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Self-assembled-monolayer-modified silicon substrate to enhance the sensitivity of peptide detection for AP-MALDI mass spectrometry

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A self-assembled-monolayer-modified silicon substrate was successfully used to enhance the sensitivity of peptide detection for atmospheric pressure-matrix-assisted laser desorption/ionization mass spectrometry (AP-MALDI/MS). The effect of surface modification of silicon wafer samples with NH_2 and OH functional groups was investigated. In addition, solvent effects for the preparation of modified NH_2-functionalized surfaces were examined. The sensitivities for the two peptides were significantly improved, increasing between 12 and 160 times, for bradykinin and gramicidin, respectively, on an NH_2-modified silicon surface prepared in toluene, over that on a conventional gold substrate. The limits of detection (LODs) for bradykinin and gramicidin using the conventional gold substrate in AP-MALDI/MS experiments were >0.011 μM and 110 μM, respectively. Using our SAM approach, the LODs for bradykinin and gramicidin in AP-MALDI/MS can be improved to 0.93 nM and 0.33 μM, respectively. This SAM approach for AP-MALDI/MS is simple and sensitive, and can be used for high-throughput analysis. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: SAM substrate; AP-MALDI/MS; solvent effect; surface modification; self-assembled monolayer

INTRODUCTION

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) was discovered by Karas *et al.*[1–4] in the 1980s. This technique uses a laser excitation source to ionize particles in a substrate 'matrix'. These particles then transfer charge to the molecules of interest through a proton transfer event, thus creating analyte ions. Because the proton transfer process requires only a single proton for each molecule, this is a soft ionization technique and has been largely used in the field of analytical biology.

Later in 1999, Wei *et al.*[5] improved the MALDI/MS technique by introducing a nonorganic matrix method. Wei used porous silicon as the substrate matrix and 'bridge' to absorb the ultraviolet laser energy, thus eliminating the need for an organic matrix such as α-cyano-4-hydroxycinnamic acid (CHCA).[6] Because this technique requires only an inorganic porous silicon wafer substrate, the mass spectroscopy output is very clean, and contains information only from the compound of interest. Because silicon is a technologically important and widely available material, this new application has drawn much attention from many fields.

Self-assembled monolayers (SAMs) on silicon[7–9] have been studied since 1985, and are used in a great number of important applications,[10] such as biosensors, molecular electronics, lubrication layers, corrosion protection, and nanocluster organization. More recently, these films have shown promise for enhancing the signal in MALDI/MS. The use of SAM-modified gold substrates for MALDI/MS has been demonstrated by several groups. Brockman *et al.*[11] have applied hydrophobic methyl-terminated SAM on a gold probe surface, and showed that the CH_3-SAM-modified gold surface was able to bind polypeptides via a hydrophobic interaction. Furthermore, the hydrophobic properties provided salt tolerance. Fukuo *et al.*[12] studied SAM of Ru complex on Au films. Stone *et al.*[13] used fluorinated SAMs to optimize the probe surface condition for matrix-assisted laser desorption ionization-ion mobility-surface-induced dissociation (MALDI-IM-SID). Min *et al.*[14] used maleimide-SAM on a modified Au surface to selectively immobilize cysteine-containing peptides. Mu *et al.*[15] studied low-density COOH-SAM on Au to selectively assemble the charged proteins, avidin, and streptavidin. Trauger *et al.*[16] showed that perfluorophenyl silylated and amine silylated porous silicon exhibit high sensitivity on Des-Arg-bradykinin and extend desorption/ionization on silicon mass spectrometry (DIOS-MS) activities to longer times.

For atmospheric pressure MALDI/MS (AP-MALDI/MS), many samples can be loaded simultaneously under atmospheric pressure, with each sample requiring an extremely short time for analysis. Therefore, it offers significant advantages over other methods by way of convenience, and rapid

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and high-throughout analysis. However, this technique has been limited by poor sensitivity compared with conventional MALDI/MS operated in high vacuum. Thus, efforts to improve the sensitivity of AP-MALDI/MS are very important for further development of this recent technique. In this study, to the best of our knowledge, we have demonstrated for the first time the use of a SAM-modified unetched silicon wafer for AP-MALDI/MS to improve the sensitivity for two peptide samples. The effects of surface modification with NH_2 and OH groups were investigated. In addition, solvent effects for the preparation of the NH_2-modified silicon samples were examined using bradykinin (1–8) and gramicidin as model compounds for this novel approach.

EXPERIMENTAL

Materials

Bradykinin fragment (1–8) (molecular weight 904 g/mol), gramicidin D (molecular weight 1882 g/mol), CHCA, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. Gramicidin has the amino acid sequence HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Xxx[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-$NHCH_2CH_2OH$, where Xxx[11] is Trp in gramicidin A, Phe in gramicidin B, and Tyr in gramicidin C. The commercial gramicidin D contains a mixture of 80, 5, and 15 parts of the components A, B, and C, respectively. Toluene, acetone, and (3-aminopropyl) triethoxysilane (APTES) were purchased from Mallinckrodt (Phillipsburg, NJ, USA). Methanol was purchased from Echo Chemical Co. Ltd. Water used for sample preparation was purified using a Milli-Q reagent water treatment system (Millipore, Milford, MA, USA).

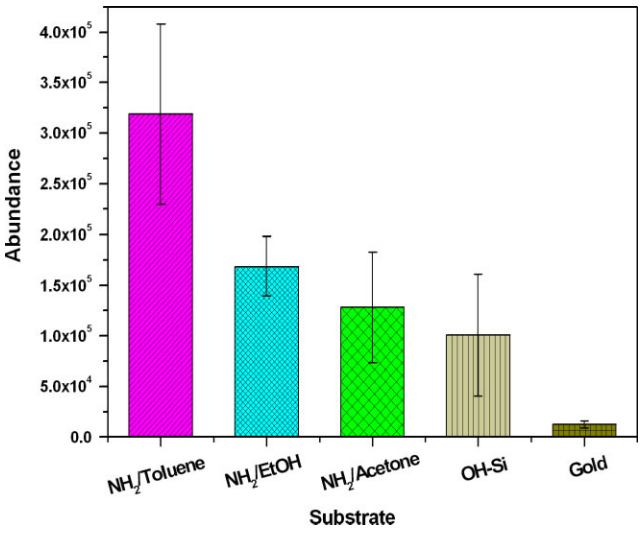

Figure 1. Bradykinin (1–8) fragment abundance from MALDI mass spectra for several NH_2-terminated SAM modified Si(100) substrates, an OH-rich Si(100) substrate, and a standard gold substrate. A total volume of 2 µl of 11 nM bradykinin solution were deposited on each substrate for MALDI analysis. The NH_2/Toluene combination provided the best and most repeatable signal and was used exclusively for all subsequent experiments.

Sample preparation

Matrix solution was prepared using CHCA, which was dissolved in a 1% solution of TFA in methanol/water (2 : 1, v/v). Stock standard solutions of bradykinin fragment (1–8) and gramicidin were prepared in methanol and stored in a refrigerator at 4 °C.

Instrumentation

AP-MALDI/MS

All AP-MALDI/MS experiments were conducted using a Finnigan LCQ ion trap mass spectrometer (Thermoquest Inc., San Jose, CA, USA) equipped with a commercial AP-MALDI source (Mass Tech., Columbia, MD, USA). A nitrogen laser with a UV wavelength of 337 nm was used for all experiments. The laser power was attenuated to 60% at 10 Hz repetitions. CHCA was used as the MALDI matrix. Mass spectra were obtained in the positive ion mode. All mass spectra were generated using 3-min laser shots. The ion injection time was 1070 ms; capillary temperature 250 °C; capillary voltage 40 V; and tube lens offset voltage 70 V. The target plate bias was 1.8 kV. Standard gold and SAM-modified silicon substrates were attached to the target plate with double-sided carbon tape (Ted Pella, Inc.). Two microliters of the matrix (CHCA) solution was added by means of the 'dried droplet' method. Samples were air-dried for 5 min at room temperature and then loaded into the AP-MALDI/MS for analysis.

FTIR

Fourier transform infrared spectroscopy (FTIR) data for the NH_2-modified silicon substrates was acquired using a Spectrum 100 FTIR Spectrometer (PerkinElmer) equipped

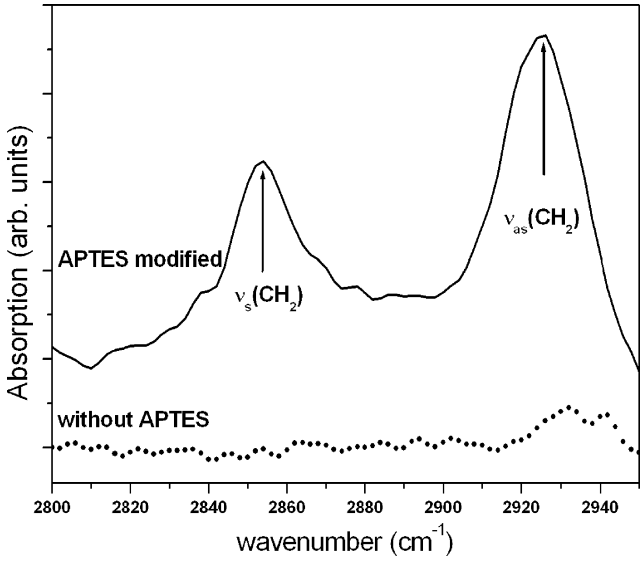

Figure 2. FTIR spectra for the NH_2-terminated SAM-modified Si(100) substrate and a clean silicon sample. The peaks correspond to the CH_2 symmetric and CH_2 asymmetric stretch positions reported in the literature.[17] The control sample was also treated with toluene to rule out contamination as the source of the CH_2 peaks. Thus, the presence of CH_2 stretching peaks confirms the growth of the APTES SAM on the Si surface.

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with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. Transmitted signal was used to detect molecular-level signal on the silicon surface.[17] The incident light was oriented along the surface normal, and spectra were collected over 1000 scans at a resolution of 8 cm^{-1}. A background spectrum from a freshly cleaned silicon substrate was collected before each measurement.

AFM

Topographic images of the samples were obtained using an atomic force microscope (AFM) (Asylum Research MFP-3D, Santa Barbara, CA, USA) operating in intermittent contact mode under ambient conditions. A silicon cantilever (Olympus, AC240) with a nominal spring constant of 1 N/m was used for all images with a scan rate of 1.0 Hz and image pixel density of 512 × 512.

Preparation of SAM-modified silicon substrate

Self-assembled monolayers were prepared on Si(100) substrates using a solution mixture of APTES, and anhydrous ethanol, toluene, or acetone. The Si(100) wafers were first cleaned using a micro surfactant followed by an ethanol rinse, and then dried under a stream of dry nitrogen gas. These wafers were then treated using a plasma cleaner (Harrick Scientific Products, Inc.) for 2 min to increase the OH concentration at the surface. Several methods for preparing NH_2-modified Si(100) substrates have been reported in the literature,[18–20] each with its own solvent mixture. We have used three of these to prepare samples in this study in order to identify the optimal preparation method for use in the MALDI/MS technique. In the first method, a plasma-cleaned, OH-rich silicon substrate was immersed in a solution of APTES/anhydrous ethanol (v/v = 1:20) for 12 h[18] and then removed and dried under a stream of dry nitrogen gas. For the second method, the OH-rich Si(100) substrate was immersed in a solution of 5.0×10^{-3} M APTES in a solvent mixture of acetone and ultra pure water (v/v = 5:1) for 12 h[20] and then removed and dried under a stream of dry nitrogen gas. For the third method, the OH-rich Si(100) substrate was immersed in a solution of 1% APTES in toluene for 12 h.[19] Following this, the sample was removed from the solution and baked in an oven at 120 °C for 5 min.

RESULTS AND DISCUSSION

Effect of surface modification with NH_2 and OH, and solvent effects

In order to investigate the solvent effect, (APTES) SAM-modified Si(100) samples were prepared using toluene, acetone, and ethanol prior to the addition of the bradykinin (1–8) or the gramicidin samples. The AP-MALDI results from these substrates were then compared with the results for samples prepared using the same concentrations of analyte on conventional gold substrate samples. In addition, the effect of the chemically functionalized surface, both NH_2-and OH-modified silicon wafer samples, were examined.

Figure 1 shows the AP-MALDI/MS ion abundance results for bradykinin (1–8) fragment deposited on NH_2-terminated SAM modified Si(100) substrates prepared from three different solvents, an OH-rich Si(100) substrate, and a standard gold substrate. A total volume of 2 µl of 0.011 µM bradykinin solution was deposited on each substrate for AP-MALDI analysis. Six samples of each substrate were analyzed and averaged. The AP-MALDI/MS ion abundance results for NH_2/toluene, NH_2/ethanol, NH_2/acetone, OH-Si, and gold, were 319 094 ± 89 013, 168 834 ± 29 313, 128 122 ± 54 789, 101 080 ± 60 238 and 12 687 ± 3502, respectively. Most notably, all the samples prepared using silicon showed higher ion abundance than the standard gold substrate. Of the silicon samples, the one prepared using an APTES/toluene solvent mixture gave the highest signal in the AP-MALDI/MS data, and was used exclusively for all subsequent experiments. The OH-Si substrate exhibited much lower abundance than that of any of the NH_2-SAM-modified silicon samples. This indicates that the SAM layer plays an important role in enhancing ion abundances for bradykinin (1–8).

The NH_2-modified silicon samples, prepared using the APTES/toluene mixture, were further characterized using FTIR spectroscopy in order to confirm the presence of an NH_2-terminated SAM monolayer. The specific method we used involved mounting the Silicon sample such that the incident IR beam was along the surface normal and the transmitted light (through the silicon sample) was

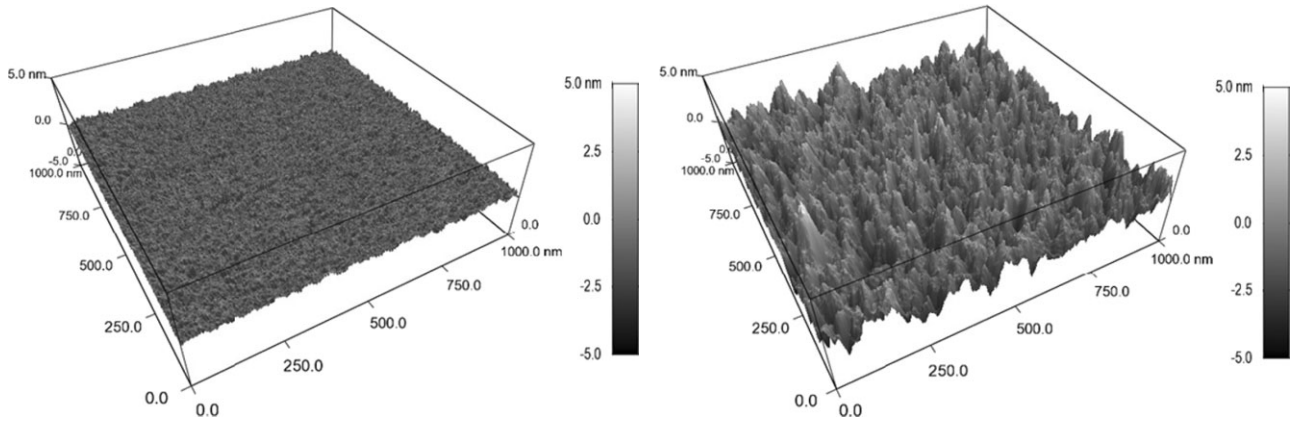

Figure 3. AFM topographical image (a) of an OH-modified silicon surface. The root mean square (RMS) roughness for the entire imaged area was 0.19 nm. AFM topographical image (b) of NH_2-modified silicon surface. The RMS roughness for the entire imaged area was 1.05 nm. Both images correspond to 1 µm × 1 µm.

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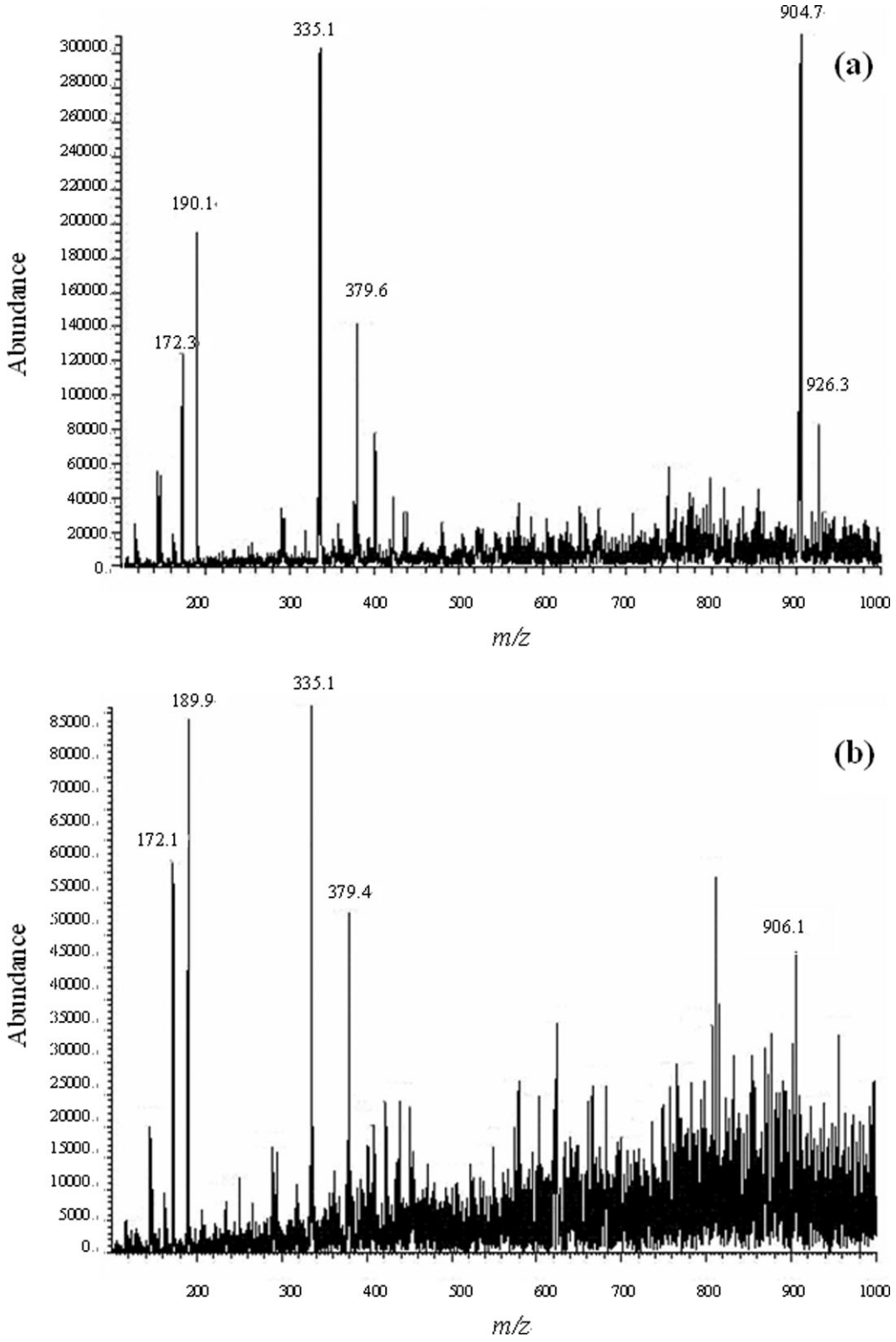

Figure 4. (a) Plot of MALDI mass spectra abundance *versus m/z* for a bradykinin solution concentration of 11 nM on the NH_2-terminated SAM Si(100) substrate. Figure (b) shows the corresponding MALDI spectrum for an 11 nM (the detection limit) bradykinin sample on a gold standard substrate.

monitored using an MCT detector. This method is a useful tool for characterizing SAM film growth on silicon substrates.

The portion of the spectrum corresponding to the CH_2 symmetric and asymmetric stretching modes ($\nu_{as}(CH_2)$ and $\nu_s(CH_2)$) is shown in Fig. 2. The dashed line represents the FTIR signal from a blank Si(100) sample that was immersed in pure toluene without APTES. This data is included to rule out contamination as the source of the CH_2 stretching signal in the APTES sample FTIR result. The CH_2 peaks were used

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rather than the NH_2 peak to confirm the SAM film growth because of the small signal of NH_2 at ~3200–3500 cm^{-1} (only one moiety per APTES molecule) and its overlap with the H_2O peak, which is present in the background. The positions of the two CH_2 peaks in the spectrum at 2854 and 2927 cm^{-1}, corresponding to the $\nu_s(CH_2)$ and $\nu_{as}(CH_2)$ stretch respectively, are in agreement with literature values[21,22] and confirm the presence of APTES on the silicon surface.

Another important characteristic of the sample substrates is the surface roughness, which may influence the binding affinity of analytes (bradykinin (1–8) or gramicidin) to the SAM surfaces. We have used AFM to characterize the surface roughness of an OH-rich Si(100) and an NH_2-terminated SAM on Si(100) surface. The resulting topographical images are shown in Fig. 3. In order to compare the relative surface features between these samples, we have displayed the images (1 μm × 1 μm) in a 3D projected view format using the same 10 nm Z scale. Qualitatively, the OH-modified silicon surface appears to be smoother than the NH_2-modified Si(100) surface. The root mean square (RMS) roughness for the two substrates, OH and NH_2 was 0.19 and 1.05 nm, respectively. Although we cannot rule it out, the increased roughness of the NH_2-SAM substrate does not account for the degree of signal enhancement observed by us. Okuno *et al.*[23] showed that roughness at the submicron scale, not the nanometer scale, was the key factor in improving the ionization efficiency for hydrophilic polymers on scratched silicon and stainless steel samples using matrix-free MALDI. Thus, we believe that chemical differences in the films rather than the small physical differences (roughness) result in the enhanced AP-MALDI/MS signal for bradykinin.

Figure 4(a) shows the AP-MALDI mass spectrum for a 0.011 μM concentration of bradykinin (1–8) on an NH_2-SAM-modified silicon wafer formed using toluene as the solvent. A total volume of 2 μl of bradykinin (1–8) solution was deposited on an area of the sample, roughly 5 mm × 5 mm, corresponding to an adsorbed surface concentration of 4.2×10^{-4} bradykinin (1–8) molecules/nm^2 (or 420 molecules/μm^2). The protonated bradykinin (1–8) molecule shown at m/z 904.7 ($[M + H]^+$) in Fig. 4(a) is present at an abundance of ~300 000. Figure 4(b) shows the AP-MALDI mass spectrum for a bradykinin (1–8) sample on a conventional gold substrate using the same concentration and preparation procedure as for the modified Si substrate. The protonated bradykinin (1–8) molecule at m/z 904.7 ($[M + H]^+$) was not detected and only the background noise (CHCA matrix ions) was observed in the spectra. This is because the limits of detection (LOD) of bradykinin (1–8) on a conventional Au substrate is higher than 0.011 μM.

Determination of sensitivity for NH_2-modified silicon surface prepared in toluene for bradykinin (1–8)

A significant signal enhancement for bradykinin (1–8) was observed in AP-MALDI/MS when it was deposited on NH_2-modified silicon surfaces compared to deposition on a conventional gold substrate. In order to determine the LOD for this approach, we selected the APTES/toluene mixture to prepare a set of NH_2-SAM-modified silicon substrates as it exhibited the highest sensitivity in our solvent effects results (Fig. 1). A series of samples with different concentrations of bradykinin (1–8) were prepared and their AP-MALDI mass spectra were collected. These results are shown in Fig. 5.

As shown in Fig. 4(a), the detection limit of bradykinin on a conventional gold substrate is >0.011 μM. For the same bradykinin concentration on an NH_2-modified Si substrate, we observed a signal of ~2.8×10^5 which corresponds to an enhancement of ~20× over the standard gold substrate. Further measurements were made (Fig. 5) by diluting the bradykinin (1–8) solutions in order to determine the detection limit for bradykinin on the NH_2-modified Si substrate. Six samples at each concentration were analyzed and the results averaged. The AP-MALDI/MS ion abundance results for concentrations of 0.93, 1.4, 2.8, 5.5, and 11 nM, were 29 052 ± 14 820, 44 622 ± 14 801, 60 072 ± 13 240, 203 445 ± 103 873 and 319 094 ± 89 013, respectively. At a concentration of 0.93 nM of bradykinin solution, one can see in the figure that the signal is at the detection limit. This amounts to a lowering of the detection limit of bradykinin by nearly 12 times using our NH_2-SAM-modified silicon samples prepared in toluene over that on conventional Au substrates.

Determination of sensitivity for NH_2-modified silicon surface prepared in toluene for the hydrophobic peptide gramicidin D

A second peptide, gramicidin D, was used to further test the NH_2-SAM-modified silicon samples prepared in toluene to determine if the enhanced AP-MALDI/MS signal could be observed for a hydrophobic peptide, since it is extremely difficult to detect hydrophobic peptides using conventional gold substrates in AP-MALDI/MS. Gramicidin is a linear polypeptide antibiotic, containing 15 amino acids (D and L forms) with an ethanolamine residue at the carboxyl end and a formyl group at the amino end. All the side chains on gramicidin are relatively hydrophobic.[24–27]

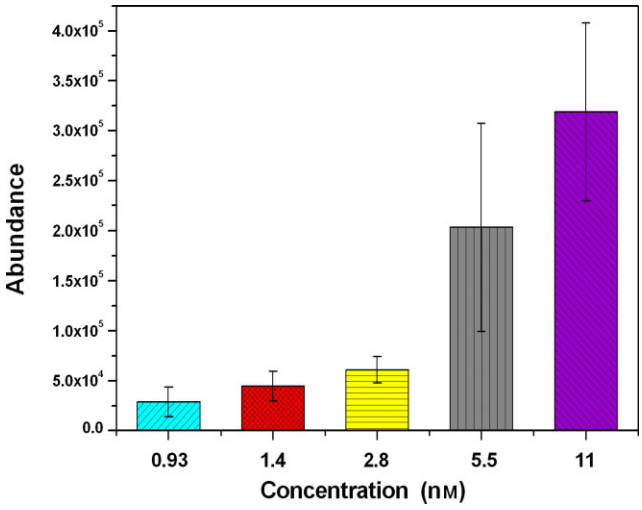

Figure 5. MALDI abundance *versus* bradykinin concentration on the NH_2-terminated SAM prepared using the APTES/toluene mixture on Si.

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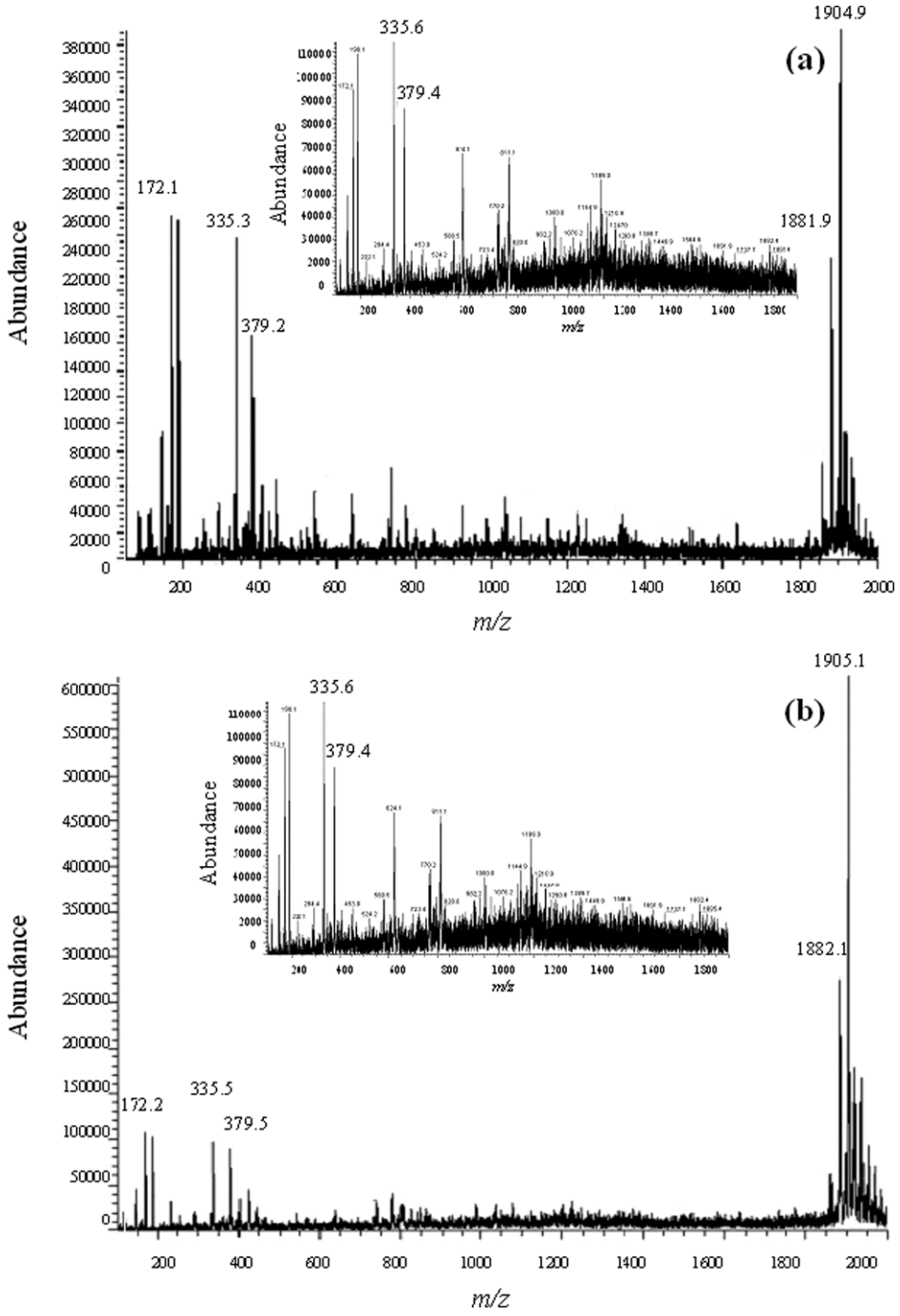

Figure 6. MALDI mass spectra of gramicidin deposited on NH_2-terminated SAM-modified Si(100) substrates: (a) 13 μM, (b) 2.7 μM, (c) 1.3 μM, (d) 0.33 μM. The 0.33 μM concentration represents a 160× reduction in the detection limit on this substrate compared to the standard gold substrate (54 μM) shown in the inserts.

Gramicidin was deposited on NH_2-SAM-modified Si(100) substrates prepared in toluene and AP-MALDI mass spectra were subsequently acquired. These results were also compared with those obtained using conventional gold substrates at the same gramicidin concentration. Figure 6(a–d) shows AP-MALDI mass spectra collected for four different gramicidin concentrations. The concentrations in Fig. 6(a–d) were 13, 2.7, 1.3, and 0.33 μM with corresponding ion abundance signals of 255 100, 284 638, 345 232 and 119 721, respectively. Since the LOD for gramicidin was 110 μM using a conventional gold substrate, all the insert figures in Fig 6(a–d) show those spectra obtained from conventional gold substrates using the same concentration of gramicidin. Only the matrix ions of CHCA can be seen in these data. Here, even though gramicidin D was used, its primary component (80% gramicidin A) was the dominant form detected by us and was observed as protonated ion and sodium ion adducts. The product ions shown at m/z 1882 and 1905 are identified as $[Val\text{-}GrA + H]^+$, $[Val\text{-}GrA + Na]^+$, respectively.

The ion abundance from AP-MALDI/MS *versus* gramicidin concentration is plotted in Fig. 7. Six samples at

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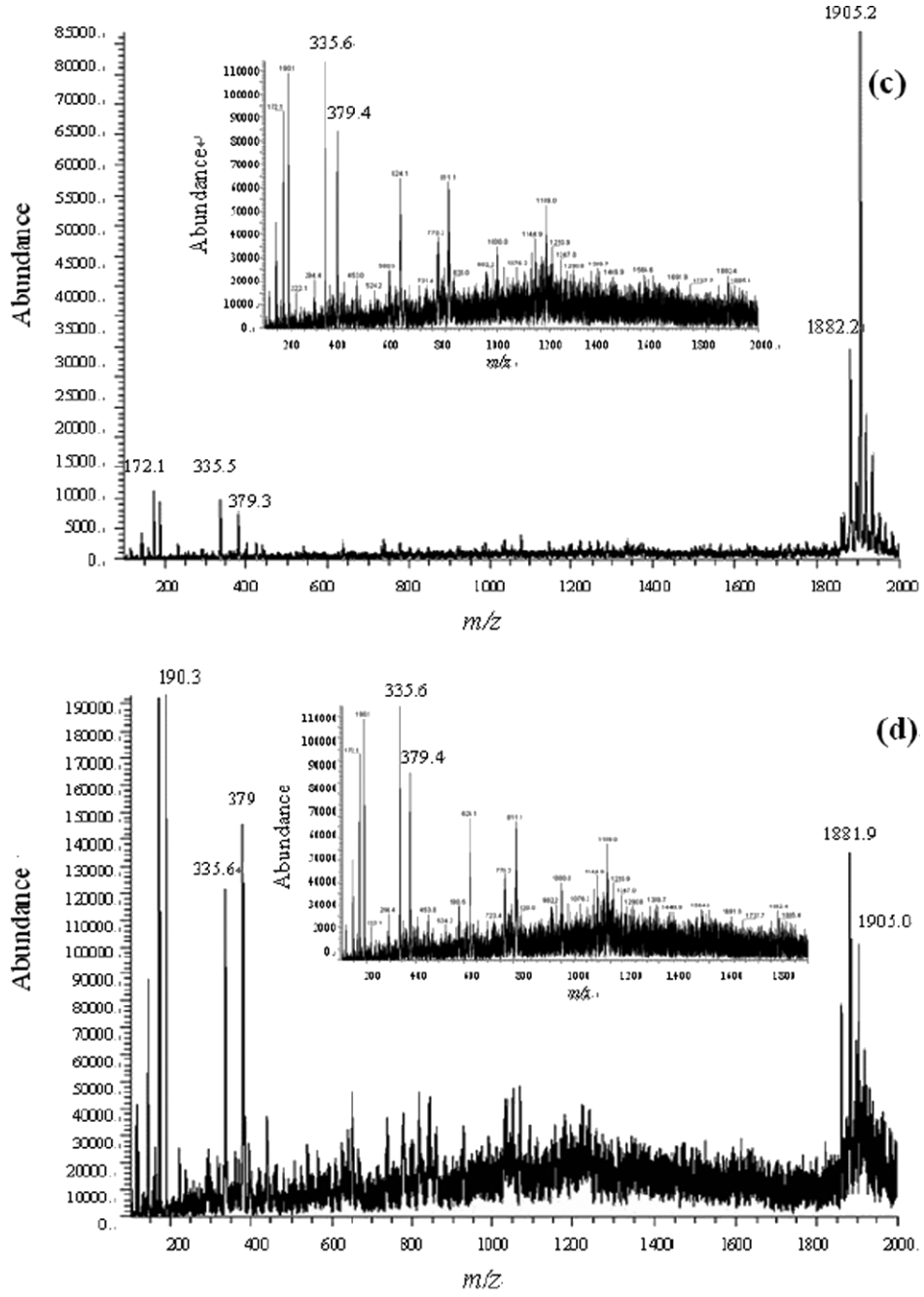

Figure 6. (*Continued*).

each concentration were analyzed and averaged. The AP-MALDI/MS ion abundance results for concentrations of 0.33, 0.67, 1.3, 2.7, 13, and 27 μM were 119 721 ± 31 267, 75 557 ± 34 885, 345 232 ± 80 754, 284 638 ± 67 271, 255 100 ± 81 453, and 170 010 ± 66 879, respectively.

As shown in Fig. 7, there is a maximum in the AP-MALDI abundance for a 1.3 μM concentration of gramicidin. The abundance decreases for both higher and lower gramicidin concentrations. A possible explanation for this is when concentrations are higher than 1.3 μM, gramicidin that agglomeration leads to stronger gramicidin–gramicidin interactions which reduce the overall AP-MALDI/MS signal. For concentrations lower than 1.3 μM, there is simply less gramicidin on the surface resulting in a lower signal. The lowest gramicidin concentration used in this study was 0.33 μM, corresponding to a surface density of 1.27×10^{-2} molecules/nm^2 for gramicidin. This concentration represents the detection limit for gramicidin on the NH_2-SAM-modified Si(100) substrates used in this study. Furthermore, this shows a reduction in detection limit of 160 fold over that for gramicidin deposited on a conventional gold substrate (54 μM). When compared with the bradykinin results, the dramatic enhancement in sensitivity of AP-MALDI/MS observed for gramicidin deposited on NH_2-terminated SAM-modified Si(100) substrates prepared in toluene has demonstrated that this approach is useful, especially for the detection of hydrophobic proteins.

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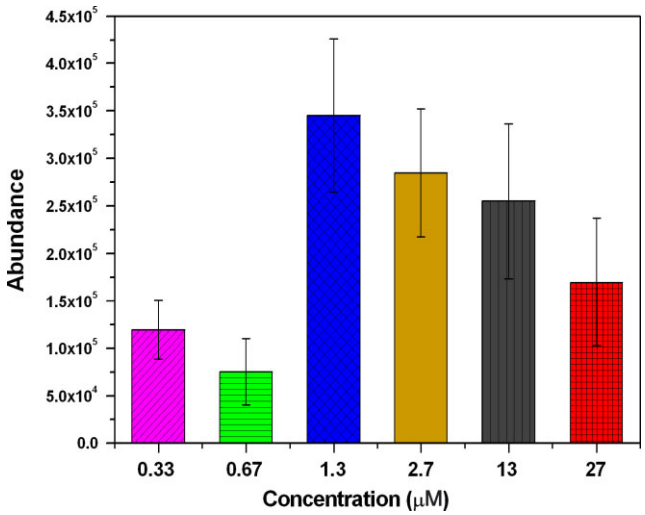

Figure 7. MALDI abundance *versus* gramicidin concentration on the NH_2-terminated SAM prepared using the APTES/toluene mixture on Si. The signal increases as the concentration decreases from 27 to 1.3 μM and then decreases as the concentration is further reduced to 0.33 μM. This behavior is discussed in the text.

CONCLUSIONS

We have demonstrated that the sensitivity of AP-MALDI/MS can be significantly enhanced, and detection limits can be greatly reduced by using an NH_2-terminated SAM-modified Si(100) substrate prepared in toluene. For bradykinin, the detection limit was lowered by a factor of 12, and for gramicidin, a 160-fold reduction was observed. The reason for this improvement is attributed to the SAM layer, which provides a more efficient pathway for energy transfer to the peptide molecules resulting in higher sensitivity in AP-MALDI/MS. This simple SAM approach has the potential to make a great impact on the development of AP-MALDI/MS, making it a more rapid, sensitive, and higher-throughput analysis tool.

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