

# Desorption/ionization mass spectrometry on nanocrystalline titania sol–gel-deposited films

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**This paper describes a matrix-free method for performing desorption/ionization directly from mesoporous nanocrystalline titania sol–gel thin films, which have good absorption capacity in the ultraviolet (UV) range and can act as assisting materials during UV matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) analysis. A high concentration of citrate buffer was added into this system to provide the proton source and to reduce the presence of alkali cation adducts of the analytes. The analyte signals appear uniformly over the whole sample deposition area. Protonated molecules (MH<sup>+</sup> ions) of analytes dominate the titania MALDI mass spectra. Surfactants, peptides, tryptic digest products, and small proteins with molecular weights below ca. 24 000 Da, are observed in the titania MALDI mass spectra. Detection limits for insulin are as low as ca. 2 fmol with mass resolution of ca. 660. Copyright © 2004 John Wiley & Sons, Ltd.**

Although matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has made enormous contributions to various fields,<sup>1</sup> matrix interference in the low-mass region and inhomogeneous distributions of analyte signals over the sample deposition area can cause problems during MALDI-MS analysis. The development of two-phase matrix systems consisting of suspended particles mixed with viscous liquids has solved some of the problems encountered during conventional MALDI-MS analysis.<sup>2–15</sup> Alternatively, matrix-free methods, such as performing the desorption/ionization on porous silicon (DIOS) in a process that is facilitated by treating the silicon surfaces electrochemically,<sup>16–20</sup> and mass analysis using silicon films that are formed from a silicon surface by plasma-enhanced chemical vapor deposition, have been applied successfully to the analysis of small molecules.<sup>21</sup> Additionally, previous studies have demonstrated the feasibility of using a film substrate to support samples during laser desorption/ionization mass spectrometric analysis.<sup>15,22–25</sup>

Recently, we proposed a new sample preparation method in which sol–gel/dihydroxybenzoic acid (DHB) hybrid materials are used as assisting materials for MALDI MS analysis.<sup>26–28</sup> The sol–gel-derived film can function as an energy absorber during laser irradiation because it contains the DHB molecules within the film. Furthermore, irradiation with a laser of average energy and power (70–110  $\mu$ J in a few ns) is not likely to generate any background ions from such a sol–gel/DHB-derived film. We have demonstrated that glass chips coated with these sol–gel-derived DHB thin films can be used for on-chip protein and bacterium enzymatic digestions.<sup>28</sup> Additionally, we have also generated sol–gel/diaminobenzoic acid (DABA) hybrid materials that display a

reasonably good capacity for desalting in the MALDI-MS analysis of oligonucleotides.<sup>29</sup> These approaches require laser-light-absorbing molecules to be doped into the sol–gels, which could be a potential drawback.

Thus, we continue to investigate other simpler alternative sol–gel systems that meet the requirements for use as the assisting material for MALDI-MS analysis. Although silica is one of the most common materials used to form sol–gels, titania sol–gels are also used frequently in many fields.<sup>30–32</sup> Pure titania sol–gels have the unique characteristic of being capable of absorbing energy in the UV region. Recently, we successfully employed pure titania sol–gel-deposited thin films as assisting materials for MALDI-MS analysis without requiring additional laser absorber species; we demonstrated the utility of this approach in molecular-recognition-based mass spectrometry.<sup>33</sup> Unfortunately, this approach suffers from interference by Ti<sub>x</sub>O<sub>y</sub> ions in the low-mass region, so titania sol–gel MALDI-MS seems limited to the analysis of molecules with masses >500 Da. Furthermore, the largest molecule that we could detect using this approach, without adding extra matrix, was ubiquitin, which has a mass of ca. 8000 Da; when glycerol was added, the largest detectable molecule was extended to cytochrome C (MW 12 360 Da). Herein, we propose an alternative titania sol–gel-based MALDI-MS analysis technique that reduces the level of background interference and extends the range of detectable masses.

Titania-sol–gel thin films can be converted into crystalline titania with an anatase framework when the titania films are calcinated at a temperature of 500°C for a suitable period of time.<sup>34–37</sup> Many studies have emphasized the optical and photoactivity properties of titania,<sup>30–33</sup> but its most interesting characteristic for use in MALDI-MS analyses is that, in its crystalline anatase framework, it possesses absorption capacity in the UV region. We generated a homogeneous crystalline distribution of titania on a solid support by a sol–gel reaction followed by heat-treatment, and then

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employed this material as the assisting material for MALDI-MS analysis. After the heat-treatment process, however, no traces of any acid remained in the titania film for use as the proton source. Thus we added a proton source, namely citrate buffer, into this titania matrix system to render it suitable for the ionization of analytes. The results were promising and are reported here.

## EXPERIMENTAL

### Reagents

Titanium(IV) *n*-butoxide, ethanol, and nitric acid were obtained from Acros (NJ, USA), Showa (Tokyo, Japan), and J. T. Baker (Phillipburg, USA), respectively. Bradykinin, citric acid, insulin, ubiquitin, trypsinogen, 2,5-dihydroxybenzoic acid (2,5-DHB) and cytochrome C were purchased from Sigma (MO, USA). Melittin and polyethylene glycol ( $MW_{ave}$  600) were obtained from Fluka (Buchs, Switzerland). Diammonium hydrogen citrate was purchased from Riedel-de Haën (Deisenhofen, Germany). Sinapinic acid (SA) and  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich (Milwaukee, USA). Double-sided carbon tape was obtained from Ted Pella (CA, USA).

### Preparation of cytochrome C tryptic digest sample

Cytochrome C ( $10^{-5}$  M) tryptic digestion (50:1, w/w) was performed in an  $NH_4HCO_3$  buffer solution for 24 h at 38°C.

### Preparation of titania thin films

Titania sol was prepared by stirring titanium(IV) *n*-butoxide (3.4 mL) and ethanol (1.6 mL) for 30 min at room temperature (ca. 27°C). A solution of ethanol (1.6 mL), water (0.18 mL), and 60% nitric acid (75  $\mu$ L) was then added slowly into the titanium(IV) *n*-butoxide/ethanol solution, which was stirred for an additional 10 min in an ice bath. Polyethylene glycol

( $MW_{ave}$  600, 15 g) was added into the mixture and stirred for ca. 30 min in a water bath maintained at 40°C. An aluminum sheet (2 cm  $\times$  2 cm  $\times$  0.2 mm) was used as the support for the titania sol coating. The aluminum support was pretreated by soaking it in acetone and then in methanol for 5 min in a sonicator to remove impurities. The titania sol solution was spin-coated onto the surface of the aluminum support using a spin coater. The titania sol solution was applied slowly to the aluminum sheet during the spin-coating process. The modified aluminum sheet, coated with a thin film of titania, was aged for 20 min at room temperature. This titania chip was calcinated at 500°C for 1 h. Fresh titania chips were quite hydrophilic; the contact angle of water on the titania film was measured as ca. 10.7°. The titania chip was stored in a desiccator before use. The thickness of the film was ca. 390 nm measured by using an electron microscope.

### MALDI-MS analyses on a titania chip

Sample preparation for the direct MALDI-MS analysis on the titania chip was straightforward. The titania chip was fixed onto a sample target using double-sided carbon tape. The protein solution (0.2  $\mu$ L) was mixed with an equal volume of citrate aqueous solution [diammonium citrate (200 mM)/citric acid (200 mM) = 5:1.1 (v/v); pH 4.5], while each solution of a small analyte or peptide was mixed with an equal volume of [diammonium citrate (50 mM)/citric acid (100 mM) = 3:1 (v/v); pH 4] aqueous solution prior to MALDI-MS analysis. A portion of this mixture (0.2  $\mu$ L) was applied directly to the modified aluminum sheet. Homogeneous analyte signals were found from the sample deposition spot.

However, for more hydrophobic samples, it is recommended to deposit the citric buffer on the titania film first. Homogenous sample/citrate distribution on the titania chip can be obtained and the analyte signal can be evenly searched over the entire spot of sample deposition. After the solvent

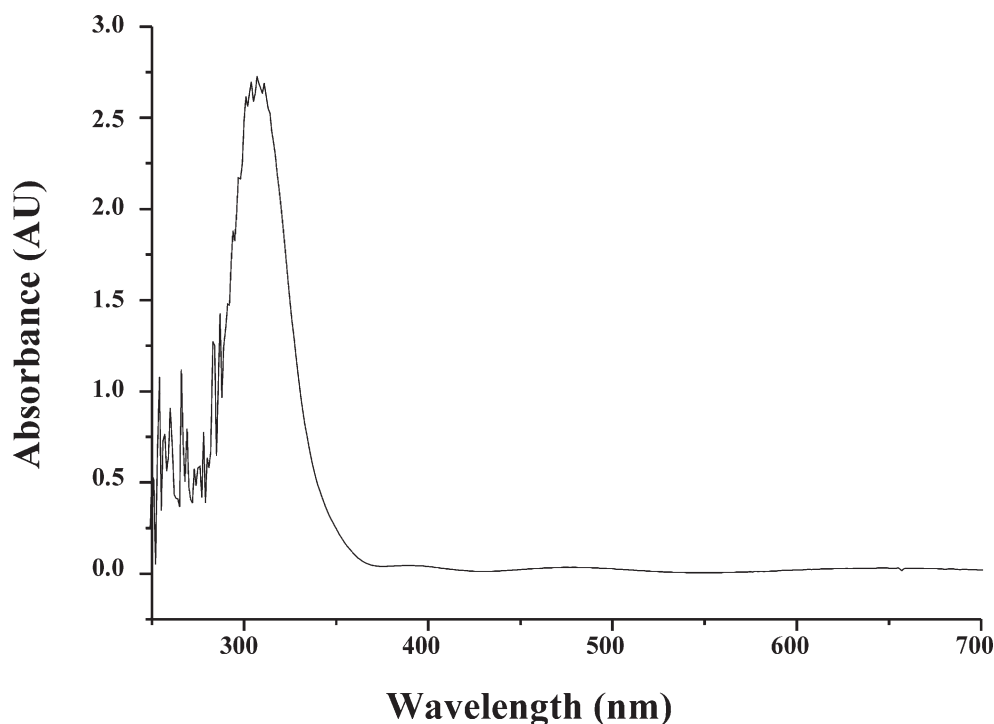
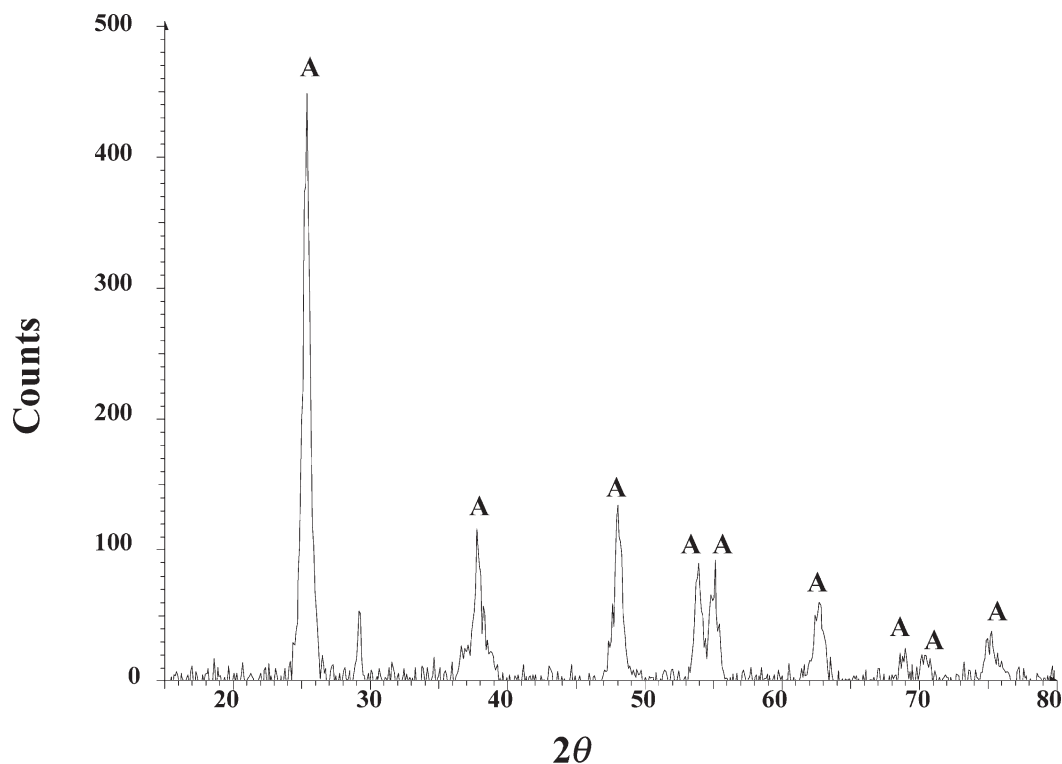


Figure 1. UV absorption spectrum of a titania sol-gel-deposited thin film.



**Figure 2.** XRD pattern of titania powder generated using the same procedure as that used to prepare the titania thin film (A: anatase).

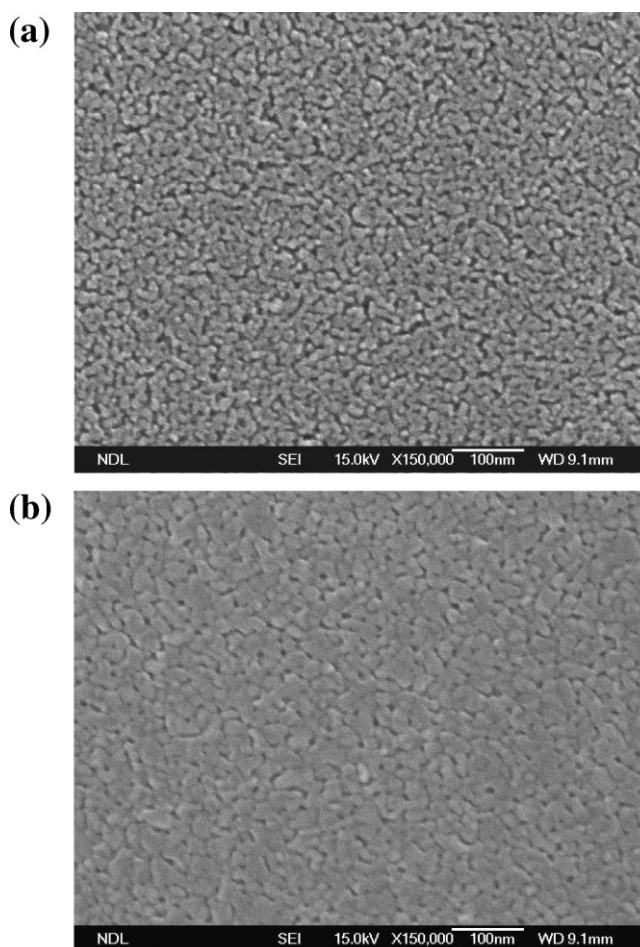
had evaporated, the sample dissolved in organic solvents was then deposited on top of the titania film treated with citric buffer. After the solvent had evaporated, the sample target was ready to be introduced into the mass spectrometer for analysis. This sample preparation step is helpful in obtaining hydrophobic analyte signals homogeneously from the sample deposition.

### Instrumentation

The experiments were performed using a Biflex III (Bruker Daltonics, Germany) time-of-flight mass spectrometer, which was operated in the reflectron mode when protein digest samples were analyzed. The mass spectrometer was equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target with the capacity to load 384 samples simultaneously. The accelerating voltage was set to 19 kV. UV spectra were obtained using a UV spectrometer (Agilent 8453, Germany). Scanning electronic microscope (SEM) images were obtained using a JEOL JSM-6500F SEM. The X-ray diffraction (XRD) result was obtained using a Bruker AXS D8 Advance instrument.

### RESULTS AND DISCUSSIONS

Figure 1 displays the UV absorption spectrum of the titania sol-gel-deposited thin film. The thickness of the glass slide was 0.15 mm, while the thickness of the sol-gel film was 390 nm. The absorbance of the titania thin film at a wavelength of 337 nm is ca.  $3.6 \times 10^6 \text{ m}^{-1}$ , which suggests that the thin film can be employed directly as an assisting material in UV-MALDI-MS analysis. Figure 2 presents the XRD pattern of the titania powder generated using the same procedure as that used to prepare the titania thin film; the XRD pattern is characteristic of anatase titania.<sup>37</sup> The fact that



**Figure 3.** SEM images of titania sol-gel-deposited thin films obtained (a) with and (b) without the addition of PEG 600 during the sol-gel reaction.

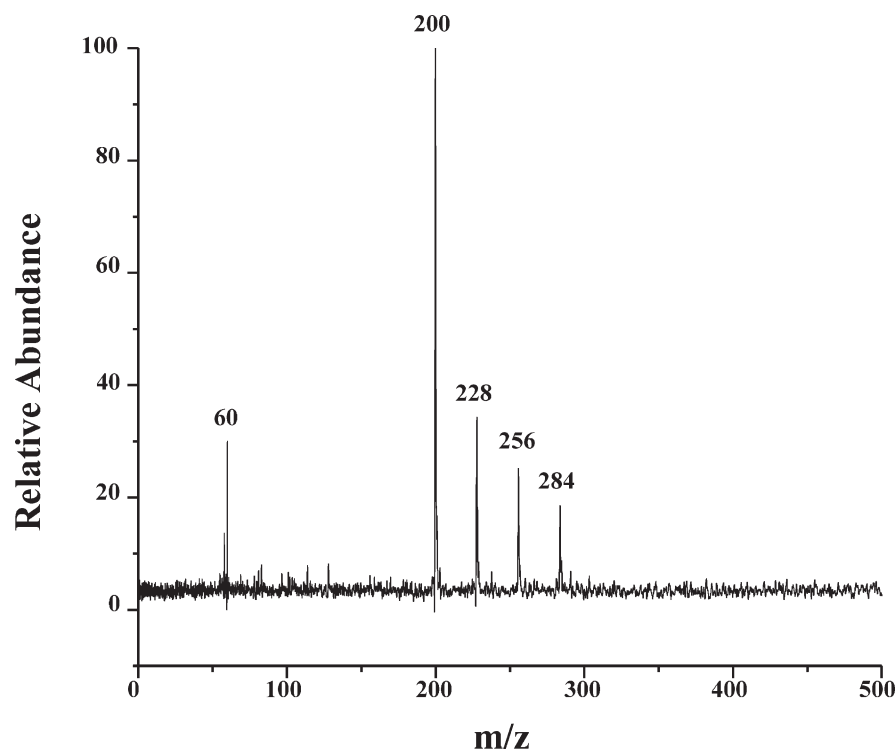
anatase titania has the capacity to absorb light in the UV region is consistent with our observations in Fig. 1.

Figure 3 displays SEM images of the titania sol-gel-deposited thin films. We observe a mesoporous morphology for the titania film with pore sizes of ca. 10 nm (Fig. 3(a)); when PEG 600 is added into the titania sols during the sol-gel process, the resulting pore sizes are larger than those obtained without the addition of PEG 600 (Fig. 3(b)). We found that the enlarged pore size is helpful in lowering detection limits and in extending the mass range in titania MALDI-MS analysis. Presumably, a larger pore size may facilitate more facile incorporation of the analyte molecules into the titania pores, which is beneficial for the energy transfer from titania to analyte molecules during laser desorption. Thus, all of the following titania MALDI-MS results were obtained using mesoporous films similar to that displayed in Fig. 3(a). In addition, we observe in Fig. 3 that the nanocrystalline titania is distributed evenly on the substrate. When sample solutions are placed on these titania films, the analyte signals are observed homogeneously over the sample spot and therefore the problem of 'sweet spots' is avoided.

Figure 4 displays the titania MALDI mass spectrum of a mixture of hexadecyltrimethylammonium bromide ( $C16^+$ , 68 fmol), tetradecyltrimethylammonium bromide ( $C14^+$ , 74 fmol), dodecyltrimethylammonium bromide ( $C12^+$ , 80 fmol), and decyltrimethylammonium bromide ( $C10^+$ , 90 fmol). The peaks at  $m/z$  200, 228, 256, and 284 correspond to the  $C10^+$ ,  $C12^+$ ,  $C14^+$ , and  $C16^+$  ions, respectively, each without its bromide counterion. In addition to these pre-charged ions, a peak corresponding to the  $NH(CH_3)_3^+$  ion, arising from fragmenta-

tion of the cationic surfactants, appears in the lower-mass region at  $m/z$  60.

No background ions arising from the titania matrix appear in this mass spectrum, but in the case of analytes that are not pre-charged it is necessary to provide an extra proton source to ionize them. Previously, we have employed traces of glycerol as the proton source for a related titania MALDI-MS approach in which the titania film was generated from a sol-gel reaction without heat-treatment.<sup>33</sup> The presence of a liquid in the matrix system, however, may limit the applications of this method to high-throughput analysis because of concerns regarding the need to maintain a high vacuum in the mass spectrometer. As an alternative, we mixed citrate buffer with the analytes during the sample preparation process and evaporated the solvent in the buffer prior to introducing the sample into the mass spectrometer. Because the citrate buffer does not absorb the light at 337 nm, we assume that it plays the roles only of a proton source and an alkali cation-sequestering reagent. Because of the addition of citrate buffer, the ions contributed by the buffer are frequently observed in the titania MALDI mass spectra. Figure 5 displays the titania MALDI mass spectra of bradykinin (940 fmol) obtained with the addition of citrate buffer. The protonated molecule of bradykinin ( $M_bH^+$ ) dominates the mass spectrum; the peaks at  $m/z$  39, 70, 231, and 269 correspond to  $K^+$  and  $Al_2O^+$  ions and to potassium adducts of citric acid ( $[M+K]^+$  and  $[M-H^++2K]^+$ ), respectively. The  $Al_2O^+$  signal may arise after the ablation of the titania layer. A weak signal corresponding to the potassium adduct of bradykinin ( $[M_b+K]^+$ ) appears adjacent to the  $MH^+$  peak for bradykinin. Additionally, the



**Figure 4.** Titania MALDI mass spectrum of a mixture of hexadecyltrimethylammonium bromide ( $C16^+$ , 68 fmol), tetradecyltrimethylammonium bromide ( $C14^+$ , 74 fmol), dodecyltrimethylammonium bromide ( $C12^+$ , 80 fmol), and decyltrimethylammonium bromide ( $C10^+$ , 90 fmol).

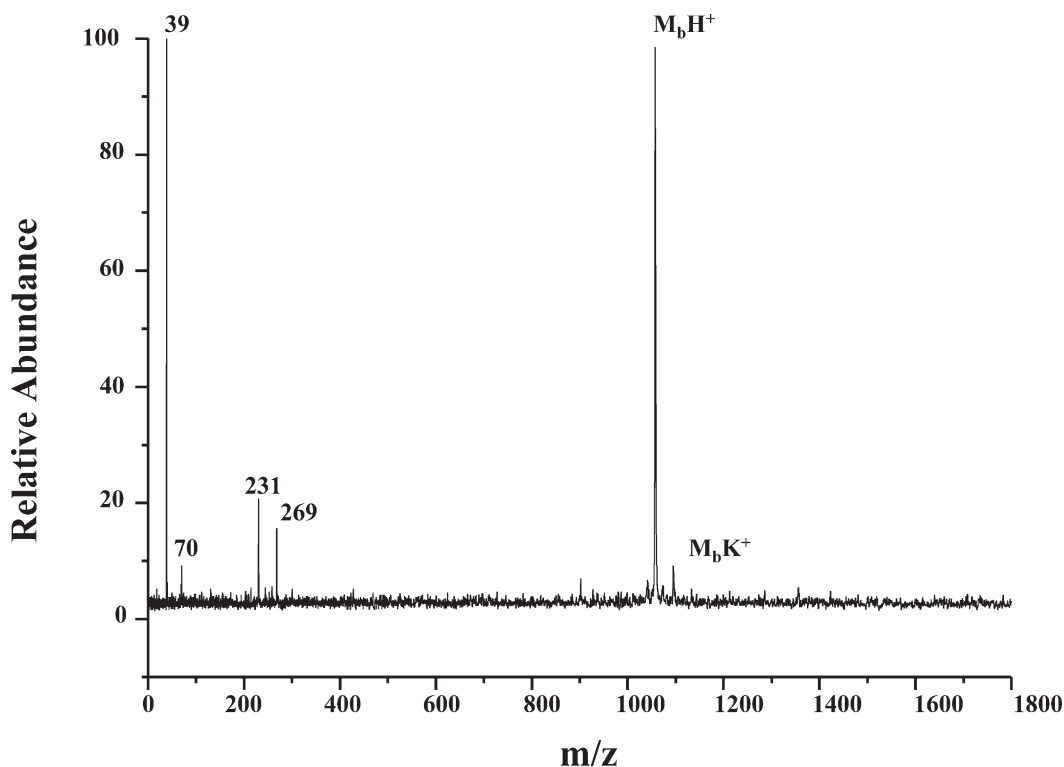


Figure 5. Titania MALDI mass spectrum of bradykinin (940 fmol).

analyte signal generally ceases growing after laser irradiation at the same spot for 10–15 shots.

Figures 6(a)–6(c) display the titania MALDI mass spectra of insulin (8.7 pmol) obtained 1, 15, and 30 days, respectively, after the titania chips were prepared. The  $M_iH^+$  ions obtained using either the 15- or 30-day-old titania chips have

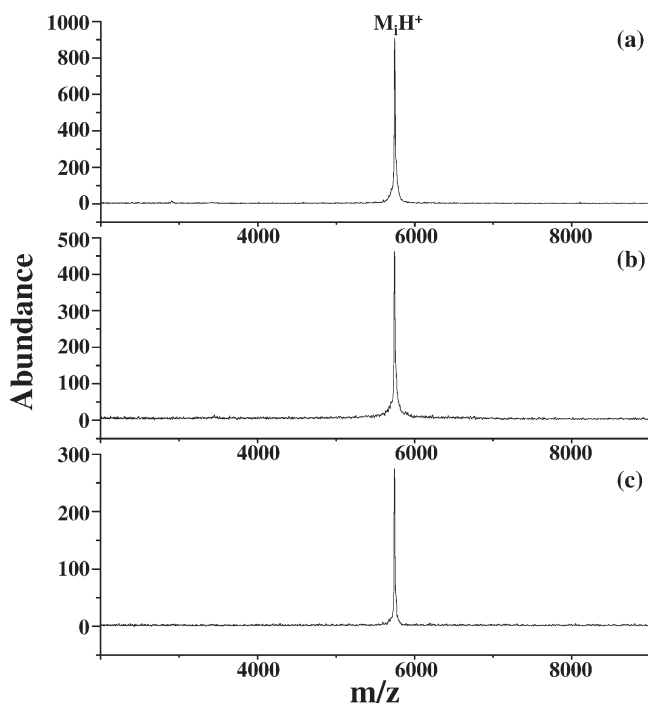
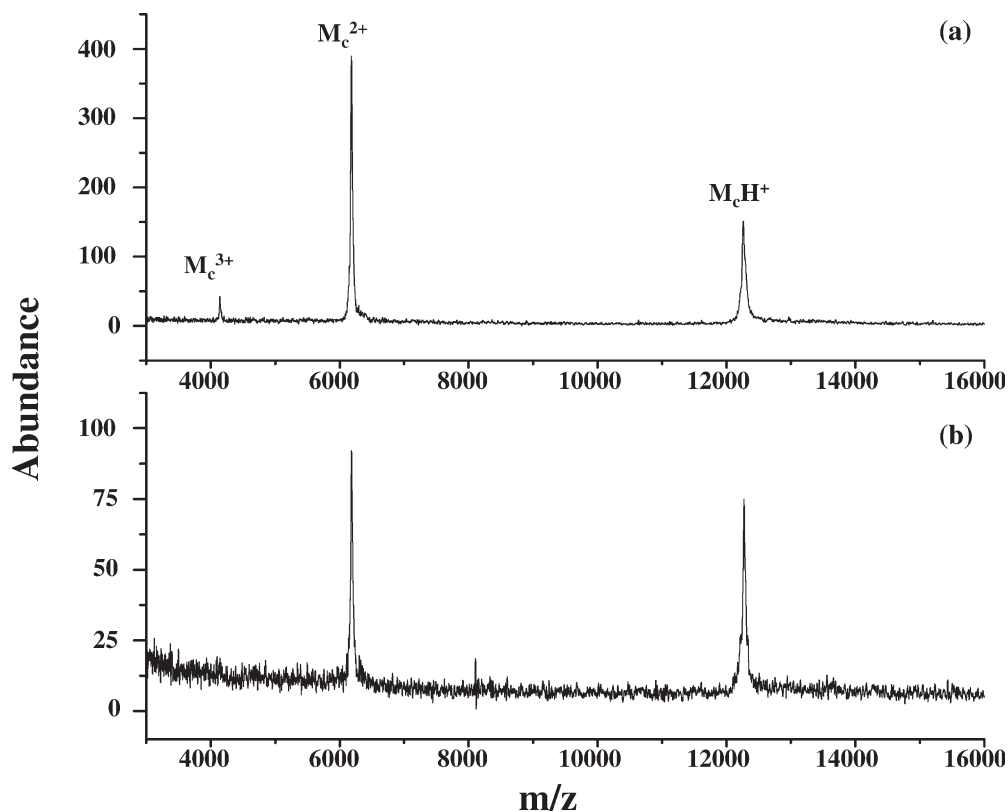


Figure 6. Titania MALDI mass spectra of insulin (8.7 pmol) obtained (a) 1 day, (b) 15 days, and (c) 30 days after the titania chips were prepared.

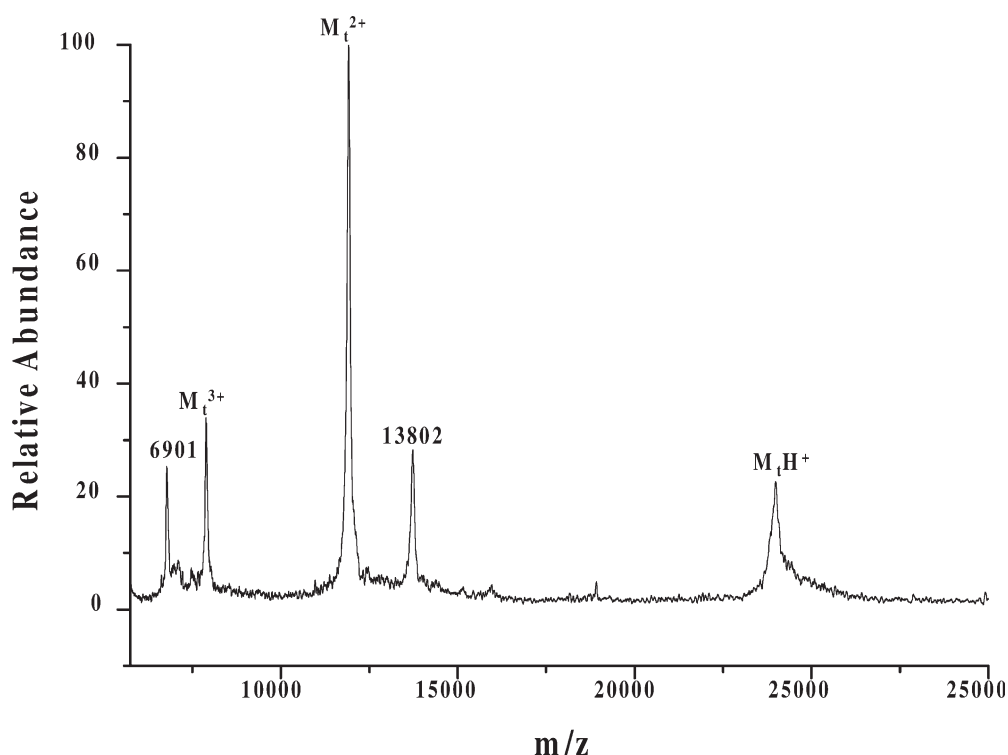
intensities similar to that obtained using the freshly prepared chip. The mass spectral quality of analyte signals for molecules of mass less than 5000 Da was unaffected by the freshness of the titania chips. However, for the analysis of larger molecules like cytochrome C, the  $MH^+$  ion of cytochrome C was appreciably less abundant when using a 30-day-old chip. This may be the result of some degree of chemical adsorption on the surface of the titania film during storage. Figure 7(a) presents the titania MALDI mass spectrum of cytochrome C (4 pmol) on a fresh titania chip, while Fig. 7(b) displays the corresponding spectrum obtained using a 30-day-old chip. Although the singly charged  $M_cH^+$  and doubly charged  $[M_c+2H]^{2+}$  ions of cytochrome C appear in both of the mass spectra in Fig. 7, the signal-to-noise (S/N) ratio in Fig. 7(b) is significantly lower than that in Fig. 7(a). Nevertheless, the analyte signal was homogeneous over the sample deposition area when MALDI analysis was performed directly on a fresh chip or on a 30-day-old chip.

Figure 8 presents the titania MALDI mass spectrum of trypsinogen (8.5 pmol), which is the largest molecule we have analyzed so far using this approach. In addition to the peak for the singly charged ion ( $M_tH^+$ ), we observe also the doubly  $[M_t+2H]^{2+}$  and triply charged  $[M_t+3H]^{3+}$  ions of trypsinogen in this mass spectrum. Trypsinogen is a proenzyme of trypsin; two other peaks observed at ca.  $m/z$  13 802 and 6901 presumably correspond to the singly charged and doubly charged ions of an autolysis product of trypsinogen.

Based on our observations thus far, multiply charged ions are observed frequently in the titania MALDI mass spectra of small proteins because of the presence of excess protons from the citrate buffer in the sample solution. However, when we used acetic acid, trifluoroacetic acid, or phosphoric acid as the proton sources, the results were much worse than those



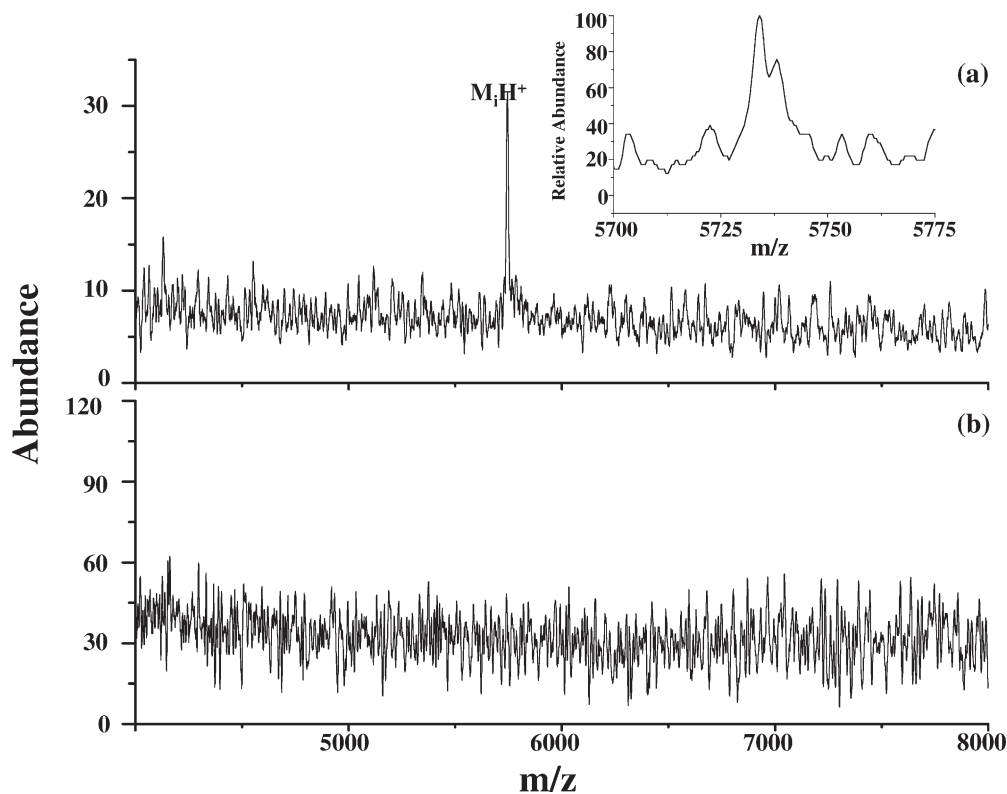
**Figure 7.** Titania MALDI mass spectra of cytochrome C (4 pmol) obtained (a) 1 day and (b) 30 days after the titania chips were prepared.



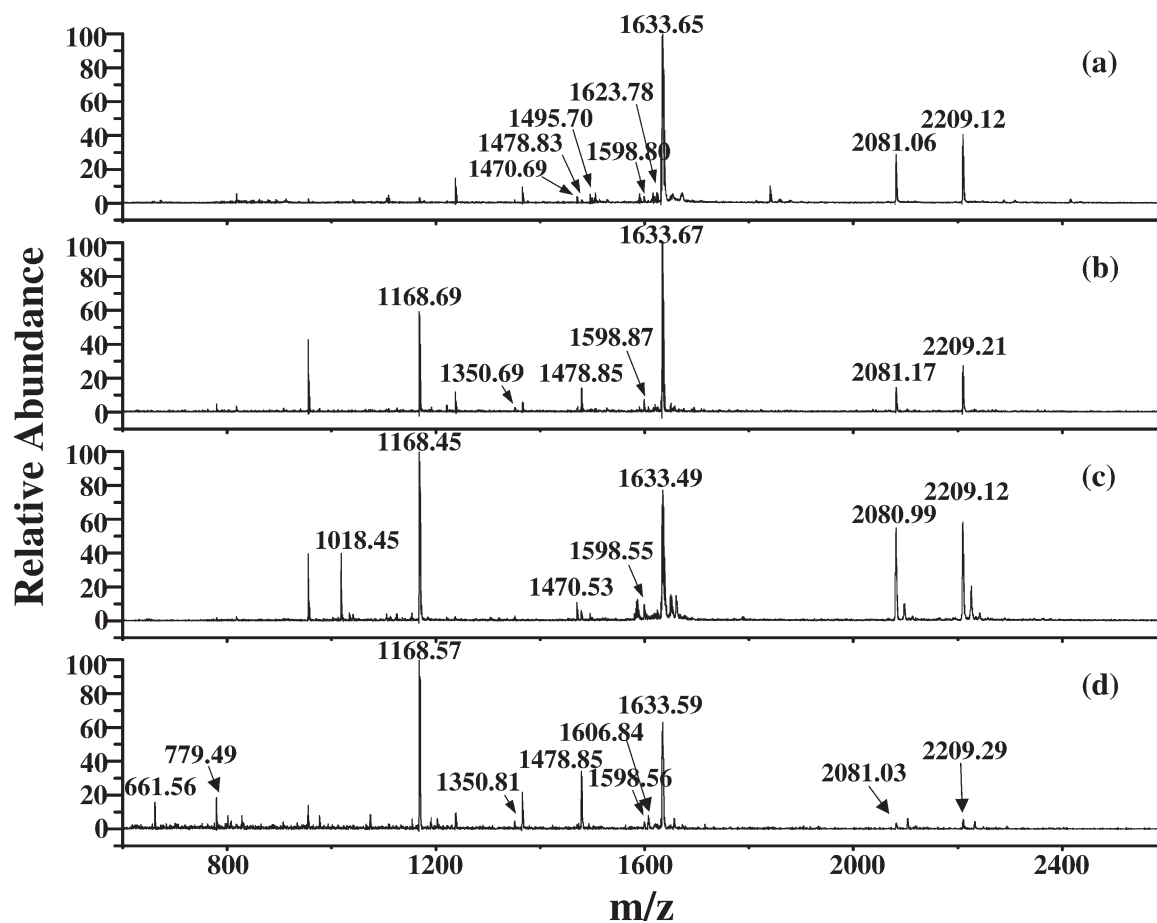
**Figure 8.** Titania MALDI mass spectrum of trypsinogen (8.5 pmol).

obtained using citric acid as the proton source. As has been reported previously,<sup>38</sup> ammonium citrate not only plays the role of a proton donor, but can also chelate alkali cations and thus prevent or greatly reduce their adduction to the analyte. We found that addition of large quantities of ammonium

citrate/citric acid to this system led to dramatic improvements in both the mass spectral quality and the detection limits. Figure 9(a) presents a spectrum, obtained using this approach, of insulin at its detection limit (1.7 fmol), while no analyte ions were observed without adding citric buffer as the



**Figure 9.** Titania MALDI mass spectrum of insulin (1.7 fmol) (a) with and (b) without adding citric buffer.



**Figure 10.** Titania MALDI mass spectra of the tryptic digest product of cytochrome C ( $10^{-5}$  M) using (a) SA (20 mg/mL), (b) CHCA (saturated), (c) 2,5-DHB (30 mg/mL), and (d) titania film as the assisting material. All the MALDI matrices were dissolved in a mixture of acetonitrile/0.1% trifluoroacetic acid/ $H_2O$  (2:1, v/v).

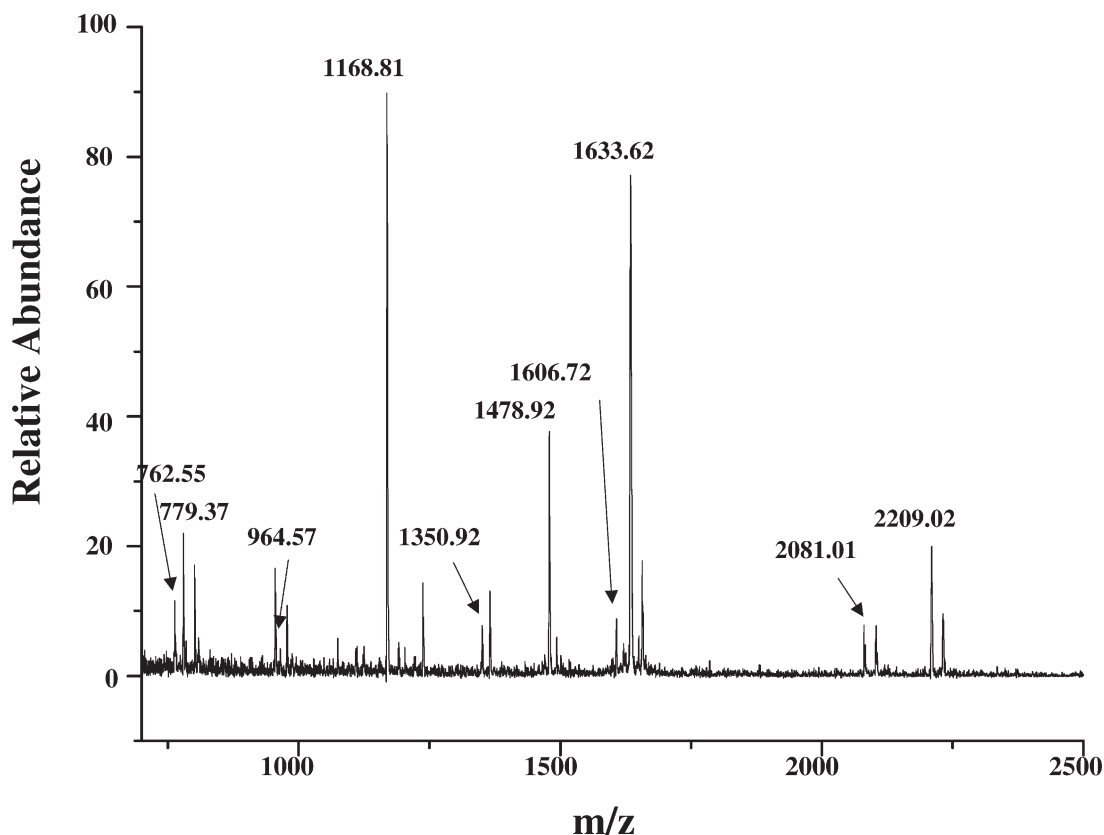
proton source (Fig. 9(b)). The inset mass spectrum shows the expanded region around the peak for protonated insulin ( $M_iH^+$ ); the mass resolution of the peak is approximately 660. The addition of a large amount of extra proton source clearly renders the MALDI-MS analysis effective in this inorganic matrix system. This result is similar to the principle that has become a commonly employed rule of thumb in conventional MALDI-MS analysis, i.e., the ratio of organic MALDI matrix to analyte must be ca. 100 to 50 000 if good-quality MALDI mass spectra are to be obtained. In this case, the inorganic titania plays the role of the energy absorber and an excess of protons is essential for obtaining good quality results.

Figures 10(a)–10(d) present the MALDI mass spectra of the tryptic digest of cytochrome C ( $10^{-5}$  M) using SA, CHCA, 2,5-DHB, and titania film as the matrices, respectively. There are more ion peaks observed in Fig. 10(d) than in Figs. 10(a), 10(b) and 10(c), suggesting that use of titania film as the assisting material in MALDI analysis involves less ion suppression effects than in conventional MALDI analysis. However, the S/N ratios in Figs. 10(a)–10(c) are appreciably better than that in Fig. 10(d). By protein database search we identified the peaks at  $m/z$  779.50, 907.71, 964.48, 1168.57, 1350.81, 1478.85, 1598.56, 1606.84, 1633.59, 2081.03, and 2209.29 in Fig. 10(d) as tryptic peptides of cytochrome C; a probability-based Mowse score of 112 based on these peaks identifies cytochrome C as the protein with good reliability.

This titania MALDI-MS approach to proteomic samples is an efficient and powerful method because only a one-step sample deposition process is required on the titania chip

before analysis. However, the present results also show that the sensitivity for the cytochrome C tryptic digest using our approach in its current state of development is about one order worse than that of conventional MALDI analysis. Nevertheless, in compensation, lower ion suppression effects and no sweet spot problems are the chief merits of this titania approach. Another potential advantage is illustrated in Fig. 11, that shows the titania MALDI mass spectrum of the very same MALDI sample as that used for Fig. 10(d), obtained when the titania chip was 1 month old; the mass spectral quality in Fig. 11 is similar to that shown in Fig. 10(d).

The results presented in this paper were obtained using aluminum sheets as the titania film support. We have found that the corresponding results obtained by using titania-modified glass chips are far worse, in terms of sensitivity and mass range, than those obtained using the aluminum-based substrates. This phenomenon may be a consequence of the better electrical conductivity of the aluminum support compared with the glass chip. Because titania is a semiconductor, however, photoelectrons may be generated during MALDI-MS analysis if the laser power used is too high. Uninterpretable signals suspected to arise as the result of photoelectrons were also observed during MALDI-MS analysis at high power when glass slides were used as the support for titania films. This is important because molecules larger than trypsinogen generally require a high laser power and, whenever these unknown signals appeared in the mass spectrum, the analyte ions were totally suppressed. The mass spectra we present here were all obtained using a laser power



**Figure 11.** Titania MALDI mass spectrum of the tryptic digest product of cytochrome C ( $10^{-5}$  M) using a 1-month-old titania film as the assisting material.



of two to three times higher than that applied in conventional MALDI-MS analysis. We did not observe the appearance of any effects attributable to results of photoelectrons at this range of laser power. However, if the laser power was adjusted to about four times higher than that used for conventional MALDI-MS analysis, signals suspected to arise due to production of photoelectrons did appear in the mass spectra.

## CONCLUSIONS

We propose a new matrix-free chip-based method for MALDI-MS analyses, using mesoporous titania films with an anatase framework as the assisting material; these films provide a low matrix background in the mass spectra and avoid the problems of 'sweet spots' during analysis. The largest molecule we have investigated successfully using this approach is trypsinogen (23 982 Da) which, to the best of our knowledge, is the largest molecule analyzed so far by MALDI-MS using chip-based inorganic films as the assisting materials. Furthermore, we have observed that a 1-month-old titania film functions exactly the same as a fresh chip does for the analysis of molecules smaller than ca. 5700 Da, so it seems that these titania films are quite stable at room temperature for at least a month. Additionally, we have demonstrated that this approach is applicable to the analysis of protein enzymatic digests. Work is in progress to further improve the method.

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