Use of a Non-porous Polyurethane Membrane as a Sample Support for Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry of Peptides and Proteins

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) of proteins and peptides was performed on samples deposited onto non-porous ether-type polyurethane (PU) membranes. Spectra obtained using PU membranes showed that mass resolution and accuracy were equivalent to values observed using a metal target, and superior to those obtained using poly(vinylidene difluoride) (PVDF) membranes. A small apparent increase in the mass of proteins and also loss of resolution were observed at very high laser irradiance due to charging, but were not observed under normal conditions. Analysis of NaCl-doped standards demonstrated that PU membranes yielded better results than a metallic target for salt-containing solutions. Relatively strong hydrophobic interactions between the proteins and peptides and the PU membrane allowed the incorporation of a washing step. This step allowed for the removal of salts and buffer components and thus provided an increase in resolution and mass accuracy. Digestion of citrate synthase (a protein of molecular weight 47 886) with trypsin was performed directly on the surface of the membrane for variable periods of time, and characteristic peptide fragments were observed by MALDI-TOFMS. Delayed extraction was used to increase the resolution and to permit more accurate mass assignments for those fragments. The use of PU membranes for MALDI-TOFMS analysis of proteins with higher molecular weights is also demonstrated. © 1997 by John Wiley & Sons, Ltd.

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS)[1–3] provides a rapid and convenient means for the characterization of proteins and peptides derived from biological samples. The method is relatively tolerant of impurities, such as salts and buffers. Molecular ions of peptides and proteins can still be produced by MALDI, even with salts or buffers at concentrations which would hamper other ionization processes such as electrospray (ES). Delayed extraction, combined with reflecting TOF mass analysis, provides high resolution and allows accurate mass measurements for sample components in the ppm range, even for fairly complex mixtures.[4]

Various methods of sample preparation using different matrices have been developed for MALDI-TOFMS applications.[5] In spite of the tolerance to impurities mentioned above, biologically derived samples must still be isolated and purified prior to analysis to obtain the best results. Purification is performed to remove salts and buffer components (among others) which may interfere with the signal during data acquisition. High salt concentrations strongly disrupt crystallization and quench MALDI signals. Smaller amounts 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-TOFMS analysis have been developed, including dialysis and chromatography with reversed phase C-18 cartridges. Both methods have limitations, such as sample loss and time-consuming sample preparation.

A different approach is to carry out the purification on the MALDI probe surface itself; this clearly avoids many sources of sample loss. For example, a small amount of powdered chromatographic packing placed on the MALDI target, allows for the selective removal of interfering components.[6] Surface modified agarose beads have been used for the same purpose.[7] An alternative technique consists of chemically modifying the probe surface by the addition of coatings such as nitrocellulose[8] and Nafion.[9] As an extension of this approach, C-18 derivatized targets have been prepared.[10] If the analyte of interest is selectively adsorbed onto the modified probe, interfering substances can be washed off, while the analyte is retained. However, in these examples mentioned above, modification of the probe surface is time-consuming, and the probes are good for only a limited number of uses. In addition, samples must still be transported to the MALDI-TOFMS laboratory by conventional means, e.g. in solution and on ice.

The use of membranes as sample supports has recently been adopted as a means of both sample purification and sample delivery into the mass spectrometer.[11,12] Membrane supports investigated include poly(vinylidene difluoride) (PVDF)[13,14] and polyether.[15] Membranes have been used to prepare samples for MALDI-TOFMS following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[13–15]

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and for collection of capillary electrophoresis samples.[16] Deposition of aqueous protein solutions onto membrane supports has been shown to enhance MALDI signals for samples containing buffer components in higher concentrations than can generally be tolerated. Subsequent purification by on-probe washing and enzymatic digestion result in low sample loss since proteins and peptides are bound fairly strongly to the membrane by hydrophobic interactions. The membranes are also very convenient for transporting samples from the preparation lab to the MALDI-TOFMS lab, and for easy introduction of the samples into the mass spectrometer.

Here we report the use of non-porous polyurethane (PU) membranes for this purpose. Our choice is based on previous studies performed in our Chemistry Department on PU foam and PU membranes which demonstrate that uptake of a neutral analyte by PU is strongly favored over uptake of a charged species.[17] PU membranes have been used previously for the separation and concentration of neutral metal complexes and organic dyes from aqueous solution.[17,18] They possess a unique two-phase structure consisting of hydrophobic soft domains and relatively hydrophilic hard domains. Proteins and lipids have been shown to adsorb through hydrophobic interaction with the soft domains of the polymer.[19]

EXPERIMENTAL

Reagents and Materials

Solutions of horse heart myoglobin (16 951 Da), bovine insulin (5733 Da) and bovine serum albumin (66 430 Da) from Sigma Chemicals (St. Louis, MO, USA), and bovine apotransferrin (78 030 Da) from Calbiochem (LaJolla, Ca, USA), were made up in water (10^{-4}–10^{-6} M), and used without further purification. No attempt was made in this initial study to work with very small sample amounts. 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, USA). Sinapinic acid, used as the MALDI matrix (saturated in 70:30 water–acetonitrile), was obtained from Sigma. The non-porous ether-type PU membrane, 50 μm in thickness (XPR625-FS), was supplied by Stevens Elastomerics (Northampton, MA, USA). The Immobilon-P membrane (PVDF) was obtained from Millipore (Malborough, MA, USA). The PU and PVDF membranes were washed with water and methanol prior to use.

Sample preparation

Sample preparation for MALDI was based on the dried-drop method.[20] The sample solution (2 μL) was placed on the membrane and allowed to dry slowly. Methanol (2 μL) was added and also dried. The matrix solution (2 μL) was then added and allowed to crystallize slowly. When incorporating a washing step, 20 μL aliquots of water were applied to the sample at intervals of one minute, and then removed prior to the addition of matrix. After the matrix had dried, the membrane was placed on a silver disk which had been coated with a thin layer of adhesive (Spraymount, 3M). Excess membrane was trimmed from the disk and the disk placed into the MALDI probe. This assembly is shown in Fig. 1.

MALDI-TOFMS

MALDI-TOFMS was performed in the linear mode on Manitoba II, an instrument constructed in our laboratory,[21] using sinapinic acid as the matrix (sat. in 70:30 H_2O: ACN),[22] and an accelerating potential of 25 kV. In order to avoid saturation of the detector by low mass matrix ions, the detector was pulsed on ~19 000 ns after each laser shot. Delayed extraction experiments were performed on the same instrument with a delay time of 700 μs with a pulse height of 3 kV and an accelerating potential of 20 kV. Spectra were obtained using a laser fluence (337 nm) adjusted slightly above threshold. Each spectrum presented here results from the sum of either 50 or 100 consecutive shots. External and internal calibration modes were used. External calibrations for measurements using the PU or PVDF membranes were performed with standards prepared on similar targets.

Proteolytic digestion of citrate synthase

Wild type citrate synthase (*E. coli* 47 886 Da, 1.0 mg/mL in 20 mM Tris-HCl and 1 mM EDTA, pH = 7.8) was supplied by A. Ayed and H. Duckworth.[23] The molecular weight was derived from the Swiss-Prot sequence[24] with the following modifications: loss of N-terminal M, post-translational modification (11:N→D),[25] conflict (289:F→V).[26] Proteolytic digestion was performed on- and off-membrane for comparison. On-membrane digestion was performed on 2 μL of protein solution, which was initially placed directly on the membrane and allowed to dry. Trypsin, (2–10 μL, 0.01 mg/mL in same buffer) was then placed onto the protein spots and the digestion was allowed to proceed for times ranging from 2 to 60 min. The digestion was stopped by adding 1 μL of a 1% solution of acetic acid. Digestion of citrate synthase was repeated in Eppendorf™ tubes under similar conditions, for time periods of 2 to 60 min and also for 3 hours, after which the digest was presumed to be complete.

Scanning electron microscopy

Scanning electron micrographs were obtained on a Cambridge Instruments scanning electron microscope.

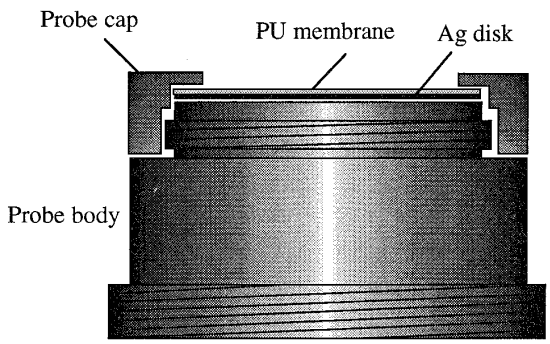

Figure 1. MALDI-TOFMS probe design, with silver disk and PU membrane.

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Samples prepared on the PU membranes and on a metallic surface were coated with gold and palladium by plasma deposition prior to analysis. Images were recorded with a magnification of 30 ×.

RESULTS AND DISCUSSION

Properties of PU membranes

The ether-type PU membrane used possesses a unique two-phase structure consisting of hard (moderately polar) and soft (non polar) domains, as shown in Fig. 2.[27] The hard domains are microcrystalline regions on the surface of the polymer, where the isocyanate portions of the polymer chains are aggregated by hydrogen bonding between the carbamate groups on adjacent polymer chains. These hard domains are relatively polar in comparison to the soft segment domains. The soft domains consist of long chain polyethers which are relatively amorphous in character compared with the hard domains. The two-phase structure of the polyurethane elastomer provides two different regions of possible membrane–protein interactions, differing in polarity and in ability to form hydrogen bonds. Hydrogen bonding with the hard domains and hydrophobic interactions with the soft domains are believed to take place between the protein and the PU membrane, resulting in relatively strong analyte binding.[18,19]

Initial work on PU membranes 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 with washing. Addition of methanol possibly causes disruption of the intermolecular forces holding the polymer chains together, allowing an increase of the effective surface area available for protein sorption. Methanol also facilitates the partitioning of proteins and peptides from more polar components, such as salts.

Preparation of samples on PU membranes and introduction of the samples into the mass spectrometer were relatively straightforward. Both steps were facilitated by the probe design (Fig. 1). Samples could be prepared on the PU membranes in our chemistry laboratory and affixed to the metallic disks allowing analysis at a later date in the MALDI-TOFMS lab. 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. The spectra of myoglobin obtained (a) using a PU membrane and (b) a metallic target are shown in Fig. 3. The spectra were essentially identical. Equivalent resolution and mass accuracy were observed for several proteins of medium molecular weight, whether the samples were deposited on a PU membrane or on a metallic target, with irradiation at 337 nm. In general, spectral acquisition using the PU membranes were more facile and reproducible than with the metal targets. Results obtained for several myoglobin samples indicate an average resolution of 200, and an accuracy of ±0.026% with external calibration.

Charging of the membrane, a phenomenon sometimes observed in MALDI,[28] occurred at laser intensities well above threshold. The build-up of a static charge on the membrane affects the potential of the target surface and thus influences the flight time of the ions. This resulted in an increase in the time of flight of bovine insulin ions as a result of charging (results not shown). Also, as the sampling frequency was increased from taking individual shots at less than 1 Hz to ~10 Hz, the charging phenomenon became more pronounced. This was attributed to shorter times being available for the dissipation of the static charge built up on the membrane. A decrease in resolution resulted, as well as longer flight times. However, the laser intensity used was substantially above the intensity required at

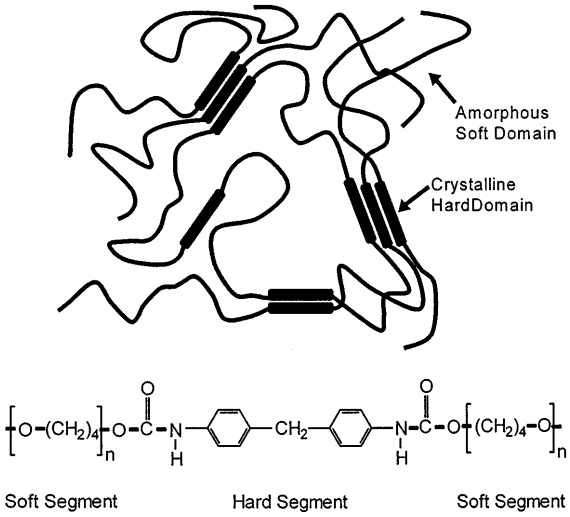

Figure 2. Structure of PU in a membrane, showing hard and soft domains.

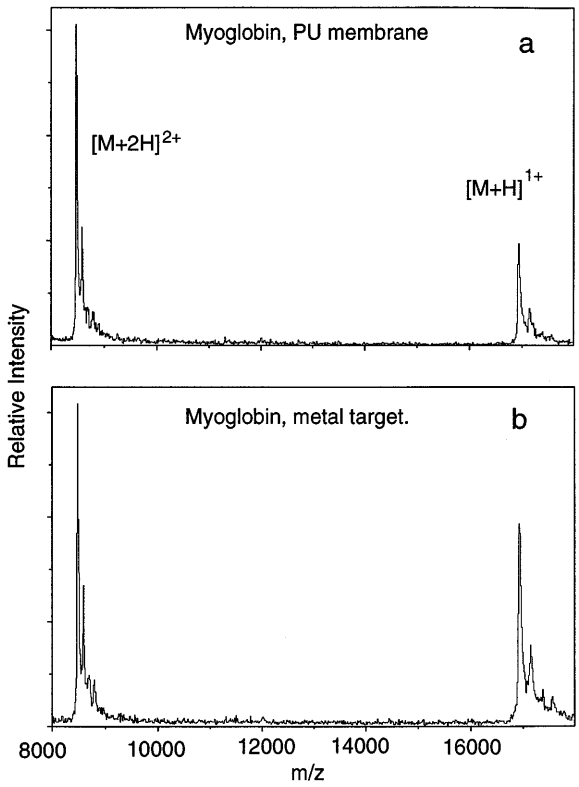

Figure 3. MALDI-TOF mass spectra of 200 pmol of myoglobin in sinapinic acid, obtained using (a) a PU membrane and (b) a metallic target; accumulation of 50 shots.

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threshold, and thus charging was not observed under normal operating conditions with PU.

Comparison between PU, metal and PVDF

Comparison between the spectra obtained for bovine insulin on a PU membrane, on a PVDF membrane and on a metallic target are shown in Fig. 4 for 50 pmol of sample. The PU membrane (a) and metallic target (c) yielded equivalent resolution and mass accuracy for 50 pmol amounts of bovine insulin. However, in our hands the mass accuracy observed with PVDF (b) was not as satisfactory with 50 pmol of sample. A comparison was also made with 5 pmol amounts of bovine insulin (results not shown). In this case, PU and the metallic target yielded comparable spectra to that observed with 50 pmol of sample while the PVDF membrane produced poor quality spectra. In the case with PVDF, the laser intensity required to obtain ionization threshold was higher than that required for PU and the metal target. This was attributed to the porosity of PVDF, which permits distribution of the analyte and matrix within the membrane.[15] The result is surface charging which causes an increase in the flight time, as shown in Fig. 4(b). In comparison, the non-porous nature of the PU membrane (or a metallic surface) favours crystal growth on the surface only. PU thus provides for enhanced spectral quality over membranes with porous structures such as PVDF.

Figure 5 shows the distribution in the flight times for bovine insulin ions desorbed from PU and PVDF membranes. This distribution for PU shows a similar variance to what is typically obtained on a metal target and a smaller variance than observed for ions desorbed from PVDF membranes. In general, peak shapes were better on PU membranes compared to metal targets, making centroid assignment more systematic. This was possibly due to partitioning of the bound protein molecules from interfering adducts (e.g. salts) which can affect the position of the peak centroid. PU-deposited samples were also tolerant of a large range of laser intensities, without observation of peak broadening due to charging or adduct formation. A larger

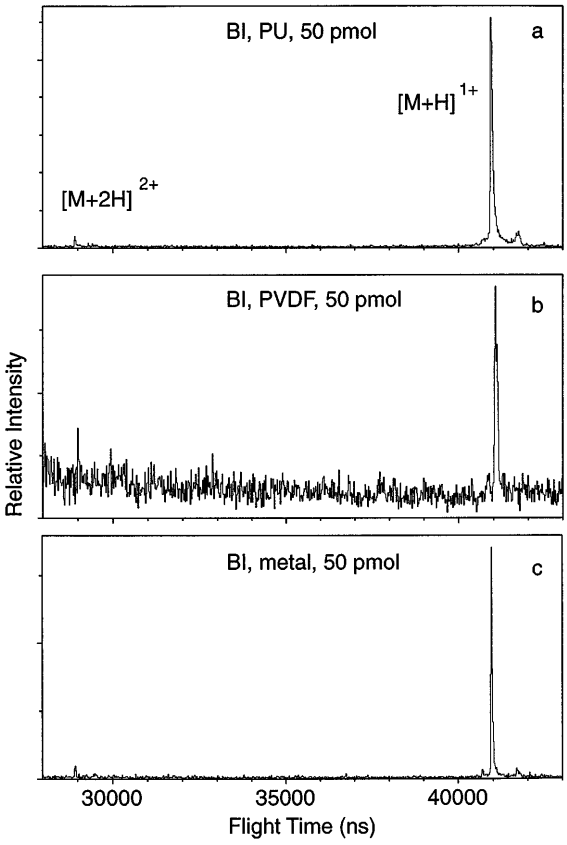

Figure 4. MALDI-TOF mass spectra of 50 pmol of bovine insulin in sinapinic acid, obtained using (a) a PU membrane, (b) a PVDF membrane and (c) a metallic surface; accumulation of 50 shots.

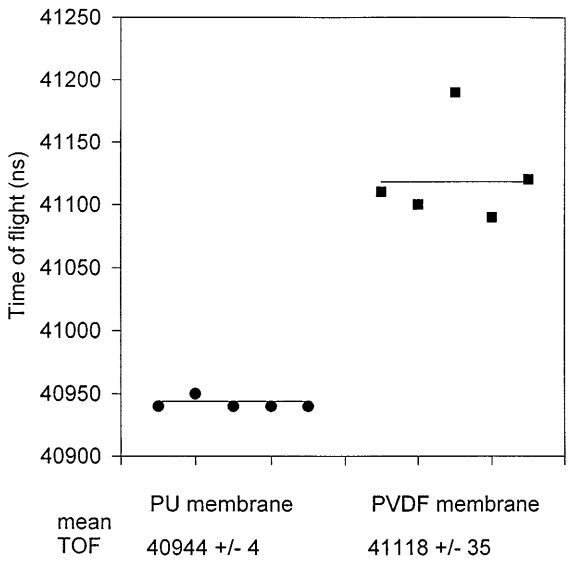

Figure 5. Replicate measurements of the flight times of bovine insulin $[M+H]^+$ ions generated by MALDI. Comparison between a PU membrane and a PVDF membrane (Bovine insulin: 5–250 pmol). Accumulation of 50 shots per measurement.

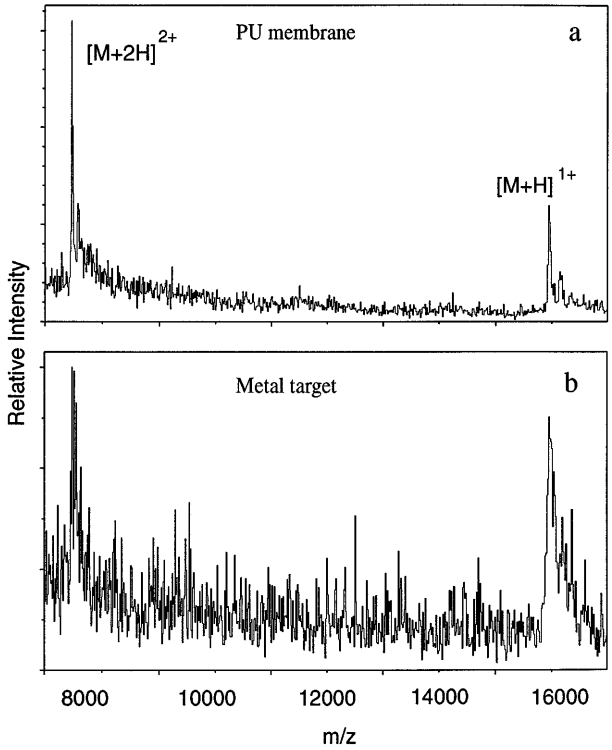

Figure 6. MALDI-TOF mass spectra of 200 pmol of myoglobin with 200 nmol of NaCl in sinapinic acid, obtained using (a) a PU membrane and (b) a metallic target. Accumulation of 50 shots.

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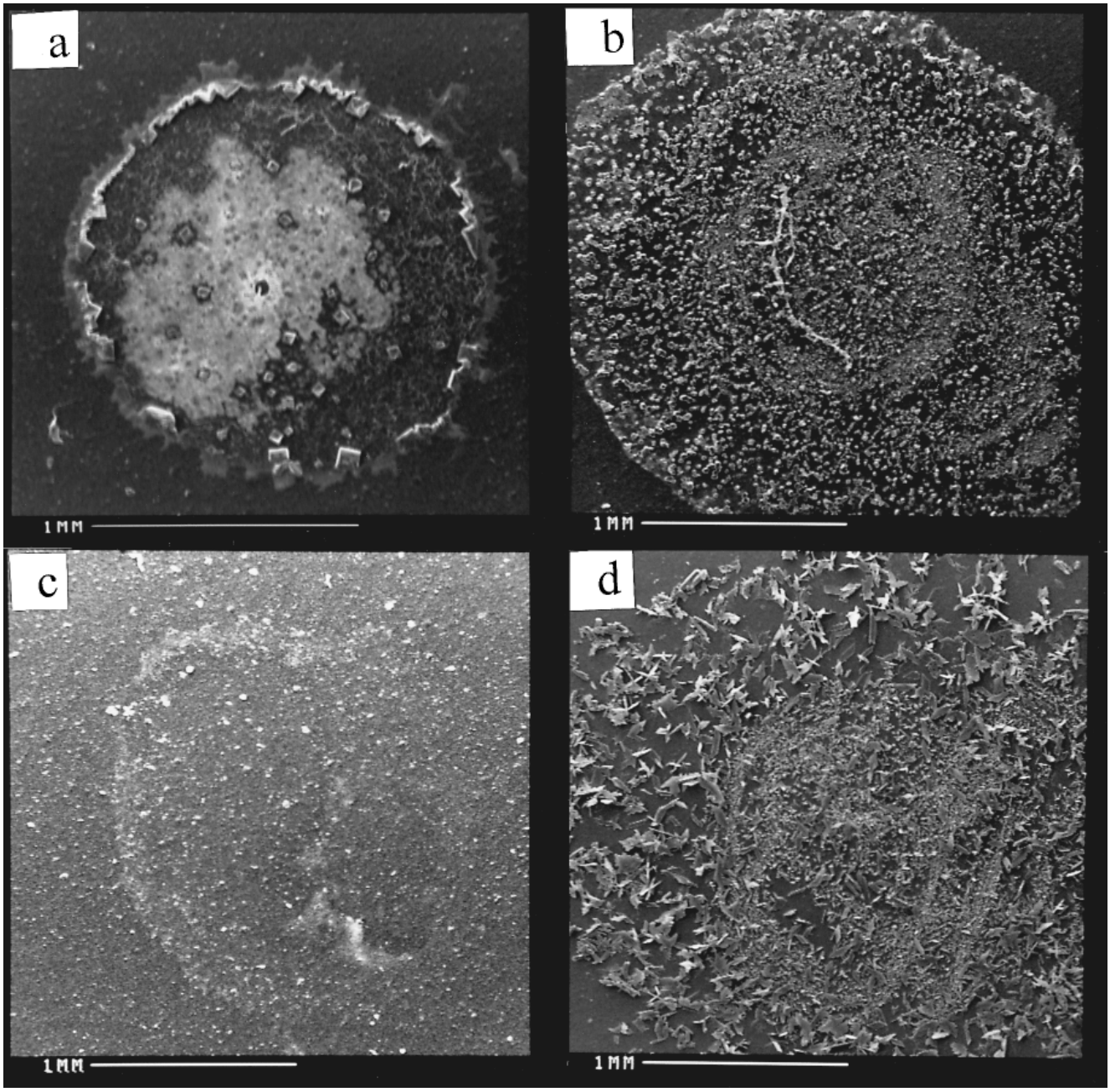

Figure 7. Scanning electron micrographs of myoglobin samples (200 pmol) in the presence of 200 nmol of NaCl on a PU membrane (30× magnification), (a) neat sample, (b) after addition of sinapinic acid, (c) after one wash and (d) after two washes and addition of matrix.

variance in flight times was observed with PVDF due to the spatial distribution of sample within the pores and the larger laser intensity required to generate spectra.

When compared with metal targets for the analysis of NaCl-doped solutions, PU membranes brought a substantial improvement to the quality of the data, as indicated in Fig. 6. Selective partitioning of the protein molecules, NaCl and matrix components between the aqueous phase and the surface of the membrane likely occurs, as some areas of the target produced good quality spectra even in the presence of excess NaCl. This was not the case with the metal target. It has been shown that differences in the crystallization of the analyte with the matrix affect the quality of the spectra.[29–31]

Development of a washing protocol

Application of NaCl-containing solutions to PU membranes resulted in a marked difference in the crystallization patterns of the protein and NaCl mixture before washing, after washing and with addition of matrix, as shown in Fig. 7. Prior to washing (a), NaCl is visible on the surface. After addition of matrix (b), disrupted crystallization is observed, due to presence of the salt. Following one washing step (c), the 'visible' amount of NaCl is removed and only a small amount of sample remains on the membrane. After two or more washing steps, protein and salt are not visible on the membrane surface. A sufficient amount of protein remains bound to the membrane as MALDI still produces strong signals. After washing, the addition of

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matrix (d), results in the formation of analyte–matrix crystals, typical of a clean sample which will produce a good MALDI spectrum.

The relatively strong interactions of the PU membrane with proteins and peptides enables the introduction of a washing step. The use of a washing step was examined for the analysis of samples with relatively high amounts of salts and buffer components. Samples of myoglobin were prepared in a 1000-fold excess of NaCl and applied to the membrane. In this case MALDI spectra were obtained using a wide laser beam to ensure sampling of the entire surface of the target including areas which contained NaCl, myoglobin and matrix. An overall improvement in peak shape and resolution was observed with increasing numbers of washes, as shown in Fig. 8. Some peaks in the spectrum of the original sample (a) correspond to Na adducts. These adducts cause peak broadening and make accurate mass assignment difficult. After successive washing steps (b)–(d), peaks become narrower as the abundance of Na adducts decreases with the removal of NaCl. The resulting increase in resolution enables correct mass assignment.

On-membrane proteolytic digestion of citrate synthase

Application of our membrane methodology to real samples was carried out by performing tryptic digests of citrate synthase directly on the membrane and comparing the results to those from samples digested in Eppendorf™ tubes. Digests were performed for periods of time varying from 2–60 min on the membrane. Good quality MALDI spectra were observed following removal of the buffer components with the washing procedure, an example of which is shown in Fig. 9(a), for the 2 min digest. Samples prepared on metallic targets did not produce spectra at all. Over the duration of the digest, the initially abundant high mass ions were replaced with lower mass ions (i.e. Fig. 9(b)). Also, the protein underwent significant digestion after only two minutes. This may indicate that the protein denatures upon sorption to the membrane, thus facilitating rapid digestion. Similar spectra were obtained for samples digested in Eppendorf™ tubes and on the PU membrane. Most segments of the protein were mapped against calculated fragments, as shown in Table 1. The entire on-membrane digestion process took less than 3 hours from the start of the series of digests to the collection of data. Most of the time was devoted to acquisition and interpretation of spectra.

The 3 hr proteolytic digestion was used to investigate the advantages of using delayed extraction with samples deposited on the PU membrane. The results presented in Fig. 9(b) indicate a peak profile similar to the earlier digest profiles. The use of delayed extraction resulted in a substantial increase in resolution as shown in the inset where the oxidation product of the compound, producing a peak at *m/z* 5759, may be observed. This enabled more accurate mass assignments as shown in Table 1.

Application to high mass proteins

Application of our PU membrane technology to MALDI analysis of higher molecular weight proteins was briefly investigated. The results obtained for bovine serum albumin and apotransferrin are shown in Fig. 10.

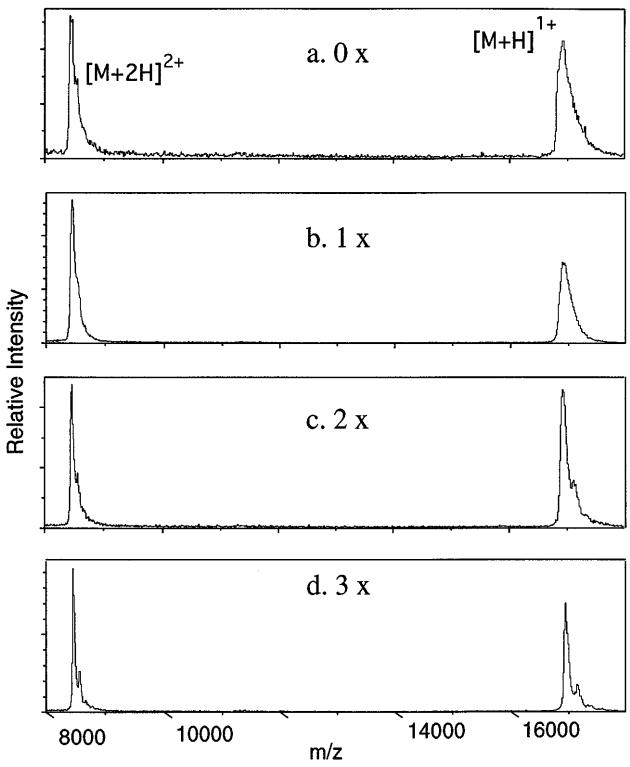

Figure 8. MALDI-TOF mass spectra of 200 pmol of myoglobin in the presence of 200 nmol of NaCl on a PU membrane, (a) neat sample, (b) washed once, (c) washed twice and (d) washed three times. Accumulation of 50 shots.

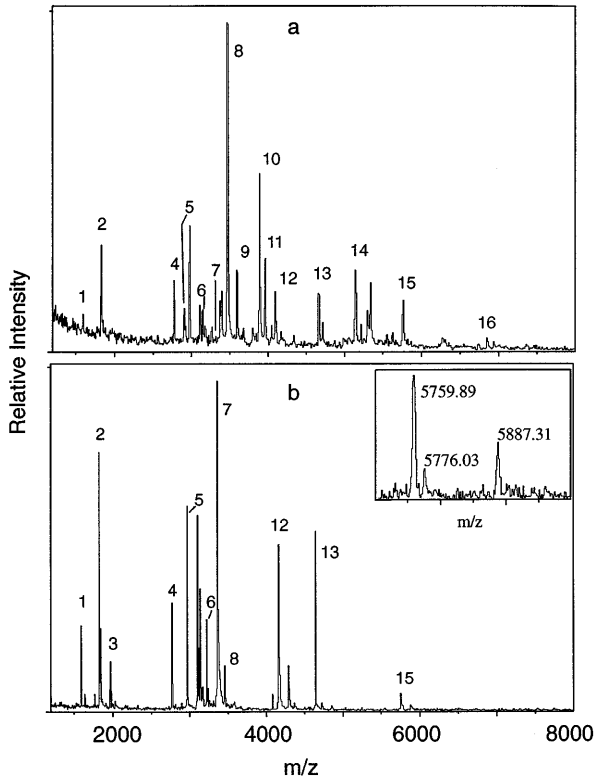

Figure 9. MALDI-TOF mass spectra of the tryptic digest products of citrate synthase (40 pmol), (a) 2 minute digest performed directly on-membrane (PU) and (b) 3 hour digest with delayed extraction. Accumulation of 50 shots.

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Table 1. Selected tryptic fragments of citrate cynthase

Peak #[a]	Sequence	TOF *m/z*	Calc. [M+H]$^+$	Error	% Error
1	57–70	1599.74	1599.83	0.09	0.01
2	372–388	1831.25	1831.26	0.01	0.00
3	389–405	1972.55	1973.25	0.70	0.04
4	8–33	2771.59	2771.14	–0.45	–0.02
5	6–33	2970.44	2970.39	–0.05	–0.00
6	241–274	3140.23	3140.51	0.28	0.01
7	190–218	3366.15	3366.83	0.68	0.02
8	39–70	3462.76	3462.87	0.11	0.00
9	357–388	3581.05	3583.37	2.32	0.06
10	301–333	3881.80	3881.55	–0.25	–0.01
11	34–70	3949.45	3949.44	–0.01	–0.00
12	321–356	4164.60	4164.75	0.15	0.00
13	71–110	4642.48	4642.16	–0.32	–0.01
14	8–56	5121.08	5120.75	–0.33	–0.01
15	308–356	5759.30	5759.61	0.31	0.01
16	106–164	6819.18	6819.84	0.66	0.01
				Accuracy	0.0074

[a] Peaks correspond to fragments in Fig. 9.

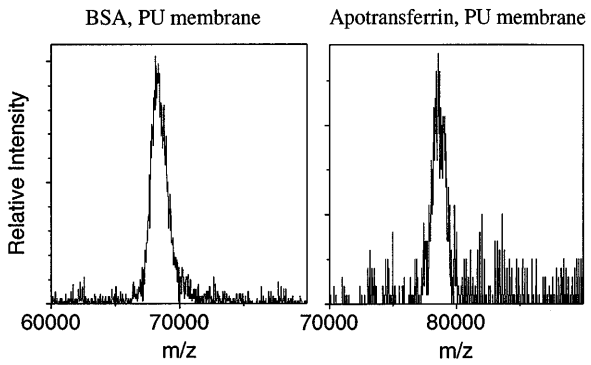

Figure 10. MALDI-TOF mass spectrum of high molecular weight proteins on PU membranes (20 pmol of protein in sinapinic acid), accumulation of 50 shots.

The observed resolution was comparable to that obtained using the metallic target. A slight increase in mass was observed for the samples deposited on PU, likely due to charging and to use of external calibration. This phenomenon was observed only for higher *m/z* values and may be corrected with calibration in similar experimental conditions.

CONCLUSIONS

The use of PU membranes as sample supports for MALDI-TOFMS analysis of proteins and peptides yields equivalent accuracy and resolution to values obtained with metal targets. The non-porous nature of the membrane facilitates crystal growth on the surface only and thus provides for enhanced spectral quality over porous membranes. The relatively strong interactions of the PU membranes with bound proteins and peptides enables the introduction of a washing step in order to remove salt and buffer components, which may interfere with MALDI analysis. Tryptic digestion of citrate synthase performed on the membrane surface yielded characteristic fragments, allowing for successful peptide mapping. As the method is simple and involves robust technology, it is now used in our laboratory on a routine basis.

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