

Iva Urbanova
Frantisek Svec

The Molecular Foundry,
Lawrence Berkeley National
Laboratory, Berkeley, CA, USA

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Research Article

Monolithic polymer layer with gradient of hydrophobicity for separation of peptides using two-dimensional thin layer chromatography and MALDI-TOF-MS detection

Superhydrophobic monolithic porous polymer layers supported onto glass plates with a gradient of hydrophobicity have been prepared and used for 2-D thin layer chromatography of peptides. The 50 μm -thin poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) layers prepared using UV-initiated polymerization in a simple mold were first hydrolyzed using dilute sulfuric acid and then hydrophilized via two-step grafting of poly(ethylene glycol) methacrylate to obtain superhydrophilic plates. The hydrophobicity was then formed by photografting of lauryl methacrylate. The exposure to UV light that initiates photografting was spatially controlled using moving shutter that enabled forming of the diagonal gradient of hydrophobicity. This new concept enables the solutes to encounter the gradient for each of the two sequential developments. Practical application of our novel plates was demonstrated with a rapid 2-D separation of a mixture of model peptides gly-tyr, val-tyr-val, leucine enkephalin, and oxytocin in dual reversed-phase mode using different mobile phases in each direction. Detection of fluorescent-labeled peptides was achieved through UV light visualization while separation of native leucine enkephalin and oxytocin was monitored directly using MALDI mass spectrometry.

Keywords: 2-D separation / Monolith / Photografting / Separation of peptides / Thin-layer chromatography
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1 Introduction

Thin-layer chromatography (TLC) is characterized by the simplicity that enables to achieve separations without any sophisticated instrumentation. TLC was first demonstrated in the 1930s [1]. However, the wide acceptance of this analytical method was noticed only after works published by Kirchner and Stahl in the 1950s [2–4]. Today, TLC is a routine tool in organic chemistry laboratories, where it enables to follow the progress of syntheses and selection of the mobile phase for the purification of compounds using flash chromatography. TLC also finds applications in fields,

such as quality control, separation of metal ions, drug and forensics testing, clinical diagnostics, and analysis of pesticide residues. Use of scanning densitometry enables quantitative analysis in TLC. The affordable TLC plates are typically disposed after the analysis. However, the plate with separated sample can be dried and kept for archiving. The thin-layer format also permits the separation of several samples in parallel lines thus significantly increasing the throughput of this method [4].

Thin chromatographic layers can be formed from different organic and inorganic sorbents and different plates are commercially available. However, the most commonly used conventional TLC plates consist of sorbent layers formed from irregular ca. 10 μm silica particles held together by a binder. The more expensive high-performance layers introduced in the mid 1970s are manufactured from sub-10 μm porous silica beads and enable better resolution [5]. All these plates contain a layer with a thickness of up to 400 μm . In contrast, Merck introduced in the early 2000s the new generation of chromatographic plates with ultrathin layer featuring 10 μm monolithic silica structure [6]. Several new developments in the layer technology appeared recently [7, 8].

Our interest in monolithic thin layers dates back to the mid 2000s when we discovered the ease of the preparation

Correspondence: Dr. Frantisek Svec, The Molecular Foundry, E. O. Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

E-mail: fsvec@lbl.gov

Fax: +1-510-486-7413

Abbreviations: **1,8-ANS**, 1-anilinonaphthalene-8-sulfonic acid; **CHCA**, α -cyano-4-hydroxycinnamic acid; **EtOH**, ethanol; **EDMA**, ethylene dimethacrylate; **GMA**, glycidyl methacrylate; **LMA**, lauryl methacrylate; **PEGMA**, poly(ethylene glycol) methacrylate

of monolithic porous polymer layers using photoinitiated polymerization in a simple glass mold. First, we have prepared thin layers from porous poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths attached to a glass plate and used them for the separation of peptides and proteins with MALDI-TOF-MS detection [9]. Similar plates with their surface modified via co-grafting of 2-acrylamido-2-methyl-1-propanesulfonic acid and 2-hydroxyethyl methacrylate were used for the faster separation of peptides in electrophoretic and pressurized planar electrochromatographic modes [10].

The 2-D character of TLC plates makes them an ideal medium for 2-D separations. In the typical implementation, the sample is applied at one corner of the standard plate, the layer developed in the first dimension followed by rotating it by 90° and development in the second direction. Use of different eluents with complementary selectivity in each dimension is the simplest way that may help to afford the desired orthogonality. More sophisticated approaches including those requiring layers with different chemistries have been summarized by Poole [11] and an extensive review of 2-D TLC has recently been published by Gocan [12]. Unfortunately, handling these less common formats is a complex task that eliminates the ease of the original TLC and new setups need to be developed to fully exploit the 2-D nature of TLC [13].

While working with some of these layers, we noticed their superhydrophobic properties [14]. These layers accommodated the combination of micro- and nano-scale roughness that is desirable to afford the superhydrophobicity. We then used this superhydrophobic polymer to prepare monolithic layers with dual chemistry enabling a new approach to 2-D thin layer chromatography [15]. First, we prepared the superhydrophobic poly(butyl methacrylate-co-ethylene dimethacrylate) layer using UV-initiated polymerization and then we photographed a superhydrophilic virtual channel along one side of the layer using a solution of monomers consisting of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and 2-hydroxyethyl methacrylate (HEMA). This virtual channel enabled the first-dimension separation of peptides in ion-exchange mode using aqueous mobile phase. The flow through this virtual channel took advantage of the surface tension-confined channel dimensions. The second-dimension separation in the reversed phase using the unmodified superhydrophobic part of the layer and water-organic solvent mixture was then achieved by simply developing the TLC plate in orthogonal direction.

Application of different solvent systems for development in each dimension is the typical way to enhance the peak capacity in typical 2-D TLC using standard plates with uniform chemistry. While 1-D TLC using one solvent separates components of the sample in a single lane, changing the mobile phase used for the development in orthogonal directions helps to separate the sample over the entire plate surface and to achieve better resolution. We

anticipated that even better separation could be achieved with plates whose chemistry is not identical all across the plate.

Our photografting process that we have developed during the last decade [16–22] can be easily adjusted to form gradients of chemistry within the monoliths. For example, we prepared monolithic column with a linear gradient of AMPS and applied it for capillary electrochromatography [23]. This report describes the use of a similar approach for the preparation of monolithic thin layers with a 2-D gradient of hydrophobicity photografted on superhydrophilic monolithic layer increasing along the diagonal direction on the plate as shown in the artistic rendition of Fig. 1. Using these plates, the separation of all analytes is achieved in a gradient of chemistry in both subsequent developments. Specifically, we demonstrate this new concept with 2-D TLC of peptides combined with MALDI-TOF-MS detection.

2 Materials and methods

2.1 Reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), lauryl methacrylate (LMA), poly(ethylene glycol) methacrylate (PEGMA), benzophenone, 2,2-dimethoxy-2-phenylacetophenone (DMPAP), cyclohexanol, 1-decanol, ethanol (EtOH), acetic acid, 3-(trimethoxysilyl)propyl methacrylate, trifluoroacetic acid (TFA), formic acid (FA), hydrochloric acid, sodium phosphate dibasic, sodium phosphate monobasic, α -cyano-4-hydroxycinnamic acid (CHCA), 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), fluorescamine, acetone, acetonitrile, and water, all of the highest available purity were purchased from Sigma-Aldrich

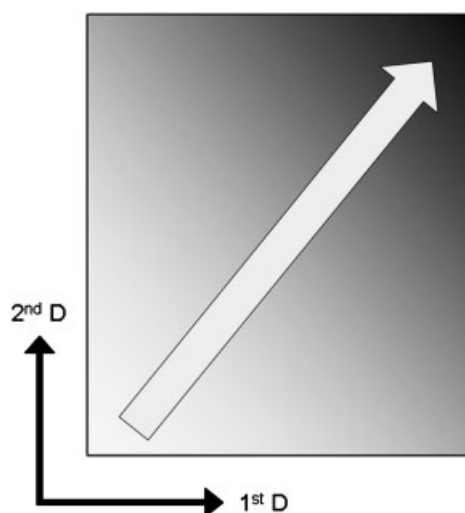


Figure 1. Artistic rendition of the gradient of hydrophobicity at a monolithic TLC plate increasing in the direction of the arrow together with suggested directions of the separations in first dimension and second dimension.

(St. Louis, MO, USA). Sulfuric acid and sodium hydroxide were obtained from Fischer Scientific (NJ, USA). The methacrylate monomers were purified by passing through a polypropylene syringe packed with aluminium oxide, (activated, basic, cat. number 199443, Sigma-Aldrich).

Borofloat glass plates 12 × 3.3 cm, 1.1 mm thick were purchased from S. I. Howard Glass (Worcester, MA, USA). The surface of the glass plates was functionalized using a procedure described elsewhere [15]. After modification, the plates were cut to four 3 × 3.3 cm pieces.

2.2 Peptide samples and matrix solution

All 0.5 mg/mL stock solutions of peptides oxytocin, leucine enkephalin, gly-tyr, and val-tyr-val (all from Sigma-Aldrich) and saturated solution of MALDI matrix (CHCA) were prepared using 0.1% formic acid solution in 30% aqueous acetonitrile as a solvent. Properties of peptides used in this study are shown in Table 1. Fluorescamine (1 mg/mL) was dissolved in acetone and the solution refrigerated. Peptides were labeled by mixing equal volumes of both fluorescamine and peptide solutions. Peptide mixture for the TLC separations followed by UV detection was prepared by mixing equal volumes of labeled peptides. For the TLC separation followed by MALDI detection, peptide mixture was prepared by mixing equal volumes of unlabeled peptides and the matrix.

2.3 Preparation of porous polymer layer

The polymerization mixture adopted from our previous works [14, 24] consisting of GMA (24 wt%), EDMA (16 wt%), cyclohexanol (20 wt%), 1-decanol (40 wt%), and DMPAP (1 wt% with respect to monomers) was deaerated by sonication and purging with nitrogen for 15 min. This mixture was filled into a mold consisting of two modified glass plates clamped together and separated with two Teflon strips (American Durafilm). The Teflon strips were 50 μm thick and defined the thickness of the monolithic layer. After filling the mold using capillary action, it was exposed to 254 nm UV light for 15 min. Once the polymerization was completed, the mold was disassembled. The monolith remained attached to the top plate. The plate with the polymer layer was immersed in methanol for 30 min to remove the porogens and then dried in a vacuum oven at 40°C for 30 min.

Table 1. Properties of separated peptides. Values of *pI* and average hydrophilicity were calculated using <http://www.innovagen.se/>.

Peptide	Sequence	Mol. mass	<i>pI</i>	Average hydrophilicity
gly-tyr	gly-tyr	238.2	5.9	−1.1
val-tyr-val	val-tyr-val	379.5	5.9	−1.8
Leucine enkephalin	tyr-gly-gly-phe-leu	555.6	5.9	−1.3
Oxytocin	cys-tyr-ile-gln-asn-cys-pro-leu-gly	1007.2	5.2	−0.8

2.4 Hydrolysis of GMA-co-EDMA layer

The glass plate with attached GMA-co-EDMA layer was immersed for 24 h into 0.5 mol/L sulfuric acid in order to open the epoxide ring and form vicinal diol functionalities. The plate was then washed with water and dried in a stream of nitrogen.

2.5 Two-step photografting of PEGMA

The first step included wetting the hydrolyzed monolith with a solution containing 5 wt% benzophenone in methanol. The wetted layer was covered with a non-functionalized quartz plate and exposed to UV light for 4 min. The plate was then washed with methanol and dried. The second step comprised wetting the layer with 0.1 mol/L solution of PEGMA in water. The layer was again covered with the quartz plate, exposed to UV light for 4 min, washed with water and dried.

2.6 Photografting of diagonal gradient of hydrophobicity

The hydrophilic monolithic layer was wetted with a solution of 0.25 wt% benzophenone in 1:5 LMA–EtOH mixture. The wetted layer was covered with the quartz plate and a mask made of a piece of a thick paper coated with a black electrical tape, which was non-transparent to the UV light. The mask was attached with a string to a syringe pump set to a constant speed and moved diagonally across the plate over the course of 5 min. After the photografting was completed, the plates were washed with methanol and dried. The gradient was confirmed by measuring the water contact angle and its profile visualized by labeling the plate with 0.01 wt% 1,8-ANS dissolved in 1:4 methanol–water. In a control experiment, LMA was homogeneously photografted on the whole plate for 5 min.

2.7 2-D separation of peptides

An aqueous solution of UV-labeled peptides (0.5 μL) was spotted onto a hydrophilic part of the plate. The separation was run in a direction as shown in Fig. 1. The mobile phase used for the separation in the first direction consisted of 0.1 vol% TFA in 30% acetonitrile–water

mixture. When the front of the mobile phase reached to ca. 4 mm away from the end of the plate, the plate was removed from the development chamber, dried, turned perpendicularly, and the separation was run in the second direction using 0.1 vol% TFA in 1:1 methanol–water as the mobile phase. Once the development was completed, the plate was dried and peptide spots detected using illumination with UV light.

2.8 Instrumentation

An OAI Model 30 deep UV collimated light source (Optical Associates, San Jose, CA, USA) fitted with a 500-W HgXe lamp was used for UV exposures. The radiation power was adjusted to 12 mW/cm², which affords 4.4 mW/cm² after passing the cover glass plate as measured using an OAI Model 306 UV meter with a 260-nm probe head. Static water contact angle was determined by using an Easy Drop instrument (Krüss GmbH, Germany). Images of fluorescent-labeled plates were scanned using Chemidoc XRS+ system (BioRad Laboratories, Hercules, CA, USA) and processed by using the MATLAB software.

2.9 TLC/MALDI-TOF-MS

MALDI-TOF measurements were carried out using Autoflex TOF/TOF instrument (Bruker Daltonics). Only positively charged compounds were analyzed. Typically, 100 laser shots applied to several positions were sufficient to obtain a good signal-to-noise ratio. Spectra were recorded in reflector mode. The Flex Analysis version 2.4 software package provided by the manufacturer was used for data processing. TLC plate was affixed using a double-sided tape to the microtiter plate (MTP) adapter for the pre-spotted anchor chip. This adapter features a pit whose depth is equivalent to the height of the quartz plate with the attached monolith.

3 Results and discussion

3.1 Preparation of monolithic plates with gradient of hydrophobicity

We observed in our previous work that the hydrophilicity of diol functionalities of poly(2,3-dihydroxypropyl methacrylate-co-EDMA) monolith prepared via hydrolysis of epoxy groups of poly(GMA-co-EDMA) in a capillary was not sufficient to avoid adsorption of hydrophobic albumin in a highly aqueous mobile phase [24]. This undesired adsorption was substantially reduced only after this monolith was further hydrophilized using photografting of PEGMA. Based on this former experience, we decided to use similar approach in the present study and to prepare an inert support layer suitable for the formation of the gradient of hydrophobicity. Hydrolysis followed by two-step photograft-

ing of PEGMA results in a monolith that exhibits water contact angle close to 0° rendering the entire layer superhydrophilic. LMA was then grafted on the top of this superhydrophilic layer. Since benzophenone that is used as a photoinitiator suitable for grafting via hydrogen abstraction [25] poorly dissolves in neat LMA, we tested various solvents to dissolve both LMA and benzophenone. Commonly used solvents for benzophenone, i.e. *tert*-butanol–water mixture and acetonitrile do not dissolve LMA. In contrast, EtOH dissolved both key compounds and was used in further experiments.

Table 2 summarizes compositions of grafting mixtures and grafting times we explored in order to create a continuous gradient of LMA. The grafting time represents the interval during which the moving shutter travels all across the plate. At time 0 s the shutter starts moving and opens one corner to UV irradiation. As it moves further in diagonal direction, larger and larger areas of the plate are irradiated until the shutter reaches the other corner and the UV light is switched off. Thus, the irradiation time is the longest at the starting corner and shortest at the other end. Since the extent of grafting is proportional to the irradiation time, gradient of hydrophobicity is formed. The extent of the LMA photografting is then determined from the measurements of water contact angle and by labeling the plate with the fluorescent dye 1,8-ANS, which is sensitive to hydrophobicity. We scanned and photographed each labeled plate in Chemidoc XRS+ system and processed it using MATLAB software that converts the picture into a “map” based on the brightness of the fluorescence.

First, we attempted grafting using higher concentrations of LMA in the EtOH solution and short grafting times of 60–90 s. Although these experiments afforded the targeted grafting, it was difficult to exactly hold always the same short times due to the need for manual manipulation within the irradiation lamp that led to poorer reproducibility of the gradients. In contrast, longer grafting times of 150–600 s provided repeatable results with an RSD <5%. However, the plate was “overgrafted” when using procedure C, D, and J comprising high LMA concentration or extended period of time. The entire plates were covered with LMA functionalities with no gradient of functionality observed.

The best combination concentration and time enabling creation of the desired gradient of hydrophobicity includes 300 s grafting of the 1:5 LMA–EtOH mixture (experiment I). Figure 2A shows a fluorescence-labeled monolithic plate

Table 2. Composition of grafting solutions and grafting time used to form a gradient of hydrophobicity at the thin layer

Experiment	A	B	C	D	E	F	G	H	I	J
LMA, vol.	1	1	1	1	1	1	1	1	1	1
EtOH, vol.	1	1	1	1	2	2	3	3	5	10
Grafting time, s	60	90	150	300	60	90	60	90	300	600

with a gradient of hydrophobic chemistry. The bottom left corner is the most hydrophilic part with a contact angle of 0° , while the most hydrophobic part at the top right corner exhibits a contact angle of 135° . Scanning intensity of fluorescence emanating from the adsorbed hydrophobicity probe processed using the MATLAB software also confirms the presence of the gradient. Figure 2B shows the picture of Fig. 2A converted into a digital format with the lines connecting points of equal hydrophobicity.

3.2 2-D separation of peptides

Performance of the TLC plates with gradient of hydrophobicity was tested using a mixture of four peptides, gly-tyr, val-tyr-val, leucine enkephalin, and oxytocin shown in Table 1 labeled with fluoescamine. To select the mobile phase used for the development, we first mixed phosphate buffers pH 3, 7, and 11 with acetonitrile in the first dimension and methanol in the second dimension expecting to observe effect of ionization of peptides on retention. However, decent separations were observed only with the acidic mobile phase while at neutral and basic pH the peptides co-eluted at the front of the mobile phase and the spot was smeared. We also found that a simple solution of trifluoroacetic acid in the

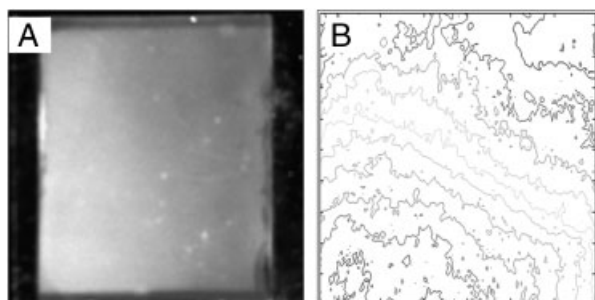


Figure 2. Visualization of the gradient of hydrophobicity using fluorescent labeling with 1,8-ANS (A). The bright area at the left down corner represents the most hydrophilic part and the dark at top right most hydrophobic part. The gradient is confirmed by the contour plot of the same plate obtained from the scanned picture using home-written routine in MATLAB program (B).

organic/aqueous mobile phase affords same good results and used this mobile phase in all further experiments.

The positive effect of TLC with the gradient of LMA is demonstrated with separations shown in Fig. 3 using different plates. Clearly, the 2-D approach using initial poly(GMA-co-EDMA) layer (Fig. 3A) does not yield any good separation. All peptides are retained close to the sampling corner and their spots after 2-D development are smeared. We next used plate with all epoxides hydrolyzed to hydrophilic 2,3-dihydroxypropyl functionalities using dilute sulfuric acid. This modification significantly reduces interactions of peptides with the layer and decreases their retention. However, the peptides move quickly through the plate without being adequately separated and the spot shapes are poor (Fig. 3B).

A better separation is achieved using monolithic TLC plate with a homogeneous grafted layer of poly(LMA) onto the monolithic layer hydrophilized with PEGMA. Figure 3C shows that the peptides are separated, with leucine enkephalin and oxytocin featuring the smallest and the largest retention factor R_F . However, val-tyr-val and gly-tyr co-elute and their spot is located between the other two peptides.

Figure 3D then shows the separation using a plate with diagonally grafted hydrophobicity. The peptide mixture is spotted again at the most hydrophilic part of the plate and the first-dimension separation is carried out using acidic aqueous acetonitrile. This separation is very fast and accomplished in <1 min. At that time, the front of the mobile phase almost reaches the end of the layer. This separation affords three distinct spots of leucine enkephalin, co-eluted val-tyr-val plus gly-tyr, and oxytocin, i.e. a result almost identical with that achieved with the homogeneously grafted plate presented in Fig. 3C. However, after turning the plate perpendicularly and developing it in the second dimension with acidic aqueous methanol, val-tyr-val and gly-tyr are clearly separated from each other with val-tyr-val having the higher R_F value. The reason for the better separation on the diagonally grafted layer is the following. At the plate grafted in the “linear” manner, the peptides come into contact with the same hydrophobicity in both the first and the second dimensions. Therefore, all the spots are lined up at the diagonal. In contrast, diagonally grafted layer provides gradient of hydrophobicity during development in both dimensions and enables fine-tuning of

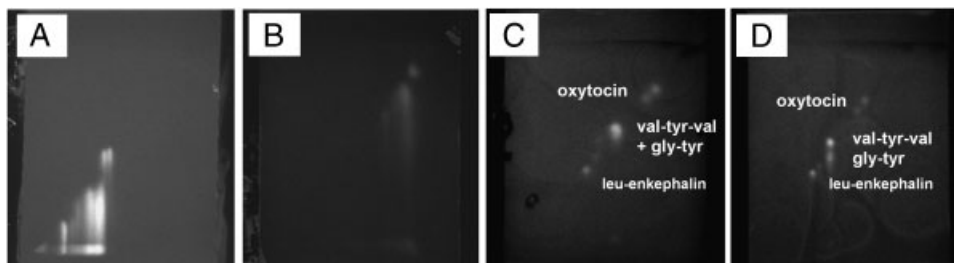


Figure 3. Separation of leucine enkephalin, gly-tyr, val-tyr-val, and oxytocin using plates consisting of a poly(GMA-EDMA) monolith (A), poly(GMA-EDMA) monolith with hydrolyzed epoxy groups (B), hydrophilized monolithic layer with poly(LMA) homogeneously photografted on the entire surface (C), and hydrophilized monolithic layer with surface photografted with a diagonal gradient of poly(LMA) (D). Conditions: Mobile phase: first dimension 0.1% trifluoroacetic acid in 30% acetonitrile–water; second dimension 0.1% trifluoroacetic acid in 50% methanol–water.

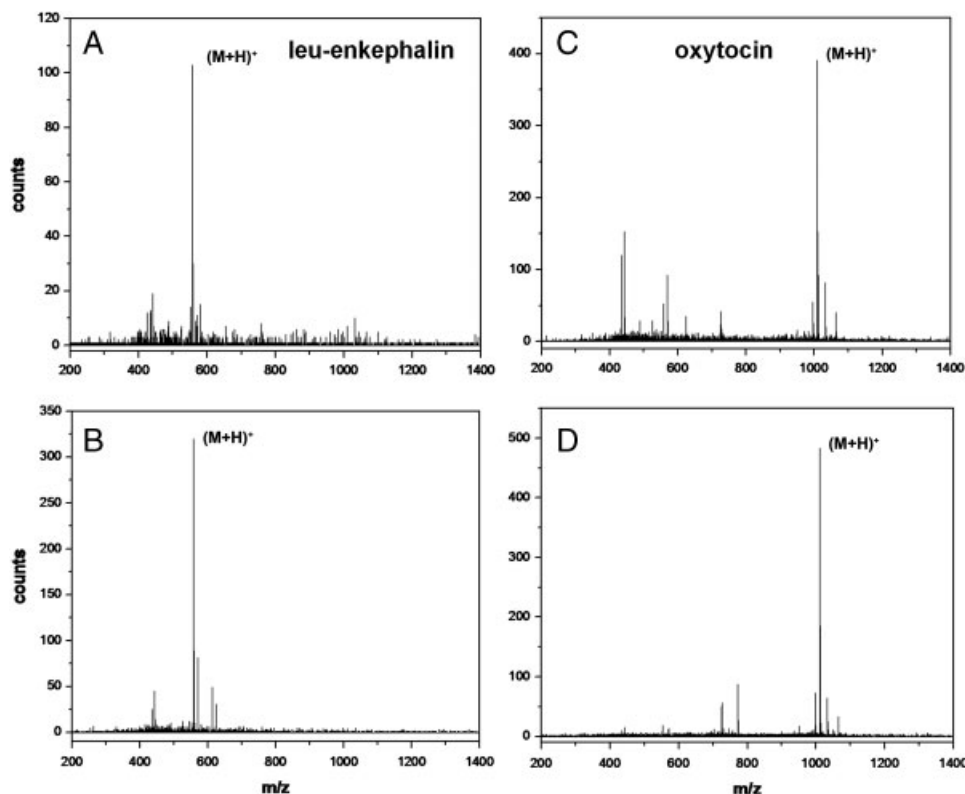


Figure 4. MALDI-MS spectra of leucine enkephalin and oxytocin obtained after ionization from the plate after separation in the first (A, C) and second dimensions (B, D). Matrix HCCA.

the separation. We observed that the oxytocin we used separated eventually into two spots. The mass spectral analysis of the commercial nonpeptide indicated that it contained molecules with the expected molecular mass of 1007 and a dimer with a mass of 2014, thus explaining the origin of the two spots. It is worth noting that the separation in the second dimension is completed in < 3 min.

3.3 MALDI-TOF-MS detection

Separation of labeled peptides is likely to be affected by the size and chemistry of fluorescamine with a molar mass of 278.3 attached to the N-terminus. In contrast, MALDI-TOF-MS detects peptides without labeling and adds another dimension to the separation since it provides information about the molar mass and enables the determination of amino acid composition of the separated peptides.

We have demonstrated that peptides and proteins can be ionized from monolithic layer attached to a stainless steel target in MALDI-MS [9, 26]. In this study, we used monolithic layer attached to a plate taped to an MTP adapter designed for pre-spotted anchor chips.

Feasibility of the MS detection is demonstrated with the larger peptides leucine enkephalin and oxytocin since the peaks of both dipeptide and tripeptide would be obscured by the peaks originating from the matrix. The two unlabeled larger peptides were mixed in equivalent quantities and their separation carried out using conditions applied to the

labeled peptides. After the separation in both the dimensions was completed, the monolith was sprayed with the saturated matrix solution and dried in air. The plate was then taped to the adapter. The known positions of the labeled peptides established in the previous experiments help to quickly find the position of the unlabeled peptides on the plate within the MALDI-MS instrument.

Figure 4 shows the mass spectra of leucine enkephalin and oxytocin, after the separation in the first and second dimensions, respectively. The signal-to-noise ratio is good after separation in both the dimensions with no signal of the other peptide observed. However, the spectra of both peptides obtained after the 2-D separation are cleaner because impurities typically contained in man-made peptides are separated farther away from the major spot. In our previous work, we observed similar “clean-up” action while carrying out the 2-D TLC separations using plates with different photopatterned chemistries [15].

4 Concluding remarks

This study demonstrates the promise of monolithic porous polymer thin layers with a diagonal gradient of hydrophobicity in 2-D TLC separations. Preparation of the hydrophilic monoliths with a density gradient of grafted poly(LMA) chains on the TLC plate is readily achieved via a simple photografting process. While labeling of peptides prior to the separations enables easy detection of spots in

UV light, MALDI-MS analysis with which the monolithic plates are fully compatible adds additional dimension to the separation. Although this initial proof-of-concept study only demonstrates the 2-D separation of a few peptides, it is obvious that these novel plates can enable the separation of other compounds. The plates are also amenable to other ionization processes used for delivering compounds from the plate directly in the mass spectrometer including desorption electrospray ionization (DESI) and laser ablation electrospray ionization (LAESI). Our current work is focused on the preparation of plates with diagonal gradients of different chemistries that are likely to enable separations in two completely orthogonal dimensions.

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The authors have declared no conflict of interest.

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