the other reactant, was dropped into the microcentrifuge tube which was not in contract with solution A. An air blower was used to drive the drop of solution B slowly towards solution A. The time at which solution A and solution B came into contact with each other was set visually as the starting point of the reaction.

All mass spectrometric experiments were carried out using an orthogonal acceleration time-of-flight mass spectrometer (AccuTOF; JEOL, Akishima, Japan). The instrumental parameters for the MS measurements were similar to those described in our previous paper[21] but were slightly different for each measurement. For clarity, the detailed parameters for every experiment are presented in the Supporting Information. All data were acquired in positive ion mode using the analogue-to-digital converter (ADC)/continuous averager ion detection system.

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All regents used in this study were of analytical grade or higher and were used without further purification. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Gramicidin S (GS), myoglobin (from horse heart), ubiquitin (from bovine erythrocytes), CH_3OD (99.5 atom %D) and ethanolamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). D_2O (99.9 atom %D) was purchased from Wako Pure Chemicals (Osaka, Japan). Angiotensin I (human) and bradykinin were obtained from Peptide Institute (Osaka, Japan). Benzaldehyde was purchased from TCI (Tokyo, Japan). Ammonium acetate, sodium hydroxide, glacial acetic acid (HAc) and methanol were provided by Kanto Chemicals (Tokyo, Japan).

RESULTS AND DISCUSSION

Real-time monitoring of the denaturation of proteins by PESI-MS

Native proteins adopt thermodynamically energy-minimized conformations. The compact shape of a protein does not allow too many charges to be incorporated on its surface, resulting in relatively low and narrow charge-state distributions in ESI mass spectra under native or near-native conditions. Non-native (denatured) proteins, however, adopt more open conformations which can accommodate a larger amount of charges. Applying ESI-MS to probe the kinetics of the denaturation process of proteins induced by acid will provide useful information for evaluation of the integrity of higher order structures of proteins.[24] Online and offline techniques can be combined with ESI-MS for studies in this field. Online methods are favored because much time-consuming and labor-intensive work can be avoided and direct observation from real-time monitoring can provide more detailed data for explaining how conformational transformation occurs.[25] Holomyoglobin (hMb), which contains a hydrophobic pocket in its structure for accommodating a heme group by non-covalent bonding, is often used for investigation of protein denaturation. Acid-induced denaturation results in drastic conformational changes in hMb and the loss of the heme group by disrupting the heme-protein interactions, thereby generating unfolded apomyoglobin (aMb).[26]

Since PESI uses the same needle for sampling and electrospraying, the latter measurement may be affected by carry-over of the previous sampling. It is necessary to adopt auxiliary methods to circumvent this kind of memory effect for real-world samples with highly concentrated analytes and not enough biofluid on the surfaces.[20,21] In this work, however, as the analytes are solutions, the carry-over effect may not be serious. In particular, when the needle penetrates into the sample solution, carry-over of the previous sampling could be combined with the higher volume sample solution, reducing the carry-over effect to a negligible level.

To demonstrate how PESI-MS can be used for kinetic studies of protein denaturation, 300 μL of 4.8×10^{-5} mol/L myoglobin water/methanol (75:25, v/v) solution was used as solution A, while 12 μL glacial HAc was used as solution B. The time-resolved mass spectra are shown in Figs. 2(A) to 2(D). The presence of aMb ions in Fig. 2(A) showed that the hMb was partially denatured. Possible reasons for this are that: (1) the hMb sample has already partially denatured during storage; (2) the HAc vaporized and caused a change in the pH of the solution near the sampling site before mixing of solutions A and B took place; and (3) the pH of an electrospraying solution can be altered due to the occurrence of electrochemical reactions[27,28] and pH-sensitive proteins

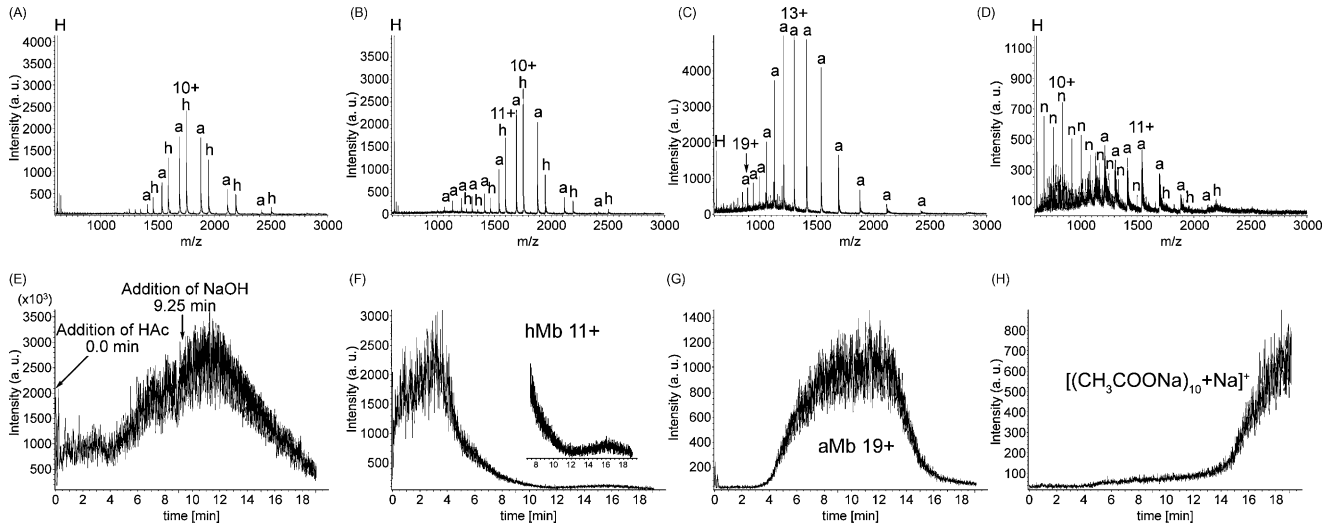

Figure 2. Denaturation of hMb in water/methanol (75:25, v/v) monitored by time-resolved PESI-MS combined with diffusive mixing strategy. At 0.00 min, 12 μL of HAc (solution B) was driven to contact 300 μL of hMb solution (solution A). At 9.25 min, 30 μL of saturated NaOH solution (solution B) was driven to contact the mixture of HAc and hMb solution (solution A). Time-resolved mass spectra at 0.00 min (A), 4.00 min (B), 12.00 min (C) and 19.00 min (D) after the contact of HAc and hMb solutions. Gross ion chromatogram is shown in (E). Extracted ion chromatograms (EICs) of three representative ions, $[hMb + 11H]^{11+}$ (F), $[aMb + 19H]^{19+}$ (G) and $[(CH_3COONa)_{10} + Na]^+$ (H) during the time course of real-time PESI-MS monitoring. Notations: H, heme; h, hMb; a, aMb; n, cluster ions of $[(CH_3COONa)_{10} + Na]^+$. Charge states of some ions are also given.

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can denature.[28,29] For PESI, because the volume of sample solution loaded each time by the needle is at the picolitre level or less,[19] the phenomenon of acidification of an electrospraying solution may be more obvious than in conventional ESI. It should be noted that even without the addition of HAc a similar spectrometric pattern was observed (shown in Supplementary Fig. S1, see Supporting Information), which indicates that reasons 1 or 3, or both, are the major causes for the initial denaturation of hMb. Because the initial denaturation of hMb was time-independent, it was simply considered to be a constant ''internal disturbance'' and was not further investigated. In order to investigate the possibility of achieving renaturation of hMb by modulating the pH value from acidic to alkaline, 9.25 min after the diffusive mixing of the hMb solution and HAc, 30 μL of a saturated NaOH solution (~18 mol/L) was added and driven into contact with solution A. The entire real-time monitoring of the denaturation of hMb lasted for 19.45 min.

Mass spectra taken at 4.00, 12.00 and 19.00 min after the mixing of the hMb solution and HAc are shown in Figs. 2(B), 2(C) and 2(D), respectively. When the glacial HAc contacted the hMb solution, the pH of the hMb solution changed gradually due to the diffusion of HAc. After 4 min, as the HAc diffused towards the sampling hole, ions with multiple charges (up to +17) of aMb were detected. As the diffusive mixing continued, higher charged aMb ions appeared at the expense of the hMb ions. After 12 min, the signals of the hMb ions decreased to the noise level while multiply charged ions (up to +22) of aMb were detected, indicating that the hMb was fully denatured. The maximum value of the total ion intensities appeared at about 11.25 min, 2 min after the mixing of the NaOH and hMb solutions (shown in Fig. 2(E)). Following this, the ion abundances decreased rapidly due to the alkalinization of the reaction system. As shown in Fig. 2(D), 10 min after the addition of NaOH solution, the intensities of the aMb ions decreased to 10% of their maximum values, while sodium acetate cluster ions $[(CH_3COONa)_i + Na]^+$ (i =8–18) appeared with similar intensities. The appearance of these cluster ions is due to the high concentration of CH_3COONa – at a final concentration of about 0.6 mol/L. The re-detection of hMb ions in Fig. 2(D) indicated that the hydrophobic pocket of myoglobin could have partially reformed due to the alkalinization and a heme group could also be reincorporated. Despite these ions being of low intensity, their detection could mean that conformational changes of proteins could reversed by changing the pH in opposite directions. The extracted ion chromatogram (EIC) of the ion at *m/z* 1598, corresponding to $[hMb + 11H]^{11+}$, shown in Fig. 2(F), consists of a rapid increase, a rapid decrease, a slow increase and a slow decrease. The change in ion intensity may be explicable in the steps of increment of the solution acidity, denaturation of hMb, renaturation of hMb and increment of the solution alkalinity, respectively. The EICs of ions at *m/z* 893 ($[aMb + 19H]^{19+}$) and 843 ($[(CH_3COONa)_{10} + Na]^+$) are shown in Figs. 2(G) and 2(H), respectively, which also present how the acidification and the subsequent alkalinization procedures affected the intensities of each ion species.

By the combination of PESI-MS and diffusive mixing, detailed stoichiometric information could be obtained on each ion species. Because our mass spectrometer does not have a tandem MS function, the identification of each peak was based on molecular weight information and previously reported spectra.[26,29] The appearance and disappearance of ions could be clearly traced in the EICs. Compared with offline monitoring, much labor-extensive work and any time lag could be avoided by using PESI-MS for real-time monitoring. The results of the real-time monitoring of the denaturation of ubiquitn, another typical example, are shown in Supplementary Fig. S2 (see Supporting Information).

Real-time monitoring of hydrogen/deuterium exchange (HDX) of peptides by PESI-MS

Hydrogen/deuterium exchange (HDX) mass spectrometry has been widely applied to investigate the structural dynamics and folding mechanism of proteins. In proteins, hydrogens which are not protected effectively by steric shielding and/or hydrogen bonding undergo HDX rapidly, and vice versa.[30] In general, sites containing more acidic hydrogen atoms undergo HDX faster. By monitoring the level of incorporated deuterium atoms, HDX can provide information about the solvent accessibility of various parts of a protein. HDX can also provide information for the identification of regions of flexibility in the protein sequence.[30] Because HDX is usually an extremely fast process, real-time monitoring of HDX is a challenge to all instrumental methods. Due to such advantages as rapid response, high sensitivity and high mass accuracy, MS is an ideal technique for the real-time monitoring of HDX kinetics because each individual exchange results in 1 Da difference in the mass of the molecule and this can be clearly detected.

Usually, two strategies are adopted for the real-time monitoring of HDX. One is to slow down the exchange rate. Under slow-exchange conditions (*viz.* 0°C, pH 2–3), Smith and coworkers have monitored the HDX of small peptides using continuous-flow fast atom bombardment (CF-FAB).[31] The other strategy is fast-mixing and fast-detection. By using continuous-flow capillary mixing systems, where two or more motor-driven syringes are used to mix reactant solutions rapidly in a mixing chamber, both continuous-labeling and pulse-labeling can be achieved.[32,33] Herein, we present our work on the combination of PESI-MS with the diffusive mixing of reactants to monitor the HDX kinetics of some peptides in real time. For example, 150 μL of a 10^{-5} mol/L gramicidin S (GS) water/methanol solution (50:50, v/v, containing HAc 0.25%) was used as solution A, and 75 μL of D-donor solution, mixture of D_2O and CH_3OD (50:50, v/v), was used as solution B. After PESI-MS measurements of HDX, the mass spectrometric data were first deconvoluted by using Magtran[34] and then analyzed using HX-Express.[35] The time-resolved PESI mass spectra and the HDX results are shown in Fig. 3.

It can be clearly seen that by adopting the method of diffusive mixing of reactants, the concentration of deuterium increased slowly which made the apparent HDX proceed slowly. Even minor changes during the HDX process could be observed clearly. For the first 1 min, no obvious changes were observed. After 1 min, the forward HDX started to dominate the whole HDX process, as shown by the increase

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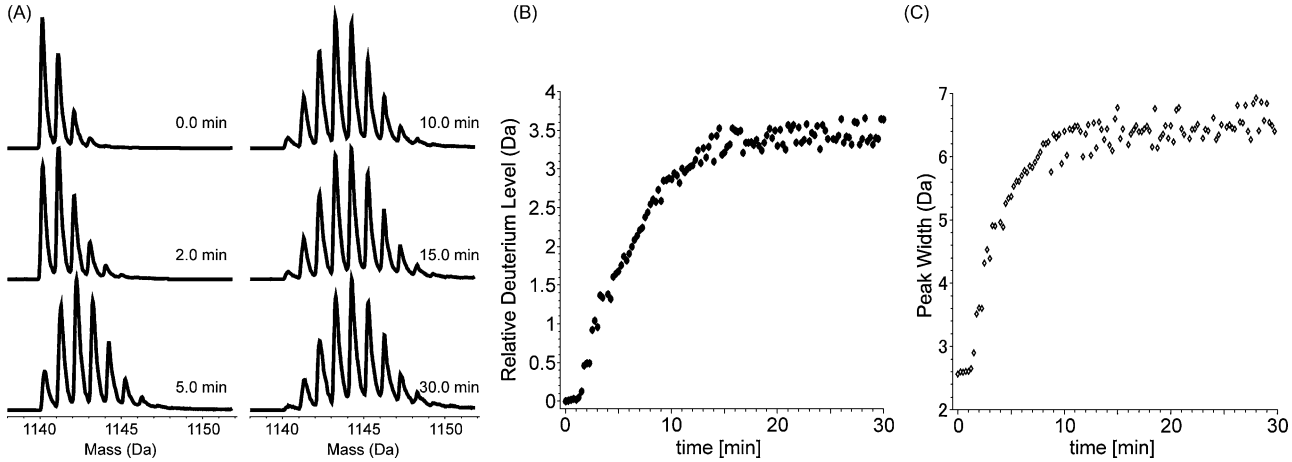

Figure 3. Deconvoluted time-resolved mass spectra of deuterium-labeled GS monitored by PESI-MS combined with diffusive mixing strategy (A). Representative labeling times are shown to the right of each spectrum. Changes in relative deuterium level (B) and peak width (C) during the time course of real-time PESI-MS monitoring.

in both the relative deuterium level and the peak width in Figs. 3(B) and 3(C), respectively. At about 15 min, both the number of deuteriums exchanged and the peak width reached a plateau, which means that equilibrium of forward and reverse HDX has been reached.

Commonly, for an entire exchange, the volume of the deuterium donor solution is many times larger than that of the peptide solution and the peak width changes from narrow to broad and back to narrow again.[36] At this stage, we did not perform this experiment for long enough to observe the whole changing process of peak width due to the possible signal instability of continuous long-time measurements and the data file size limitation. Our purpose is to demonstrate that PESI-MS could be a tool for the real-time monitoring of HDX reactions especially for the initial steps of HDX, which might be helpful for the investigation of higher-order structures of proteins. From another aspect, by using this method, peptides with different number of active hydrogens could give different HDX patterns with relative deuterium levels. In addition, different structural specificities might be reflected by different HDX patterns in a fixed time course. HDX results for two other small peptides, bradykinin and angiotensin I, are depicted in Supplementary Figs. S3 and S4, respectively (see Supporting Information). These compounds presented different HDX behavior from that of GS with different relative deuterium levels due to their different amino acid sequences.

Real-time monitoring of the reaction of Schiff base formation by PESI-MS

One of the best known organic chemistry reactions is that of aldehydes or ketones with primary amines to form imines or azomethines, also known as Schiff bases, which contains a C = N functional group. Schiff bases play an important role in bioinorganic chemistry, where their transition metal complexes provide a synthetic model for the metal-containing sites of metallo-proteins and metallo-enzymes.[37] Formation of a Schiff base is important for the synthesis of secondary amines by catalytic hydrogenation. At room temperature, reversible addition of alcohols to the carbonyl function of aldehydes takes place rapidly. The unstable addition products, hemiacetals, usually revert back to the original reactants or react further with other alcohol molecules to form acetals. Water is produced in the latter reaction. Herein, benzaldehyde and ethanolamine were chosen as starting materials for the demonstration of Schiff base formation. The reaction leading to Schiff base formation and the well-accepted mechanism[38] of acetal formation are shown together in Scheme 1.

In this experiment, 200 μL of 0.01 mol/L benzaldehyde solution (CH_3OH/H_2O, 7:3, v/v) was placed inside the microcentrifuge tube as solution A, while 10 μL of 0.1 mol/L ethanolamine solution (CH_3OH/H_2O, 7:3, v/v) was used as solution B. Representative PESI mass spectra recorded at 0.00 (the starting point), 0.30, 0.50, 1.50, 5.00 and 9.00 min are shown in Fig. 4. At the starting point, as the diffusion of the solution B did not cause any changes in solution composition near the sampling hole, only the protonated benzaldehyde (*m*/*z* 107) and the dehydration product of the methyl hemiacetal of benzaldehyde (*m*/*z* 121) were detected together with some chemical noise. At 0.30 min, the diffusive mixing of the two reactants led to the detection of the protonated ethanolamine monomer and dimer at *m*/*z* 62 and 123, respectively. From 0.50 min, the signal of the protonated Schiff base product (*m*/*z* 150) was detected and the signal at *m*/*z* 121 decreased to the noise level.

The EICs of the ions at *m*/*z* 121, 62 and 150 are shown in Figs. 4(G), 4(H) and 4(I), respectively. It was found that the diffusion rate in this reaction was higher than in the other reaction systems mentioned above, which might be due to the high methanol content in the sample solution. The intensity of the ion at *m*/*z* 121, corresponding to the starting reactant benzaldehyde, decreased rapidly to the noise level at about 0.30 min. The intensity of ethanolamine showed a contrary tendency, but the intensity of the final product increased continuously over the same time period. By using PESI-MS, changes in each chemical species could be monitored directly. Although the ion intensities provided by real-time MS monitoring are not necessarily proportional to the absolute intensities of the compounds due to their

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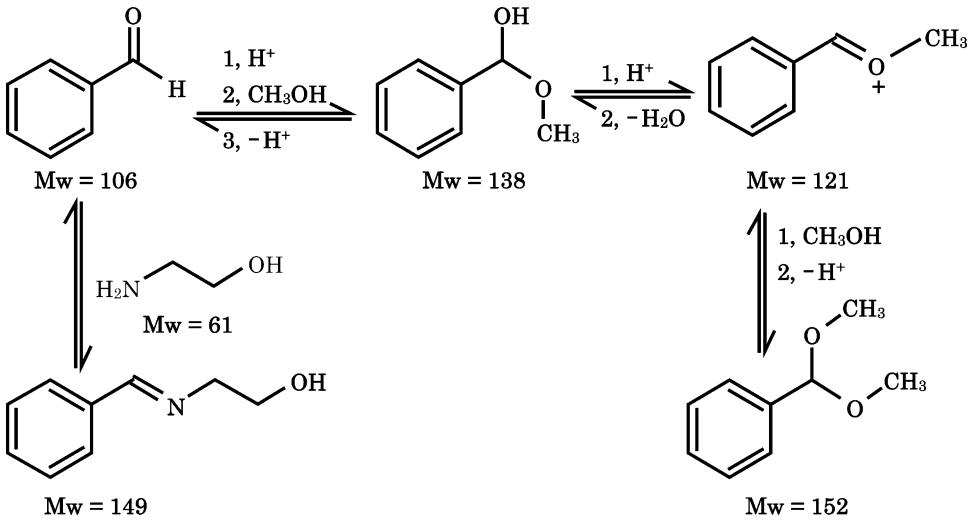

Scheme 1. Acetal formation from benzaldehyde and methanol and Schiff base formation from benzaldehyde and ethanolamine.

different physicochemical properties such as hydrophilicities, proton affinities, etc., PESI-MS could provide helpful information for the investigation of chemical kinetics. The merits of rapid response, high sensitivity and high accuracy highlight the application of PESI-MS to the real-time monitoring of reactions. Applying PESI-MS for research into the mechanism of organic reactions could be worthwhile. It should be noted that the curves shown in Fig. 4 are

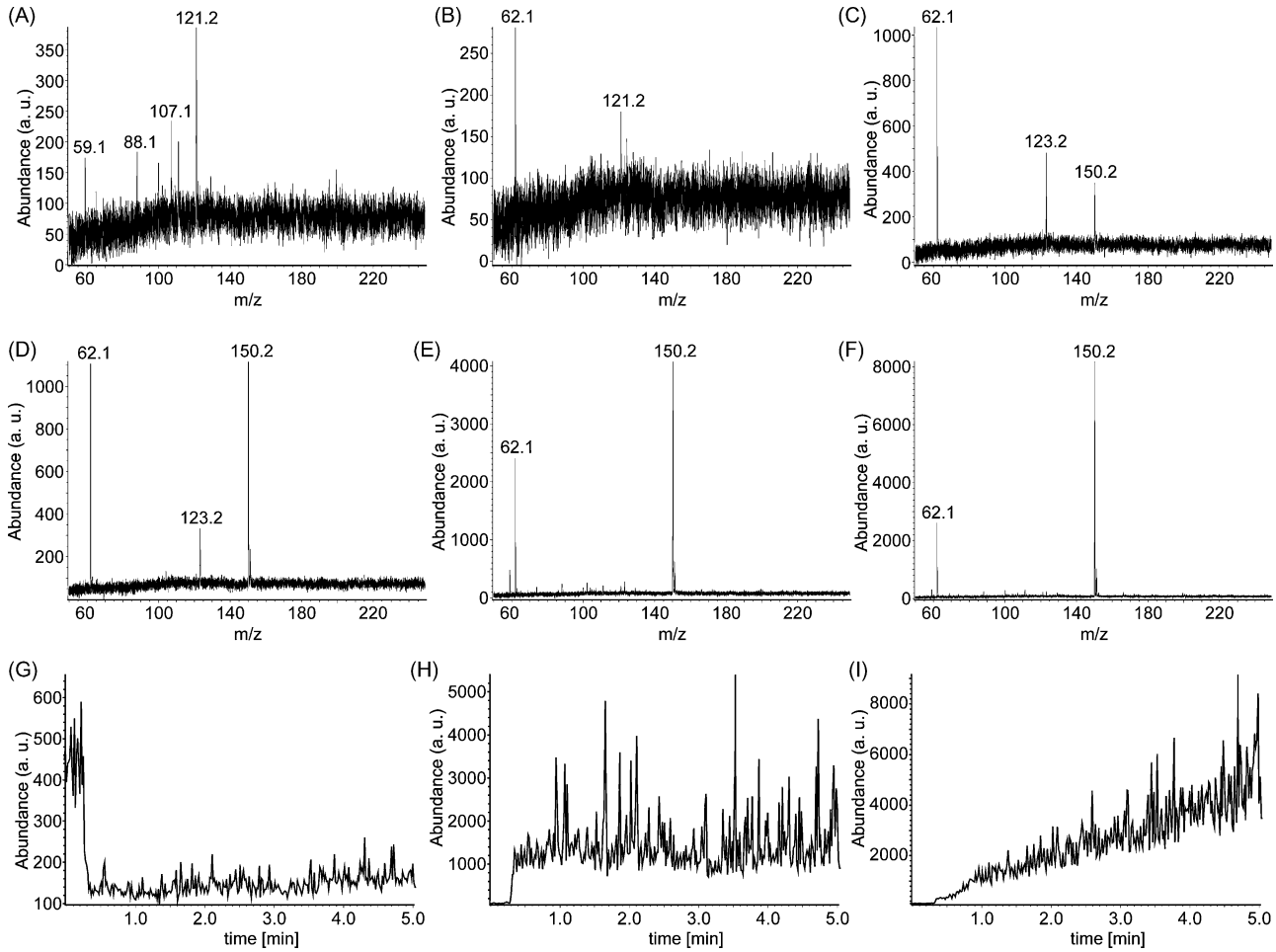

Figure 4. Time-resolved mass spectra of Schiff base formation monitored by PESI-MS combined with diffusive mixing at 0.00 min (A), 0.30 min (B), 0.50 min (C), 1.50 min (D), 5.00 min (E) and 9.00 min (F) after the ethanolamine solution (solution B) contacted the benzaldehyde solutions (solution A). EICs of three representative ions at *m/z* 121.2 (G), 61 (H) and 150 (I) during the time course of real-time PESI-MS monitoring.

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not as smooth as those in the other figures perhaps due to the high methanol content of the solution. During the motion of the needle, a small amount of high methanol concentration solution loaded on the needle tip can readily evaporate, leading to instability in the ion chromatograms. One possible approach to circumventing this difficulty is to introduce an auxiliary vapor to the needle tip.[20,21]

CONCLUSIONS

In this study, PESI-MS was utilized for the real-time monitoring of three kinds of reactions: acid-induced denaturation of proteins, HDX of peptides, and Schiff base formation. The simple instrumental configuration and easy operation made PESI-MS suitable for both laboratory and industry usage. With its merits like direct ambient sampling, low sample consumption with minimum sample pretreatments and high tolerance to salts, PESI-MS is useful in research on the kinetics of chemical and biological reactions in complex matrices. The time-resolved mass spectra and ion chromatograms provided by PESI-MS reflect changes in each chemical species and, moreover, the progress of the whole reaction. The reaction kinetics may be elucidated by the application of this technique.

At the present stage, a time resolution of 0.33 s can be achieved due to the 3 Hz driving frequency of the needle. A time resolution of 0.1 s or less may, however, be achievable by utilizing a higher frequency of needle motion. As PESI-MS has provided a window into kinetic research, further research focused on the real-time monitoring of in vivo chemical/metabolic changes in living biological systems is in progress in our laboratory.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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