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Protein UTLC-MALDI-MS using thin films of submicrometer silica particles

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Abstract

Slides for ultra thin-layer chromatography (UTLC) slides were made by coating nonporous silica particles, chemically modified with polyacrylamide, as 15 μm films on glass or silicon. Three proteins, myoglobin, cytochrome c and lysozyme, are nearly baseline resolved by the mechanism of hydrophilic interaction chromatography. A plate height as low as 3 μm , with 3,900 plates, are observed in 14 mm. Varying silica particle diameter among 900, 700 and 350 nm showed that decreasing particle diameter slightly improves resolution but slows the separation. Matrix-assisted laser desorption/ionization (MALDI)-MS of the proteins after separation is demonstrated by wicking sufficient sinapinic acid into the separation medium.

Keywords

Thin-layer chromatography; sub-micrometer; silica colloidal; proteins; hydrophilic interaction chromatography

1. Introduction

Thin layer chromatography (TLC), also called planar chromatography, is one of the simplest and the most popular methods for separating small molecules. The movement of mobile phase in TLC requires no pump; mobile phase is moved through the thin layer coating by capillary forces. Due to its simplicity, TLC is widely used in organic and pharmaceutical laboratories to monitor the progress of syntheses or quickly assess sample purity [1].

Currently, the most widely used TLC plates have consisted of a flat solid substrate (glass or aluminum) which is coated by a layer of sorbent made from silica gel. A typical early TLC plate was coated by a 0.25 mm thick layer of irregular 10 μm silica particles, which were held by a binder. As with HPLC, the trend in the development in TLC has been toward smaller particles [1,2]. In 1995, a high-performance (HP) TLC plate coated with 5-8 μm porous silica spheres was developed for a higher speed and more compact spots [2]. In 2001, the ultrathin layer chromatographic (UTLC) plate coated with a 10 μm thick monolithic silica structure was introduced, which had 3-4 nm mesopores and 1-2 μm macropores, while avoiding the need for a binder in the layer [3]. This is the latest technique that has been commercialized. Since then, three new nanoporous monolithic materials have been developed: a porous nanostructured film of silica prepared by glancing angle deposition [4],

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a nanofibrous stationary phase prepared by electrospinning [5,6], which gives extraordinary resolution of small molecules, and a porous polymer monolithic layer on glass plates, which was used for the separation of proteins [7,8].

Despite the popularity of TLC for the separation of small molecules, there are only a few reports on the separation of proteins due to the complex structure of proteins [7,9-14]. In 1995, Arkedy [13] reported TLC experiments of several proteins (bovine insulin chain B, insulin, horse heart cytochrome c, and myoglobin) combined with matrix-assisted laser desorption ionization (MALDI) MS analysis. They demonstrated on-plate MALDI-MS detection, but the separation of proteins could not be achieved with the conventional silica gel and cellulose TLC plates available at that time. Thin-layer anion-exchange chromatography was developed by Luo [14] as an alternative to conventional TLC for protein separation. A mixture of four proteins, BSA, human holo-transferrin, bovine milk lactoferrin and hen egg-white lysozyme, was separated using a three-step elution process. MALDI was not done; instead, protein labeling was performed after solvent development, and the proteins were identified by R_f value. In 2007, Bakry [7] used a porous poly(butyl acrylate-co-ethylene dimethacrylate) monolith layer to separate a mixture of four proteins: insulin, cytochrome c, lysozyme and myoglobin. They successfully separated all the four proteins, albeit with limited resolutions for all but insulin. This was the first work combining a successful TLC separation with MALDI-MS spectra obtained for each protein directly from the TLC plate.

In this paper, we report the preparation and performance of a UTLC stationary phase composed of uniform nonporous silica nanoparticles less than 1 μm in diameter, joined together by trifunctional silanes to make a monolithic material. A novel stationary phase is used: a brush layer of polyacrylamide, to increase the capillary forces that draw the mobile phase into the material. The coupling to MALDI-MS for identification after protein separation is studied.

2. Experimental

2.1. Reagents and materials

Silica particles (900, 700 and 350 nm in diameter) were purchased from Fiber Optic Center, Inc. (New Bedford, MA), and then calcined at 600 °C for 12 hours. Single side polished P-type silicon wafers were obtained from Wafer World Inc. (West Palm Beach, FL). Soda lime glass plates were purchased from a local glass store. Proteins including myoglobin from equine skeletal muscle, cytochrome C from bovine heart and lysozyme from chicken egg white (Sigma-Aldrich, St. Louis, MO) were labeled with Alexa Fluor 546, Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen Co., Carlsbad, CA) according to the labeling kit manual for fluorescence imaging. Trichloromethylsilane (Gelest, Inc., Morrisville, PA), (chloromethyl)phenylethyl-trichlorosilane (Gelest, Inc., Morrisville, PA), acrylamide (Sigma-Aldrich, St. Louis, MO), CuCl (99.999%; Alfa Aesar, Ward Hill, MA), CuCl₂ (99%; Acros Organics, Morris Plains, NJ) were used as received.

2.2. Fabrication of UTLC plate

Slurries of the silica particles in ethanol (50% w/w) were prepared and sonicated. Freshly cleaned glass slides or silicon wafer slides were coated with slurry (about 43 μL per square inch) using Auto Draw III automatic drawdown machine equipped with #20 rod (Industry Tech, Oldsmar, FL) which made the coatings 15 μm in thickness. The coated slides dried quickly in air and were cut into 1" wide strips. A 2% (v/v) solution of (chloromethyl)phenylethyl-trichlorosilane and trichloromethylsilane were mixed at a volume ratio of 1:20 in dry toluene. The slides coated with silica particles were then immersed in the trichlorosilane solution and allowed to react overnight under nitrogen. The slides were then

rinsed with dry toluene and allowed to dry in an oven at 120 °C for 2 h. A dexoygenated solution containing acrylamide, CuCl, CuCl₂ and Tris (2-dimethylaminoethyl) amine in N,N-dimethylformamide (DMF) was prepared as described earlier [15]. The silane-modified slides were then reacted in this solution under an inert atmosphere of pure argon for 3 h. (CAUTION: acrylamide is a neurotoxin. Handle with care and avoid breathing the powder or contact of the solution with skin.) This modification method gives dense growth of a low-polydispersity chains from a surface [16-18], so called brush layer polyacrylamide, which was characterized in our previous work [15]. The modified slides were then rinsed with DMF, water and ethanol, and kept dry in a vacuum desiccator. Scanning Electron Microscopy (SEM) images of the slides were obtained using a JCM-5000 NeoScope benchtop SEM (JEOL USA, Inc., Peabody, MA).

2.3. UTLC separation of protein mixture

UTLC plates were cut into 1" squares. For protein separations, a mixture of 0.05 mg/mL myoglobin, 0.04 mg/mL cytochrome c and 0.07 mg/mL lysozyme, all of which were labeled with Alexa Fluor 546, was spotted onto a UTLC plate with a microarray pin (Stealth microarray pin, Arrayit, Sunnyvale) with a delivery volume of 5.1 nL and spot diameter of 800 μm. The small delivery volume and small spot size was chosen to reduce the contribution of injection to plate height. The developing chamber was conditioned with the mobile phase for 30 min before the UTLC plate was placed inside. After development, the UTLC plate was dried briefly in air. An upright optical microscope equipped with a Hg lamp (Eclipse 80i, Nikon Instruments Inc. Melville, NY) and a CCD camera (Cascade II: 512, Photometrics, Tucson, AZ) was used for fluorescence detection. Fluorescence of labeled proteins was excited and then filtered using filters designed for the respective dyes (QMAX-Fred for Alexa Fluor 647, QMAX-Green for Alexa Fluor 488, and QMAX-Yellow for Alexa Fluor 546, Omega Optical, Brattleboro, VT). The emission light was collected through a 2× objective and a 0.5× coupler (CFI Plan Apo 2X, Nikon Instruments Inc., Melville, NY) for the CCD camera. Winview software (Version 2.5.22.0, Princeton Instruments, Trenton, NJ) was used for data acquisition. Origin® was used to fit the curves to Gaussians.

2.4. On-plate MALDI-MS analysis for protein separation on UTLC

UTLC procedure was modified when coupled with MALDI-MS analysis afterwards. UTLC plates were made from silicon wafers rather than glass slides to impart conductivity. A higher concentration mixture of unlabeled cytochrome c, myoglobin and lysozyme (1 mg/mL for each of the protein) was prepared together with the same proteins labeled with Alexa Fluor 546. The labeled proteins were in the same concentrations as previously described, 0.04 mg/mL for cytochrome c, 0.05 mg/mL for myoglobin and 0.07 for mg/mL lysozyme, and used as marker to locate the unlabeled proteins for MALDI-MS analysis after the development. A MicroCaster Arrayer pin (Whatman, Piscataway, NJ) was used to deliver the protein solution by a larger volume (20-70 nL) to facilitate viewing during MALDI-MS analysis. The mobile phase used was the same as optimized compositions, 85% MeOH with 0.5% TFA, but saturated with sinapinic acid. The solvent development was repeated after the separation to increase the amount of sinapinic acid crystals.

On-plate MALDI-MS analysis was carried out by a Voyager DE-Pro MALDI-TOF instrument (Life Technologies, Carlsbad, California). For acquisition of the mass spectra, 100 laser shots were typically applied at the region of the fluorescently labeled proteins. The silicon wafer was put into a milled out region of a stainless steel MALDI plate (AB Sciex, Foster City, California) so that its surface was flush with the stainless steel surface, and carbon tape was used to secure the UTLC plate in the inset.

3. Results and discussion

Fig. 1A shows a photograph of a typical 1"×1" slide coated with the submicrometer silica particles. These slides are opaque due to the high scattering of randomly packed spheres in a layer that is 15 μm thick. A typical SEM image is shown in Fig. 1B, confirming that the packing of silica particles is disordered, in contrast to our earlier work that shows (111) or (100) crystalline packing [19].

The polyacrylamide stationary phase has not previously been used for retention of proteins, therefore, the first step in evaluating the new separation medium is to optimize the mobile phase. Five mobile phase mixtures were studied: 70, 80, 85, 90, and 100% (v/v) MeOH in water, each with 0.5% (v/v) TFA. A UTLC slide with a 900 nm particle size was used. In each separation, the protein was identified from its R_f value, which was determined independently by spotting individual proteins. When optimizing mobile phase composition, in the cases where the proteins overlapped, proteins labeled with different dyes were used: Alexa Fluor 488 labeled myoglobin, Alexa Fluor 546 labeled cytochrome c and Alexa Fluor 647 labeled lysozyme. In the case where proteins were resolved, the same Alexa Fluor dye was used for the convenience of imaging with one filter set. The fluorescence images of the UTLC slides are shown in Fig. 2 for each mobile phase composition. Replicate measurements showed that, although the traveling distance of a given protein varies 20% from run-to-run, the relative positions of the proteins remain consistent. The images in Fig. 2 are all on the same size scale, and in each case, the solvent front was allowed to move the same distance, 19 mm, thus facilitating comparisons. The results show that the proteins advanced further with more water in the mobile phase, which is expected because polyacrylamide is very hydrophilic. The chromatographic behavior shows a clear optimum at 85% methanol, with the optimized resolution for a fixed solvent front. For methanol percentages below the optimum, i.e., more water, there is less retention of all three proteins, and they co-migrate in 70% methanol. For methanol percentages above the optimum, i.e., less water, there is more retention of all three proteins, and they do not migrate at all in 100% methanol. This behavior is consistent with the mechanism of hydrophilic interaction chromatography (HILIC) [20,21]: the stationary phase has adsorbed water, therefore, the greater the water percentage in the mobile phase, the less the retention. HILIC has previously been demonstrated using polyacrylamide in a monolithic capillary column for small molecules by Ikegami [22,23]. A stationary phase containing a block co-polymer with polyacrylamide as a component was used to study the glycopeptides [24]. As for proteins, a commercial polyamide column was used for the separation of protein acetylated isoforms [25], but this is the first report of using polyacrylamide for HILIC separations of proteins. The results also suggest that there is no significant sieving because there is no separation under strong eluting conditions: at 70% MeOH: all of the proteins moved the same distance. This is not surprising since the interstitial spaces between particles are on the scale of hundreds of nm, as is evident in the SEM image of Fig. 1B, is much larger than the protein diameters, which are on the scale of a few nm. The separations achieved are thus attributed to the HILIC mechanism rather than sieving.

Fig. 3 gives a plot of the chromatogram for the optimal mobile phase composition, which allows exploration of the separation performance in more details. The intensity data from the fluorescence image of Fig. 2C are used to plot the chromatogram, which is for the optimal case of the 85% MeOH with 0.5% TFA. The fluorescence image is aligned under the intensity data for reference, illustrating that image tends to exaggerate resolution. The peaks in the chromatogram are fit to a sum of Gaussians to characterize the efficiency and resolution. Myoglobin moved the furthest among the three proteins, reaching a distance of 14 mm, followed by cytochrome c and then lysozyme, whereas the solvent front moved to 19 mm in 10 min. Near baseline resolution of three proteins is shown in the chromatogram,

representing the first time that has resolved a mixture of three proteins by UTLC. In addition, with Equation 1 (where σ was the standard deviation from the Gaussian fit and L is the distance that the protein spot moved),

$$H = \sigma^2 / L \quad (1)$$

a plate height as low as 3 μm for myoglobin is determined from the Gaussian fit, compared to the UHPLC separation of proteins, which is in the range of 6-8 μm [26,27]. The injection width from the Arrayit pin is included in the peak width for the plate height calculation. The commercial protein samples used here are themselves mixtures, so the plate height is an upper limit. The plate heights for lysozyme and cytochrome c are more difficult to discern due to multiple impurities, and their upper limits from the Gaussian fit are shown to be 7 μm . For all of the proteins, thousands of plates were observed for distances of no more than 14 mm. The lowest plate height, 3 μm for myoglobin, represents remarkable efficiency for UTLC of proteins.

The possibility of obtaining better resolution by using even smaller particles was tested by comparing protein separations for particle diameters of 900 nm, 700 nm and 350 nm. The same protein mixture (myoglobin, cytochrome c and lysozyme) was separated on each of the UTLC slides of different particle sizes under the same optimal condition, 85% MeOH with 0.5% TFA. The results are shown in Fig. 4A. On all three UTLC slides, the migration distance for the solvent was kept the same: 19 mm. The proteins moved further with larger particle size. Since the draw-down coater deposits a constant thickness of silica, the total volume of the solvent is the same for different particle diameters. But the surface area increased for smaller particles (k is the same value for unit area), thus retention increases. This possibility can be tested quantitatively. Table 1 lists the R_f values for each of the proteins and particle sizes, along with the corresponding value of retention factor, k , calculated from the relation in Equation 2.

$$k = \frac{1}{R_f} - 1 \quad (2)$$

The retention factor, by definition, is proportional to the ratio of surface area to interstitial volume, which is inversely proportional to d_p . Hence, a plot of k vs. $1/d_p$ should be linear with an intercept of zero because the extrapolation to the case of $d_p = \infty$ would give a negligible surface area compared to these submicrometer particles. Fig. 4B shows such a plot, and the dependence is indeed linear with an intercept of zero. The results support the notion that the increased migration distance with increasing particle diameter is due to decreasing surface to volume ratio.

To determine whether smaller particles give better resolution, chromatograms for the data from each image of Fig. 4a are plotted in Fig. 5. According to the manufacture of Arrayit pin, the variance of the spotting diameter is about 0.6%. Therefore, the injection variation should not prevent a comparison of plate heights for different particle sizes. Comparing the first two peaks in each chromatogram, lysozyme and cytochrome c, the resolution, which is calculated from equation 3 (where W is the baseline width of the Gaussian peak, D is the distance between two protein peaks), increased from 1.2 to 1.3 to 1.4 for 900 to 700 to 350 nm particle sizes, respectively. The dashed line on the figure shows visually that the resolution between the peaks for lysozyme and cytochrome c increases with decreasing particle size.

$$R_s = \frac{2D}{W_1 + W_2} \quad (3)$$

One can also see from the figure that a reason for better resolution is the increased retention: the spacing between myoglobin and lysozyme increases with smaller particle size despite the fact that the overall distance migrated is decreasing. The increased resolution is apparently due to increased surface to volume ratio. Surface to volume ratio appears in the equation for resolution as the term $k_2/(1+k_2)$ in Eq. 4.

$$R_s = \left(\frac{\sqrt{N}}{4} \right) \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k_2}{1 + k_2} \right) \quad (4)$$

Table 2 lists the contribution of each term in Eq. 4 to the resolution, showing that the efficiency decreases with particle size, but this decrease is more than offset by their higher retention to give slightly higher resolution for the smaller particles. One usually expects higher efficiencies with smaller particles. A possible reason for the lower efficiency of the smaller particles is that these are packed less uniformly. It is also possible that they are just as efficient, but that their higher retention gives rise to peak broadening by separating, but not resolving, impurities. Evidence of impurities for Alexa Fluor 546 labeled lysozyme and cytochrome c was demonstrated in our previous work [28]. Other possible reasons for the non-Gaussian peak shape are mixed-mode separation, irregular packing and scattering of the fluorescence. In all, we can report a slight gain in resolution with smaller particle sizes. The separation time was longer for the smaller particles: from 900 nm, 700 nm to 350 nm, the developing times are about 10 min, 12 min and 15 min, respectively. The results thus show that the resolution per time is not significantly different among the particle sizes.

UTLC of proteins would be most useful if no labels were required, and especially if the separation were readily coupled to mass spectrometry to identify proteins. To investigate whether MALDI could be used for detection and identification, single-side polished silicon wafers were used as substrates for the UTLC plates to give good conductivity. Saturated sinapinic acid was added to the optimal mobile phase, and comparison experiments showed that there were no differences in UTLC performance. The saturated sinapinic acid only slightly increased the retention factor for each protein. Using a MicroCaster microarray pin, unlabeled proteins containing 2.6 pmol of myoglobin, 3.1 pmol of lysozyme and 3.7 pmol of cytochrome c were spotted along with tracers of the fluorescence-labeled proteins to allow for visual confirmation of spot positions. The fluorescence-labeled proteins were at trace in concentration to serve as markers for the peak locations, thus their intensities were in the noise range. Weak MALDI signals were detected for each protein. It was found that the signal intensity was significantly improved if the plate was redeveloped by first allowing the plate to dry and then allowing fresh mobile phase to wick into the plate to deliver more sinapinic acid. The height at which the sinapinic acid solution was wicked was just above the myoglobin, and this only slightly advanced the protein positions. After redevelopment, very tiny sinapinic acid crystals ($\sim 5 \mu\text{m}$) were observed on the surface of the plate, as shown in the SEM images of Fig. 6. MALDI for each protein was carried out at the region marked by the fluorescently labeled proteins under a microscope, and the mass spectrum for the center of each spot is shown in Fig. 7. The mass spectrum in each case agrees with the expected molecular weight for each protein. Peaks for both $z=1$ and $z=2$ are observed in each case. Each mass spectrum has a peak only for that protein, consistent with the near baseline resolution of the separation. No change in peak shape was observed for the

fluorescently labeled proteins in the TLC run, therefore, it is unlikely that the proteins were overloaded. The results represent the first time that UTLC has resolved three proteins.

4. Conclusions

This study demonstrates that high resolution can be achieved on the sub-micron silica nanoparticles coated with polyacrylamide for the separation of proteins in ultra-performance TLC, and that this material is compatible with MALDI-MS detection. It is also one of the very few reports on proteins being separated by a hydrophilic interaction mechanism. Separation efficiencies with varying particle sizes show that smaller particles are not significantly advantageous. UTLC of proteins could be useful as a companion of UHPLC for high-throughput methods development separation, as well as for rapid assessment in quality control of manufacturing.

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Fig. 1. (A) Photograph of a 1''x1'' UTLC slide, in this case a silicon wafer coated with a 15 μm film of 900 nm particles, illustrating its size relative to a coin. (B) Typical SEM image of a UTLC slide, in this case with a coating with 900 nm silica nanoparticles.

Fig. 2.

Fluorescence images of 3 proteins, myoglobin, cytochrome c and lysozyme, after separation using different mobile phase compositions: (A) Images of the same strip with detection at three different wavelengths after using a mobile phase of 70% MeOH; (B) same as A but 80% MeOH; (C) Images for one detection wavelength after using 85% MeOH; (D) Same as D but 90% MeOH; and (E) Images at three different wavelengths after using a mobile phase of 100% MeOH. All solvent mixtures were also with 0.5% TFA. Myo* represents myoglobin labeled with Alexa Fluor 488, and lyz* stands for lysozyme labeled with Alexa Fluor 647. All the other proteins were labeled with Alexa Fluor 546 if no specific notation was made. All UTLC slides were coated with 900 nm silica particles and also in the same dimensions, and solvent front moved to the same distance on each slide.

Fig. 3.

A. Plot of image intensities to give the chromatogram for a mobile phase of 85% MeOH/ 0.5% TFA (circles), and fit of the chromatogram to a sum of Gaussians (—). B. Overlay of image on the same distance scale.

Fig. 4. Separations of proteins on three UTLC slides coated with 900 nm, 700 nm and 350 nm silica particles, respectively. (A) Fluorescence images of the slide showing the protein separations. The three UTLC slides were the same dimensions and the solvent front moved to the same distance on each slide. (B) Using the data in part A, a plot of the retention factor vs. inverse particle diameter (circles), and a line forced through the origin and the data (—).

Fig. 5.

Plots of intensities to give chromatograms from the images of Fig. 4. The circles are the experimental data and the solid red curves are the best fits to sums of Gaussians. The dashed line is a guide to show that resolution increases from left to right, and the arrows are a guide to show that peak spacing increases from left to right.

Fig. 6. SEM of UTLC slide after 2nd solvent development, showing sinapinic acid crystals, with a small region shown on an expanded scale.

Fig. 7. MALDI mass spectra obtained from the spots of each of the three proteins after UTLC separation: (A) myoglobin, (B) cytochrome c and (C) lysozyme.

Table 1
Summary for each particle size of R_f and k for each of the proteins

d_p	R_f			k		
	myo	cyt	lyz	myo	cyt	lyz
900	0.74	0.57	0.51	0.35	0.75	0.98
700	0.67	0.45	0.37	0.49	1.2	1.7
350	0.53	0.32	0.24	0.89	2.1	3.1

Table 2
Summary for contributions of each terms, N , α and k_2 (for lysozyme) to resolutions between cytochrome c and lysozyme

d_p (nm)	N	$(\alpha-1)/\alpha$	$k_2/(k_2+1)$	R_s
900	1650	0.235	0.495	1.2
700	738	0.294	0.630	1.3
350	505	0.323	0.756	1.4