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Microscale sample deposition onto hydrophobic target plates for trace level detection of neuropeptides in brain tissue by MALDI-MS

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A sample preparation method that combines a modified target plate with a nanoscale reversed-phase column (nanocolumn) was developed for detection of neuropeptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). A gold-coated MALDI plate was modified with an octadecanethiol (ODT) self-assembled monolayer to create a hydrophobic surface that could concentrate peptide samples into a $\sim$200–500-μm diameter spot. The spot sizes generated were comparable to those obtained for a substrate patterned with 200-μm hydrophilic spots on a hydrophobic substrate. The sample spots on the ODT-coated plate were 100-fold smaller than those formed on an unmodified gold plate with a 1-μl sample and generated 10 to 50 times higher mass sensitivity for peptide standards by MALDI-TOF MS. When the sample was deposited on an ODT-modified plate from a nanocolumn, the detection limit for peptides was as low as 20 pM for 5-μl samples corresponding to 80 amol deposited. This technique was used to analyze extracts of microwave-fixed tissue from rat brain striatum. Ninety-eight putative peptides were detected including several that had masses matching neuropeptides expected in this brain region such as substance P, rimorphin, and neurotensin. Twenty-three peptides had masses that matched peaks detected by capillary liquid chromatography with electrospray ionization MS.[1,2] Copyright © 2005 John Wiley & Sons, Ltd.

INTRODUCTION

Neuropeptides constitute an important family of bioactive compounds that are involved in many physiological functions including learning, metabolism, appetite regulation, mood, and sensory perception.[3,4] Expression and processing of neuropeptides may change with physiological condition; therefore, detection of such compounds in tissue samples is of interest for neuroscience applications. Neuropeptides tend to be present at low concentrations, creating a significant analytical challenge for detection and identification. The difficulty in detecting small amounts of peptide is aggravated by *in vivo* processing of peptides, which results in many related peptides being colocalized in tissues.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become an important tool for the analysis of neuropeptides because of its high sensitivity and mass accuracy.[5–9] The quality of MALDI-MS data, including sensitivity and reproducibility, is critically dependent upon

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sample preparation and deposition. The most commonly used sample preparation method, the dried-droplet,[10] can usually only achieve detection limits in the low femtomole range for peptides. The relatively poor sensitivity is partially due to the large mismatch between sample spot size (1–4 mm) and laser irradiation spot size (50–200 μm), resulting in only a small fraction of the sample being exposed to the laser for ion generation. This method also suffers from poor shot-to-shot and sample-to-sample reproducibility due to the heterogeneity of sample crystallization.

Recent work has demonstrated several methods for improving mass sensitivity for MALDI-MS by miniaturizing the sample spot size.[11–17] One approach has been the confinement of samples to small areas using modified target plates such as picoliter silicon vials,[11] hydrophilic sample wells in a hydrophobic coating,[12] patterned monolayer/polymer films,[13] and hydrophobic surfaces.[14] In these methods, samples ranging from 250 pl to 1.5 μl are deposited on the target. As solvent evaporates, the sample is confined to a small surface, typically 100–400 μm in diameter. Such confinement has led to a limit of detection (LOD) of 2.5 to 100 amol and concentration LOD range from sub-nanomolars to 10 nM. Samples

may also be confined by using specialized sample deposition methods such as a piezoelectric flow-through microdispenser that deposits 60-pl spots,[15] electrospray sample deposition,[16] and a three-layer sample preparation protocol in combination with microspot sample deposition.[17] These methods can achieve attomole or better LODs; however, they are of limited utility for practical analysis of complex tissue samples if the sample is not concentrated and desalted prior to deposition.

An approach to microscale sample deposition that incorporates sample purification has been to utilize a 30–50-nl bed of reversed-phase chromatography particles packed into a pipette tip as a solid-phase extraction cartridge.[5,18–20] This so-called 'nanocolumn' method allows samples to be preconcentrated, desalted, and eluted in low volumes (50–100 nl) as small spots. Using solutions with a few hundred picomolar concentration of peptide, this method has been reported to yield mass LODs of low attomole for peptide standards, which is a considerable improvement over more commonly used solid-phase extraction methods.[21] The nanocolumn has been used to analyze tryptic digests of protein and immunoprecipitation-purified neuropeptides with LODs in the low femtomole range.[5,20]

In this work, we test the hypothesis that combining the nanocolumn technique with modified surfaces that confine samples to small spots would improve the detection limit over either approach alone. The sample confinement method used is to coat the MALDI target plate with a hydrophobic monolayer of 1-octadecanethiol (1-ODT). The results demonstrate significant improvement in sensitivity with a 20 pM concentration LOD for 5-μl samples. As a result of the improved LODs, we were able to successfully utilize the method to detect neuropeptides extracted from rat brain tissue, resulting in a simple method for analyzing these complex samples.

EXPERIMENTAL

Materials

All solvents and chemicals used were of analytical grade. All organic solvents and water were from Burdick & Jackson (Muskegon, MI, USA). α-cyano-4-hydroxycinnamic acid (CHCA), 5-dihydrobenzoic acid (DHB), peptide standards, ODT, trifluroacetic acid (TFA), and sodium dodecylsulfate (SDS) were purchased from Sigma (St Louis, MO, USA). Potassium chloride was obtained from ICN Biomedicals Inc (Irvine, CA, USA). Photoresist (AZ 9260) and developer (AZ 400) were from Clariant Corp. (Summerville, NJ, USA). Poly(dimethylsiloxane) (PDMS) (RTV 615A and RTV 615B) was obtained from GE silicones (Waterford, NY, USA). GELoader tips were purchased from Brinkmann Instruments (Westbury, NY, USA). POROS 20 R2 was purchased from Applied Biosystems (Foster City, CA, USA). The stainless steel MALDI target plate was from Micromass (Milford, MA, USA).

Preparation of reversed-phase nanocolumns

Reversed-phase nanocolumns were prepared as previously described.[20] Briefly, Porous R2 Resin (∼50 mg) was added to 1-ml isopropyl alcohol to form a slurry. Approximately 2 μl of the slurry was aspirated into a GELoader tip. The end of the tip was gently flattened, but not closed, using fine tweezers that had been preheated over a flame. By pushing liquid through the flattened tip with a 3-ml disposable syringe, a packed bed ∼1.5 mm in length was formed near the outlet of the GELoader tip.

Fabrication of patterned/nonpatterned ODT-coated plates

MALDI target plates were coated with ODT in complete coverage or in a pattern. For both procedures, stainless steel MALDI target plates were first sputter-coated with a 200-μm-thick gold layer using a Denton Desk II sputter coater (Denton Vacuum, NJ, USA).

For preparation of a nonpatterned plate, the gold-coated plate was dipped into 50 mM ODT in ethanol solution for 24 h, then sequentially rinsed with methanol and water, and air-dried.

Patterned plates consisting of 200-μm gold spots separated by 4.5 mm on a background of ODT coating were formed by microcontact printing as outlined in Fig. 1.[22] The desired pattern was printed on a negative transparency film
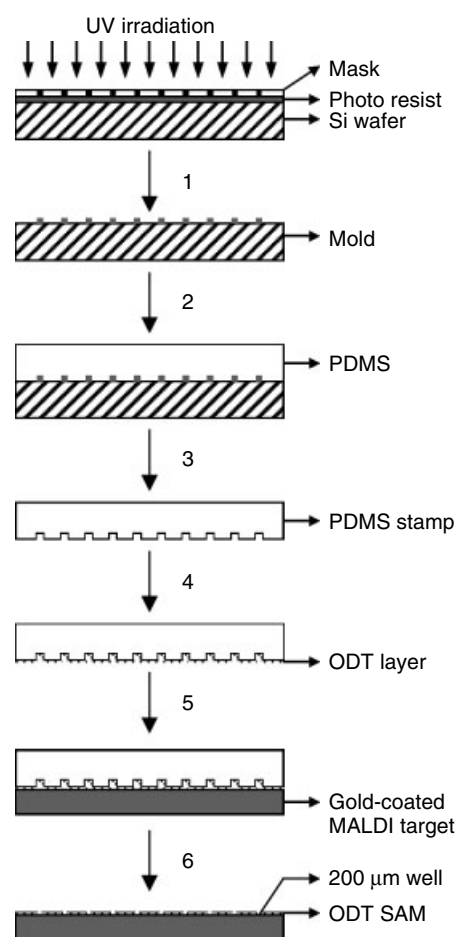

Figure 1. Outline of procedure for preparation of a patterned ODT plate. Desired pattern is obtained by UV irradiation of photoresist on Si through a mask (1); PDMS is cast over the pattern (2) to give a stamp with complementary pattern of relief (3); the stamp is moistened with ODT (4) and pressed against a gold plate (5) to form a SAM of ODT on the plate with patterned 200-μm bare gold surfaces (6). Details are given in the text.

by a high-resolution printer to create a mask. A photoresist-coated Si wafer was selectively exposed to UV light through the mask and then immersed in developer to remove the exposed photoresist. This procedure yielded a mold consisting of the Si wafer with 200-μm-diameter photoresist protrusions in the desired pattern. A PDMS solution (RTV615A : RTV615B, 5 : 1, v/v) was poured onto the photoresist template and baked for 1–2 h at 80 °C until the PDMS was cured. The PDMS slice bearing 200-μm-diameter indentations was peeled off the template and used as a microcontact-printing stamp. The stamp was immersed into 80 mM ODT ethanol solution for 5 s and then dried with a stream of N_2 gas until only slightly moist. The stamp was placed onto the gold substrate and pressed gently to remove air bubbles. After 20 s, the stamp was carefully removed, leaving an ODT self-assembled monolayer (SAM) with 200-μm-diameter bare gold spots. Pattern formation was confirmed by rinsing the plate with water and observing that water droplets adhered to the plate in the defined pattern.

Solutions for sample preparation

CHCA was recrystallized in ethanol/water (50 : 50, v/v) and dissolved in acetonitrile (ACN)/0.1% aqueous TFA (50 : 50, v/v) to yield a stock solution of 10 mg/ml and stored at −80 °C. One mg/ml CHCA was used for samples eluted onto gold plates and 0.5 mg/ml CHCA was used for samples eluted onto ODT-modified plates. The peptides were prepared as 1 mg/ml stock solutions in water and stored at −80 °C until used.

Sample preparation using nanocolumns

Solutions were transferred to the inlet (i.e. the wide end) of the nanocolumn using a pipette tip. Solutions were forced through the column using a disposable syringe attached to the column inlet.[20] Prior to loading samples, the column was wetted with 15 μl of ACN/0.1% TFA (8 : 2, v/v) and then equilibrated with 10 μl of 0.1% TFA. For analysis of standards, 5–10-μl peptide solutions diluted in 0.1% TFA were loaded onto the column and passed through it by gentle pressure applied using the syringe. Solutions were eluted from the column by passing 1 μl of CHCA solution in ACN/0.1% TFA (50 : 50, v/v) slowly through the column to form a droplet, which was directly deposited onto the MALDI plate. For brain tissue samples, 10-μl peptide extract solutions were loaded and the column was then rinsed with 50 μl 0.1% TFA to desalt the samples. CHCA in ACN/0.1% TFA (75 : 25, v/v) (1 μl) was used to elute the sample.

Sample preparation using C$_{18}$ Ziptips

A C$_{18}$ Ziptip (Millipore, Billerica, MA, USA) was initially wetted by aspirating with ACN/water (50 : 50, v/v) twice, followed by rinsing with 0.1% TFA solution twice (10 μl each rinse), as instructed by the manufacturer. Peptide standard solutions diluted in 0.1% TFA (10 μl) were then aspirated into the tip and dispensed out 7–10 times to bind the peptides to the stationary phase in the Ziptip. Peptides were eluted by aspirating and dispensing 5 μl of ACN/0.1% TFA (50 : 50, v/v) through several cycles into a sample tube and then

mixed 1 : 1 (v/v) with CHCA solution in ACN/0.1% TFA (50 : 50, v/v) before deposition onto a MALDI plate. For brain tissue samples, 10-μl solutions were loaded and the Ziptip was washed by aspirating and dispensing to waste 10 μl of 0.1% TFA three times before the elution step. For elution, 1 μl CHCA in ACN/0.1% TFA (75 : 25, v/v) was used.

Microwave-fixed tissue preparation and neuropeptide extraction

Male Long Evans rats, 4–8 weeks of age (Charles River Laboratories, Raleigh, NC, USA) were sacrificed by focused microwave irradiation (~3.5 kW applied power for 1.5 s) using a Muromachi Microwave Fixation System model TMW (Stoelting Company, Wood Dale, IL, USA). Whole striatum samples were removed by free-hand dissection and stored at −80 °C until peptide extraction. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. The animal facility was accredited by the American Association for Accreditation of Laboratory Animal Care.

Striatal tissue (50 mg) was placed into 250 μl of cold (4 °C) homogenization solution containing 145 mM KCl, 8 mM SDS, and 0.25% acetic acid (v/v). The tissue/homogenization solution was set on ice for 1 h. Throughout the 1-h period, tissue homogenization and cell lysis were aided every 10 min by 5-s sonication using a sonic dismembrator (Fisher Scientific, Chicago, IL, USA) on power-setting 3 followed by 30 s of vortexing. The homogenate was centrifuged at $20\,000 \times g$ for 30 min at 4 °C. The supernatant containing both proteins and neuropeptides was removed from the resulting tissue pellet and filtered using a Microcon YM-10 centrifugal filter (Millipore, Billerica, MA, USA) fitted with a 30-kDa membrane to separate proteins from peptides.[1] The filter device was then placed into a centrifuge and spun at $14\,000 \times g$ for 45 min. The peptide-rich filtrate was frozen by liquid N_2 and stored at −80 °C until analysis. A portion of the homogenization solution without the added tissue was retained and used as control sample for spotting onto the MALDI plate.

Mass spectrometry

Mass spectra were acquired with a MALDI TOF Spec 2E mass spectrometer (Micromass, Milford, MA, USA) equipped with a delayed extraction source and 337-nm pulsed (4 ns) nitrogen laser operated in the reflectron mode. All mass spectra were obtained in positive ion mode with 20 kV source voltage, 2.2 kV extraction pulse voltage, 19.98 kV extraction voltage, 16 kV focus voltage, and suppression mass of 500 m/z with scan extended to 5000 m/z. The size of the laser spot was 150×250 μm. Mass spectral data were generated by summing 10 to 50 scans (each scan corresponds to five laser shots) into a single spectrum. External calibration was performed on all spectra.

Scanning electron microscopy

A Hitachi S3200N scanning electron microscope (SEM) (Hitachi Ltd., Tokyo, Japan) operated in high-vacuum mode

with 3.5-nm resolution using a secondary electron scintillator detector was used to collect images of MALDI sample spots. SEM images were obtained using medium beam current and 20 kV accelerating voltage with 2000× magnification.

RESULTS AND DISCUSSION

Patterned *versus* nonpatterned ODT plates

Initial experiments were performed to evaluate the use of patterned surfaces as sample supports. Because the spot size of the lasers commonly used in MALDI instruments is between 50 and 200 µm, it is useful to generate small sample spots that are roughly equivalent to the size of the laser spot so that most of the analyte molecules can be ionized upon laser irradiation.[20,23] The patterned ODT plates made by the microcontact printing method have bare gold spots surrounded by a hydrophobic thiol layer similar to prestructured supports[12,24–26] that should allow sample confinement to the size of the well. The ability of these structures to control sample spot size was confirmed by observing that crystals formed from deposition of a 1-µl droplet of 2.5 mg/ml aqueous CHCA solution onto a plate were confined to a 200-µm-diameter circle matching the size of the gold spots (Fig. 2(A) and (B)). These spot sizes are 1/10 times the diameter of those obtained for the same volume by dried-droplet method. As summarized in Fig. 3, the smaller sample spots obtained from aqueous samples deposited onto a patterned ODT plate generated about 10 to 50 times higher signal for four peptide standards than the same samples deposited onto a gold plate. To avoid bias in this comparison, when acquiring data from dried-droplet samples, the laser in the mass spectrometer was scanned manually to search for 'hot spots'; while for samples deposited on ODT plate, laser spot irradiated upon the center of the spots where the samples were deposited.

We then investigated the use of nonpatterned surfaces coated with ODT. Because of the hydrophobicity of the ODT-modified surface, aqueous sample spots deposited onto a nonpatterned ODT plate were confined to spot size nearly identical to that of the patterned surface (Fig. 2(C)). The sample manually deposited on nonpatterned ODT was not always in the center of the spot; therefore when acquiring data by MALDI-TOF MS, the laser was moved to irradiate right on top of the sample spot. The resulting

signal intensities for peptide standards in aqueous solutions were similar to those samples deposited onto the patterned surface, although some variation was seen with different peptides (Fig. 3). Except for vasopressin (VP), the signal intensities obtained from nonpatterned ODT plate were not significantly lower than those obtained from patterned ODT plate ($p < 0.05$), and were even higher for neurotensin 1–11. (All statistical comparisons were performed using a one-tailed t-test.) Both patterned and nonpatterned ODT plates generated significantly improved signal intensities over bare gold plates ($p < 0.05$), presumably due to the concentration effect of confining the samples to smaller spots. The patterned surface did not appear to offer significant advantages relative to the simpler-to-prepare nonpatterned plate; therefore, further experiments utilized nonpatterned ODT-coated plates.
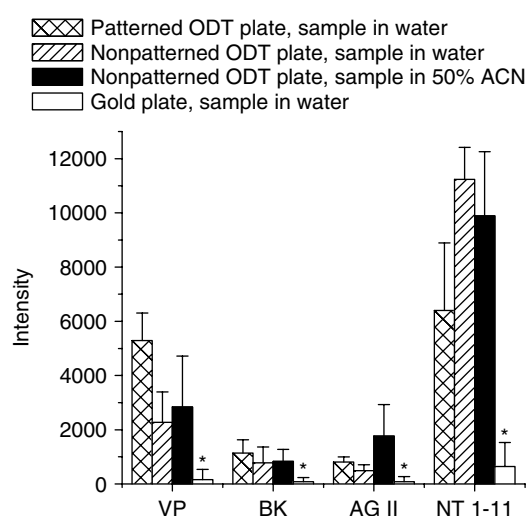

Figure 3. Comparison of the intensity of mass spectral signal generated for deposition of 1 µl of neuropeptide mixture onto a patterned ODT-coated plate, a nonpatterned ODT-coated plate, and a gold plate with dried-droplet method. Four neuropeptides were in the mixture: 20 nM vasopressin (VP), 20 nM bradykinin(BK), 20 nM angiotensin II (AG II), and 50 nM neurotensin 1–11 (NT 1–11). All results are the average with error bar equal to 1 standard deviation ($n = 6$). * indicates significantly lower S/N than the rest of the methods for each individual peptide.
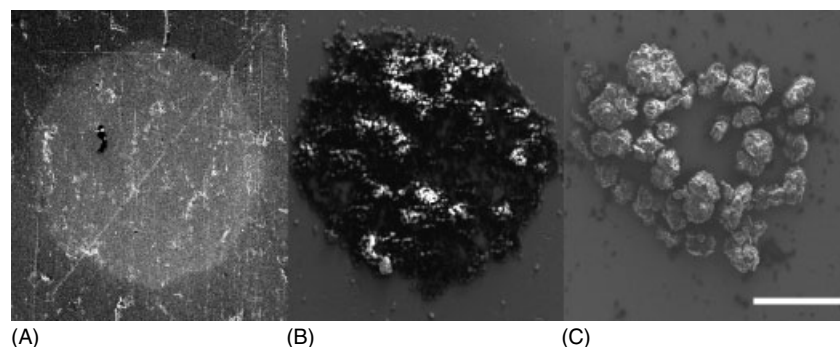

Figure 2. SEM image of patterned ODT plate with 200-µm hydrophilic spot (A); crystals formed from deposition of 1 µl of 2.5 mg/ml CHCA solution deposited on a hydrophilic spot of the patterned ODT-modified plate (B); and crystals formed from deposition of 1 µl of 2.5 mg/ml CHCA solution deposited onto nonpatterned ODT plate (C). Scale bar corresponds to 100 µm.

Further experimentation with ODT-coated plates revealed that as the percentage of ACN in the sample increased, the resulting sample spot size also increased. Using a solvent containing 50% ACN, the spot size of 1-µl sample solution on a nonpatterned ODT-coated plate was as large as 1 mm in diameter. (This effect was also found with the ODT-plate patterned with hydrophilic spots similar to that reported previously.[13]) Because we anticipated using the plates to capture samples eluted from a solid-phase extraction cartridge with high organic solvent content, this effect was limiting. Using DHB as matrix allowed samples to be confined to small spots with up to 75% ACN as solvent; however, DHB as matrix generated much lower signal intensities than CHCA as matrix for the neuropeptides we tested.

To diminish sample spreading when using high organic solvent content, samples were deposited in multiple steps. In this approach, a fraction of effluent was deposited and allowed to evaporate before the next fraction was added. Using 2–3 deposition steps for an eluent volume of 1 µl, spot sizes of ∼500 µm could be obtained on the ODT plate with 50% ACN in the sample. When the same deposition procedure was used on an unmodified gold plate, the sample spot could not be confined to such small size and the resulting signal intensity was lower. For example, the signal for neurotensin 1–11 in 50% ACN on the ODT plate was ∼12 times higher as compared to that obtained with the gold plate. As shown in Fig. 3, using this multistep deposition method for peptide standards (in 50% ACN) deposited on a nonpatterned ODT plate resulted in signals not significantly different from those achieved for aqueous solutions deposited on the same plate (except for AG II), despite slightly larger sample spots (500 µm compared to 200 µm for the aqueous samples). It is possible that differences in crystallization rate allowed the signal to be maintained despite the larger, and presumably less concentrated, spot. Water is less volatile than ACN; therefore, CHCA crystallizes more slowly in aqueous solution. Slower crystallization usually results in more crystal aggregation within sample spots, thus affecting the signal intensities.[27] From these results, we conclude that ODT-coated plates can be used to obtain good sensitivity for samples with relatively high organic solvent content as long as multistep deposition is used.

The effect of matrix concentration on sensitivity was tested for neurotensin 1–11 samples deposited on ODT-coated and unmodified gold plates. We found that 0.5 mg/ml

CHCA and 1 mg/ml CHCA gave the best sensitivities for neurotensin 1–11 deposited on ODT plate and on gold plate, respectively (data not shown). These concentrations were used for all further experiments.

Improved LOD combining nanocolumn with ODT plates

We evaluated if the ODT-coated plates would be of benefit for improving sensitivity when combined with solid-phase extraction methods by determining the LODs for vasopressin and neurotensin 1–11 using the dried-droplet, Ziptip, and nanocolumn methods (see 'Experimental' section for details on use of these methods) with samples deposited onto a bare gold plate or an ODT-coated plate. LODs were determined by calculating the concentration that would give a signal-to-noise ratio (S/N) of 3 assuming a linear relationship between S/N and concentration. To minimize error from this assumption, the concentration of sample used in different deposition methods was adjusted to obtain S/Ns in the range of 3–10. Noise was measured as the average peak-to-peak noise at 50 m/z adjacent to the peak. LODs were calculated on the basis of the initial sample volume, i.e. 1 µl for dried-droplet, 10 µl for Ziptip, and 5 µl for nanocolumn sample preparation. Using these criteria, we found that the ODT-coated plates significantly improved ($p < 0.05$) the LOD 6- to 60-fold over gold plates for each individual deposition method and peptide (Table 1), which reaffirmed experiments summarized in Fig. 3. The performance of the ODT-coated plates is comparable to other modified or prestructured surfaces when used with similar deposition methods.[12]

These results also allow an evaluation of the effectiveness of the use of nanocolumns for sample extraction and deposition. A direct comparison among the deposition methods is not valid because different starting volumes were used; however, the sample volumes used represent the maximum that is conveniently used with each method and therefore provide a comparison under best case for all methods. As expected, the methods using solid-phase extraction were significantly improved over the dried-droplet method (Table 1). The nanocolumns and Ziptip extractions provided similar mass LODs of ∼100 amol on the ODT-coated plates; however, the nanocolumns provide statistically significant ($p < 0.05$) improvements in concentration LOD of about five-fold over the Ziptip method. Detection of peptides at low picomolar concentration by this method is illustrated by the MALDI-TOF mass spectrum obtained from 5 µl of a mixture of vasopressin

Table 1. LOD for neurotensin 1–11 (NT 1–11) and vasopressin (VP) deposited via various methods on different plates

Peptide	LOD	Gold plate			ODT-coated plate		
		DD	Ziptip	Nanocolumn	DD	Ziptip	Nanocolumn
NT 1–11	mLOD (amol)	6000 ± 4000	4000 ± 2000	3000 ± 1000	500 ± 300	100 ± 70	100 ± 50
	cLOD (nM)	6 ± 4	4 ± 2	0.6 ± 0.2	0.5 ± 0.3	0.1 ± 0.07	0.02 ± 0.01
VP	mLOD (amol)	3000 ± 1000	800 ± 500	1000 ± 500	400 ± 200	100 ± 30	80 ± 4
	cLOD (nM)	3 ± 1	0.8 ± 0.5	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.03	0.02 ± 0.001

DD indicates dried-droplet deposition; mLOD and cLOD indicate mass LOD and concentration LOD respectively. All LODs are the average from 6 repetitions calculated as described in the text.
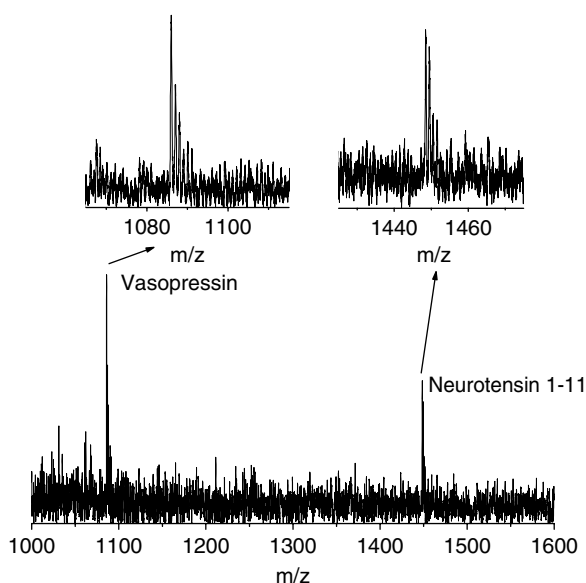
Figure 4. MALDI-TOF mass spectrum of vasopressin and neurotensin 1–11 mixture (20 pM, 100 amol each), prepared with nanocolumn/ODT-plate method. The spectrum is a sum of 10 scans.

and neurotensin 1–11 at 20 pM each in Fig. 4. The low picomolar concentration LOD of the nanocolumn/ODT-plate method is better than other deposition methods utilizing specialized surfaces that achieved sub-nanomolars to 10 nM concentration LOD.[11–14]

The much-improved concentration LOD by the nanocolumn over the Ziptip (Table 1) is largely due to the ability to use a low elution volume (1 μl) which can be entirely deposited into a single small spot. In contrast, the Ziptip required a larger volume to elute all sample (5 μl), preventing deposition into a single, small spot. This difference is due to the lower volume of the packed bed in the nanocolumn (30–50 nl)[20] compared with the Ziptip (600 μl). Another reason for improved LOD with the nanocolumn is that the narrow outlet of the GELoader tips, combined with multistep deposition, allows formation of small sample spots (200–300 μm), so that most of the sample can undergo ionization upon laser irradiation. As a result of this combination of factors, the nanocolumn is more useful for achieving low concentration LOD with limited sample volumes. This advantage is significantly heightened by using an ODT-coated plate. The trace level detection capability combined with sample purification suggested that the method could be used for determination of neuropeptides in complex media, such as brain tissue extracts.

Brain tissue study

'Neuropeptidomics', wherein many peptides are detected or identified in nervous tissue samples, is rapidly becoming a valuable tool for neurochemistry. Methods utilized for such analyses include capillary liquid chromatography-electrospray ionization mass spectrometry (cLC-ESI-MS),[1,28] direct MALDI-MS analysis of tissue[8,29] and LC-MALDI-MS.[6,30,31] Such methods have been valuable for identifying novel neurotransmitter candidates, determining the

distribution of neuropeptides, and studying peptide processing. In view of the good sensitivity of the nanocolumn/ODT-plate method, we evaluated its use for neuropeptidomic analysis of mammalian brain tissue.

Tissue extracts from striatum of rats sacrificed by microwave irradiation were used for this study. Microwave irradiation has been shown to fix tissue[32] and cause heat-induced denaturing of peptidase activity, resulting in an improved yield of neuropeptides over other methods of sacrifice.[33] Figure 5 compares spectra obtained with the nanocolumn/ODT-plate method, wherein 30 peaks were detected, with those obtained with the Ziptip/gold-plate method, wherein only 4 peaks were detected. These results illustrate that the combination of miniaturized columns, controlled deposition, and ODT-coated plates gives a significantly higher coverage of the neuropeptidome than the more conventional approach.

In analysis of extracts from two brains, we detected a total of 98 peaks that were not found in spectra obtained from the control homogenization solution. Without MS/MS capability, we had insufficient information to confidently identify the peptides from the masses detected. Pilot experiments using postsource decay on the MALDI-TOF MS instrument failed to generate reliable spectra, possibly because of the low levels. In a parallel study in our laboratory, the same samples were also analyzed using
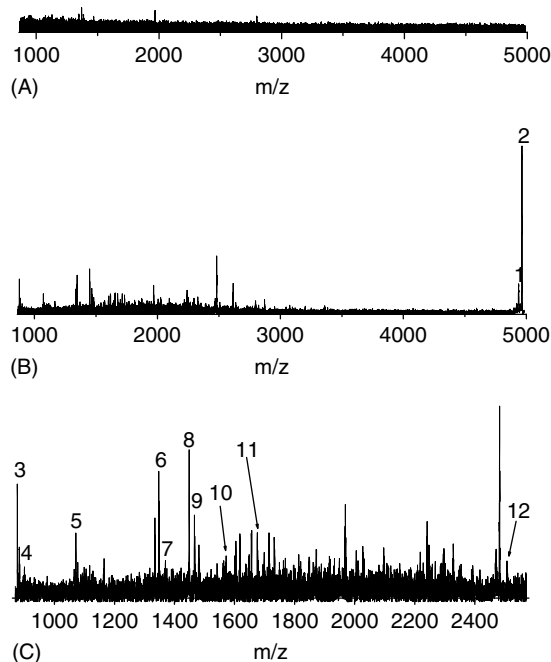

Figure 5. MALDI-TOF mass spectra of extract from microwave-fixed brain tissue samples prepared by Ziptip/gold-plate method (A) and nanocolumn/ODT-plate method (B). An insert of spectrum (B) is shown in spectrum (C). Labeled peptides correspond to: 1. thymosin beta-10; 2. thymosin beta-4; 3. YGGFMRF; 4. YGGFMRGL; 5. TEEELSPTEEE; 6. substance P; 7. RPKPQQFFGLM(O)-amidation; 8. neuropeptide E-I; 9. VGRPEWWMDYQ-amidation; 10. rimorphin; 11. acetyl-SGSAKVAFSAIRSTNH; 12. CLIP. Each spectrum is a sum of 50 scans. M(O) indicates methionine sulfoxide.

Table 2. Peptides detected in fixed tissue sample from striatum

Mass (detected)	Mass (calculated)	Sequence	Peptide	Precursor/protein	Sources
877.42	877.40	YGGFMRF	Met-enkenphalin RF	Proenkephalin A precursor	1,3
900.48	900.44	YGGFMRGL	Met-enkenphalinRGL	Proenkephalin A precursor	1,3
1149.65	1149.60	VAYWRQAGLS	–	Neurogranin	3
1165.77	1165.73	TEEELSPTEE	–	Protein-tyrosine phosphatase 1B	2
1228.69	1228.68	YGGFLRKYPK	α-Neo-endorphin	β-Neo-endorphin-dynorphin precursor	2
1347.78	1347.74	RPKPQQFFGLM-amide	Substance P	Protachykinin 1	1
1363.70	1363.73	RPKPQQFFGLM(O)-amide	Substance P	Protachykinin 1	2
1385.70	1385.69	SPQLEDEAKELQ	–	Proenkephalin A	3
1447.78	1447.71	EIGDEENSAKFPI-amide	Neuropeptide E-I	Pro-MCH precursor	1
1466.69	1466.65	VGRPEWWMDYQ	–	Proenkephalin A	1,3
1483.84	1483.85	FAVLKLM(O)GRGTKF	–	Myelin proteolipid protein	2
1505.70	1505.71	EIGDEENSAKFPIG	–	Pro-MCH precursor	1
1570.91	1570.89	YGGFLRRQFKVVT	Rimorphin	β-Neo-endorphin-dynorphin precursor	2
1637.93	1637.96	AKVAVLGASGGIGQPLSL	–	Malate dehydrogenase, mitochondrial precursor	3
1675.80	1675.86	Acetyl-SGSAKVAFSAIRSTNH	–	Cerebellin	3
1744.76	1744.82	EAEERGDLGEGGAWRL	–	Thyroliberin	3
1817.90	1817.82	QDNEPEKPVADSETKM	–	Excitatory amino acid transporter 2	3
1847.87	1847.95	PSSISWDGLDPGKLYTL-amide	–	Phosphatidylethanolamine binding protein	1,3
2132.02	2132.12	LVQTQAATTDSDKVDLSIAR	–	Fibrinogen beta chain precursor	3
2506.11	2506.26	RPVKVYPNVAENESAEAFPLEF	CLIP	Pro-opiomelanocortin	1
2617.18	2617.20	EEEEKDIEAEERGDLGEGGAWRL	–	Thyroliberin	3
4934.7	4934.53	Acetyl-ADKPDMGEIASFDKAKLKKT ETQEKNTLPTKETIEQEKRSEIS	Thymosin beta-10	Thymosin beta-10	1
4961.7	4961.49	Acetyl-SDKPDMAEIEKFDKSKLKKT ETQEKNPLPSKETIEQEKQAGES	Thymosin beta-4 short form	Thymosin beta-4	1

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2. Minamino *et al. J. Chromatogr. B* 2003; **792**: 33–48.

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Mass indicates $[M + H]^+$. M(O) indicates methionine sulfoxide.

capillary liquid chromatography-electrospray ionization-quadrupole ion trap mass spectrometry (cLC-ESI-QIT-MS) operated in the data-dependent mode. This method applied to similar tissue allowed acquisition of MS/MS spectra and peptide identification using the database-searching algorithms SEQUEST and Mascot (data not shown), yielding 95 identified peptides. Furthermore, similar samples have been previously analyzed by cLC-ESI-QTOF-MS with 89 peptides identified.[1,2] Comparison of the 98 compounds detected in our samples with those detected in the previous studies revealed that 23 peptides had masses that were within the accuracy of the instrument (difference between calculated and measured monoisotopic mass $\leq$ 50 ppm) as summarized in Table 2. These peptides include several known neuropeptides or related compounds such as substance P, neuropeptide E-I, and corticotrophinlike intermediate lobe peptide (CLIP).

While these assignments should be considered tentative, the results strongly suggest that the MALDI-TOF method combined with the nanocolumn/ODT plate can be used to detect endogenous neuropeptides in tissue samples. Use of tandem mass spectrometry is expected to allow identification from MALDI-TOF MS data. More selective sample preparation procedures, such as affinity purification[34] or chromatography separation,[15,35] may be used to target specific peptides for analysis. The combination with chromatographic separation may decrease competitive ionization and improve the yield of peptides detected.

CONCLUSION

We have demonstrated that combination of a nanocolumn with hydrophobic ODT-coated sample plates allows sensitive detection of neuropeptides in complex samples by MALDI-MS. While nanocolumns and hydrophobic or prestructured supports have both been previously used, the combination of these methods is revealed to offer significant sensitivity improvements, especially in concentration LOD of real samples, over either method independently. The sensitivity of the method, which allowed concentration LOD as low as 15 pM, is attributed to combining preconcentration and desalting with plate modifications to decrease the size of the resulting sample spot. The method is simple to implement on any TOF-MS and applicable to complex analyses where the sample is limited. The simplicity and sensitivity of the method should make it useful for numerous applications. The demonstration of neuropeptide detection in select brain regions in this work is an example of a demanding application made possible by this sample preparation method. Combination with tandem mass spectrometry the method may enable this method to be used for confident peptide identification as well.

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