Porous Anodic Alumina Membrane as a Sample Support for MALDI-TOF MS Analysis of Salt-Containing Proteins

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) analysis of proteins in salt-containing solution was performed for the first time using porous anodic alumina (PAA) membrane as sample support. The resulting spectral quality of proteins under standard sample preparation conditions was superior to that of normal metal sample stages. Analysis of phosphate-doped protein solutions indicated that porous anodic alumina membranes as a target yielded better results than a metallic target for salt-containing solutions. Because of the biocompatibility of the PAA, proteins can be adsorbed on the PAA and thus a washing process can be introduced to remove the salts from the PAA target before MS analysis. This desalting step significantly enhanced spectral quality, and better signal-to-noise ratios were obtained. The present technique is promising for proteomics research. (J Am Soc Mass Spectrom 2005, 16, 1488–1492) © 2005 American Society for Mass Spectrometry

Since the pioneering work by Karas and Hillenkamp, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been proven to be a powerfully widespread technique in analytical chemistry attributable to its high capacity to rapidly analyze minute quantities of materials [1], and a rapid and convenient method for the characterization of peptides and proteins from biological samples [2–4] because of its compatibility with biological buffers or other interferences [5, 6]. However, MALDI-MS is not immune to interferences in complex biological samples [7]. Smaller amounts of salts usually results in formation of adduct on the molecular ions of analytes and thus produce broadened and poorly resolved peaks [8]. Since adducts increase peak width and decrease resolution, desalting process before MALDI analysis has been used to increase mass accuracy and spectral quality [9]. The removal of the interfering species such as salts and buffers from protein samples, metallic supports modified with functionalized self-assembled layers, [7, 10, 11] or other types of supports with highly binding affinity for proteins have been suggested [12–19]. The other types of supports are nitrocellulose [12], poly(vinylidine difluoride) [15, 16], polyethylene [17], nylon [18], and polyurethane [14, 19].

Porous anodic alumina (PAA) membrane is an inorganic material with self-ordered nanoporous pores. It has a packed array of columnar hexagonal cells with central, uniformly sized holes ranging from 4 to 200 nm in diameter, pore density as high as 10^8 to 10^{11} pores/cm^2 [20–22]. The PAA has been extensively used in fabricating nanometer-sized tubes, rods, and wires of various materials with a great flexibility [23–26]. Because the PAA membrane has nano-sized pores and enormous pore density, it may be used in dispersing samples in MALDI analysis. In addition, PAA has a good biocompatibility and can adsorb protein via supramolecular interactions [27, 28], which permits a washing step for the removal of contaminants from protein samples. The PAA with high density of pores and biocompatibility might be used in MALDI analysis of proteins for enhancing spectral quality.

Here, we report on the use of porous anodic alumina (PAA) membrane as sample supports for MALDI-TOF

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MS analysis of proteins. MS results showed that the PAA-based spectral qualities were better than those with metal targets. Adsorption of proteins on the PAA was characterized by using infrared spectroscopic technique. The morphology of the protein deposited on the PAA was examined with scanning electron microscopy. Application of the biocompatible PAA film as support allows the introduction of a washing step before MS analysis for removal of salts and buffer components, resulting in an enhanced spectral quality and better signal-to-noise (S/N) ratios. The present protocol may be used in analysis of complex mixture of proteins.

Experimental

Myoglobin, cytochrome *c*, and insulin solutions (Sigma, St. Louis, MO) were prepared with a mixture of water and acetonitrile (vol:vol = 70:30). A phosphate-doped lysozyme (Yuanju Biological Co., Shanghai, China) solution was prepared with 70:30 (vol:vol) phosphate buffer and acetonitrile. Polyurethane adhesive (NIPPOLLAN-DC 205, Nippon Polyurethane Industry Co., Ltd., Tokyo, Japan) was diluted with ethyl acetate until colorless for further use. Porous anodic alumina (PAA) (diameter: 13 mm, pore size: 0.1 μm) was obtained from Whatman International Ltd., (Maidstone, England). Saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma, St. Louis, MO) in a mixture of 70:30 water/acetonitrile (vol:vol) with 0.1% trifluoroacetic acid (Tedia Company, Inc., Fairfield, OH) was used as matrix for MALDI-TOF MS analysis.

The porous alumina membrane was cleaved into small pieces as MALDI targets. After drying at room-temperature, PAA piece deposited with 1.0 μl of freshly prepared protein and matrix mixture (1:25, vol/vol) was adhered to a stainless steel target probe (MALDI target) using 1.0 μl of polyurethane adhesive solution. For comparison, direct deposition of the same amount of the protein and matrix mixture to a metal target was also performed. When incorporating a washing step, the protein loaded PAA piece was washed with 20 μl of 20:80 methanol/water (vol:vol) solution for a few times before addition of the matrix.

MALDI-TOF MS analyses were performed employing delayed extraction in positive ion mode on a time-of-flight mass spectrometer (Voyager-DE STR, Applied Biosystems, Framingham, MA) using an accelerating potential of 25 kV. Spectra were obtained using a nitrogen laser (337 nm) adjusted to slightly above threshold. The spectra presented in this paper generally represent the sum of 120 laser shots. Transmission FT-IR spectra (128 scans at a resolution of 8 cm^{-1}) were recorded on a Tensor 27 Fourier transform infrared spectrometer (Bruker, Karlsruhe, Germany) equipped with a liquid nitrogen cooled DTGS detector. Morphology of the metal and the PAA target deposited with phosphate-doped lysozyme was examined on a scanning electron microscope (Hitachi SEM-X650, Hitachi Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

Results and Discussion

Adsorption of Lysozyme on PAA Membrane

To understand the biocompatibility of the porous anodic alumina film, we used lysozyme as a model biomolecule to study its adsorption on the PAA membrane with the help of an infrared spectrometer. Spectra of 40 μl lysozyme (5 mg/ml) deposited on a PAA film followed by washing 1 to 3 times were collected. All the spectra showed that lysozyme adsorbed on the PAA membrane as indicated by the two-amide adsorption bands at 1540 cm^{-1} and 1655 cm^{-1} [29]. A significant decrease in the band intensities of the sample was observed upon washing the first time (Figure 1), indicating that most of the proteins were washed away from the membrane. However, further washing of the sample only decreased the band intensities slightly, demonstrating that only the lysozyme directly interacting with the PAA surface remained on the surface. These results demonstrate that a washing protocol to remove contaminants from complex protein samples can be introduced before MALDI-TOF MS analysis.

MS Analysis of Proteins on a PAA Membrane

Comparison between the MS spectra of 10 pmol lysozyme with CHCA as matrix on a porous anodic alumina membrane and a metallic target were performed. Both targets gave signals corresponding to a single charged peak and a doubly charged peak (results not shown). Careful examination of the spectra using automatic S/N calculation software of the spectrometer showed that the S/N ratio for the proteins deposited on the PAA target was about three times better than that

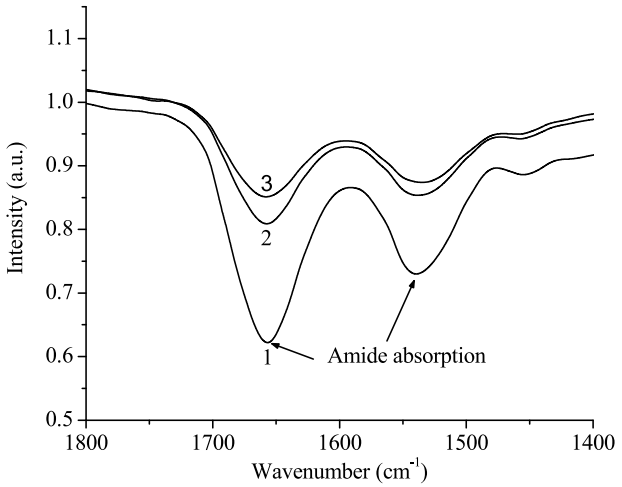

Figure 1. Transmission FT-IR spectra of 40 μl lysozyme (5 mg/ml) deposited on a PAA membrane (1) and followed by washing with water once (2), and twice (3), respectively. Spectrum of a pure PAA membrane was used as reference.

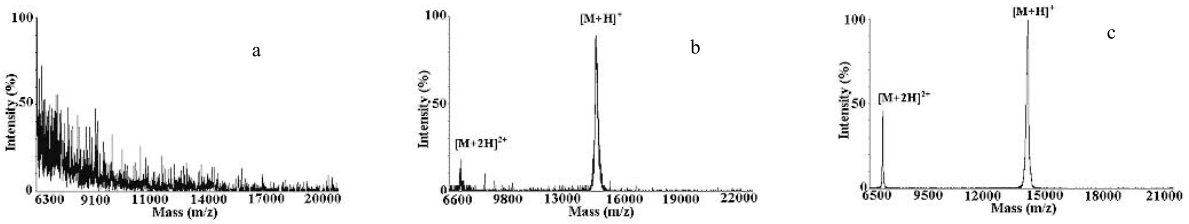

Figure 2. MALDI-TOF mass spectra of lysozyme (3 mg/ml) in a solution of phosphate buffer and acetonitrile (7:3, vol/vol) deposited on a metallic target (**a**); a PAA target (**b**). Spectra (**c**) correspond to the mass spectra of sample (**b**) washed with methanol/water (1:4, vol/vol) three times. CHCA was used as the matrix.

on the metallic target. This could be attributable to the high pore density and good biocompatibility of the PAA membrane that dispersed the protein sample well on its surface, resulting in an enhanced spectral quality and better S/N. The large surface area associated with the pore structure of the PAA membrane allowed the sample to disperse into or out of the pores, also resulting in an increased MS signal. The present PAA target could be applicable to other proteins with similar molecular weight such as myoglobin, cytochrome *c*, and insulin. The S/N ratios for myoglobin, cytochrome *c*, and insulin obtained on the PAA targets were 2.86, 2.14, and 2.70 times better than that on the metallic targets, respectively. Average of the six different locations for one sample offered all the results.

Incorporation of a Washing Protocol Before MS Analysis

Although MALDI-TOF MS is relatively tolerant of impurities such as salts and buffer components, removal of these species will yield higher resolution and mass accuracy. To accomplish this, a desalting step before MS analysis with PAA as support was used for protein analysis with relatively high amounts of salts and buffer components. In our experiments, samples of lysozyme were prepared in 350 mM phosphate and acetonitrile (7:3, vol/vol). As expected, at significantly higher concentrations of phosphate, no sample signal was observed on a metal target (Figure 2a). In contrast, a weaker signal clearly appeared for a PAA target (Figure 2b), although the peak was wide and the S/N ratio was low because of the salt adduct of the analyte molecular ions. The S/N ratio was 6.0 for the phosphate-doped lysozyme deposited on the PAA target. An overall improvement in peak shape was achieved with successive washing steps; peaks became narrower after washing three times (Figure 2c). The reason for the improvement in the peak shape must be due to a decrease in the salt adduct for the molecular ions of the analyte after washing. This result implied that the proton adduct of the molecular ions of the analyte was in the majority. In addition, the intensity of the doubly charged peaks increased and the S/N ratio was better than that without washing. The calculated S/N ratios were 28.8, 89.2, and 479.1 for washing the phosphate-doped lysozyme deposited onto the PAA target once, twice, and three times, respectively. This washing step could also be applied to the phosphate-doped cytochrome *c*. As expected, the results were similar to lysozyme. The calculated S/N ratios were 584.6, 625.4, and 988.8 for washing the phosphate-doped cytochrome *c* deposited onto the PAA target once, twice, and three times, respectively. Obviously, introduction of a washing step before the MS analysis could significantly increase the mass spectral quality of the salt-containing proteins.

SEM imaging of the morphology of the supports deposited with protein was also carried out to understand the improved MS spectral quality. For a metallic target (Figure 3a), a phosphate salt layer was clearly visible besides some branched salt crystals. The protein molecules and matrix were imbedded in the salt layer. The salt layer will shield or scatter the laser beam, resulting in inefficient ionization of the proteins. So, no sample signal was observed on a metal target for a phosphate-doped protein solution (Figure 2a). For a PAA target (Figure 3b), proteins, matrix, and salt dispersed on the surface and only discontinued salt blocks appeared as indicated by arrows. The salt blocks would shield or scatter the laser beam, resulting in inefficient ionization of the proteins beneath them. However, the proteins exposed on the PAA surface could adsorb laser beam for ionization. This was the reason that MS signal of proteins without washing was observed (Figure 2b) although the S/N ratio was relatively low. Following the first washing step of the PAA target (Figure 3c), most of the salt blocks disappeared, as indicated by the significant increase in the number and size of pores. Obviously, most of the discontinued salt blocks were removed by washing, resulting in the exposure of proteins adsorbed on the PAA surface for ionization. Therefore, MS spectra with a better S/N ratio could be obtained. Although the washing process could remove not only the salt but also the proteins deposited on the PAA (as described in Figure 1), sufficient amounts of protein remaining on the PAA membrane as MALDI still produced strong signals. This result was in good agreement with that obtained with the infrared spectroscopic measurement. These results showed that use of PAA membrane as target was an excellent means to avoid the problems of high amount of salts in protein mixture analysis.

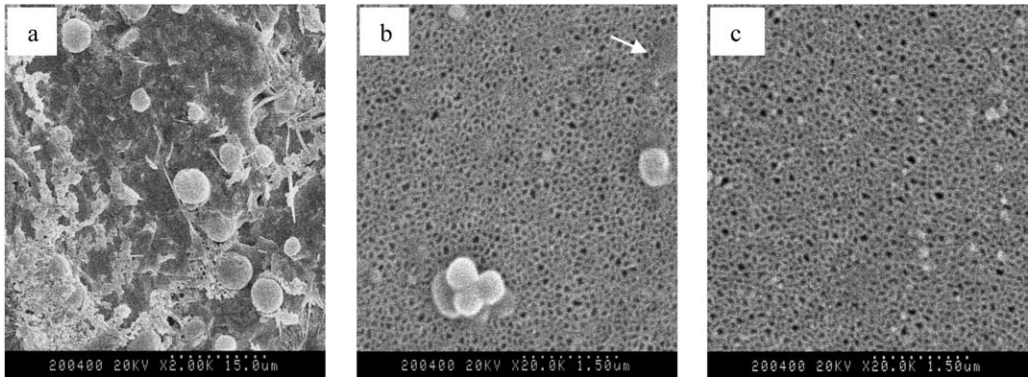

Figure 3. SEM images of a metallic target (**a**) and a PAA target (**b**) deposited with lysozyme sample (200 pmol) dissolved in a solution of phosphate (350 mM) buffer and acetonitrile with CHCA. Image (**c**) corresponds to the sample (**b**) washed with methanol and water (1:4, vol/vol) once.

Conclusions

We have first demonstrated the use of porous anodic alumina (PAA) membrane as sample support for MALDI-TOF MS analysis of proteins. The resulting spectral quality of proteins with standard sample preparation conditions is better than that of normal metal sample stages. Because of the affinity of PAA membrane toward biomolecules, as shown by FTIR measurement, the present technique allows us to introduce a desalting step before MS analysis. Therefore, high quality spectra of proteins on the PAA target can be achieved simply by a washing protocol. The present technique is promising for proteomics research.

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