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Development of Nanomaterial Supports for the Study of Affinity-Based Analytes Using Ultra-Thin Layer Chromatography

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DEVELOPMENT OF NANOMATERIAL SUPPORTS FOR THE STUDY OF AFFINITY-BASED ANALYTES USING ULTRA-THIN LAYER CHROMATOGRAPHY

By

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DEVELOPMENT OF NANOMATERIAL SUPPORTS FOR THE STUDY OF AFFINITY-BASED ANALYTES USING ULTRA-THIN LAYER CHROMATOGRAPHY

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Ultra-thin layer chromatography (UTLC) is a growing field in analytical separations. UTLC is a branch of planar and liquid chromatography that is related to thin layer chromatography. The main advantage of UTLC compared to other techniques is it uses much less material, allowing for faster and more sensitive separations to take place. The UTLC devices fabricated in this project used either silicon oxide or silicon nanopillars deposited on a glass slide using glancing angle deposition (GLAD). Even a thin layer of these nanopillars deposited on a glass slide provide a large surface area for the analyte to be separated. GLAD is a physical vapor deposition technique that allows, in this case, silicon oxide or silicon to be vaporized by an ion source and deposited in slanted pillar structures onto a glass substrate.

The overall goal of this thesis is to develop and optimize a nanomaterial support/stationary phase for a UTLC device that can be utilized for affinity chromatography. The studies performed in this thesis provide proof-of-concept that SiO_2 nanopillars can perform efficient separations and that protein can also be immobilized onto the surface of the nanopillars. With further studies, protein immobilization can be fully optimized and affinity separations performed on these UTLC devices.

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CHAPTER 1: GENERAL INFORMATION

1.1 ULTRA-THIN LAYER CHROMATOGRAPHY

Although it takes its roots from some of the earliest methods in chromatography, ultra-thin layer chromatography (UTLC) has seen much interest and development in recent years. UTLC, in its most basic form, is simply planar chromatography. Simple planar chromatography, such as thin layer chromatography (TLC), has been used as a preparative technique for thousands of years and has been commonly used for analytical separations for the past hundred years.[1,2] Advantages of using planar chromatography, as compared to column chromatography, are its simplicity, cost-efficiency, and compatibility with most off-line detection methods on the market.[3,4] Therefore, the further development of UTLC is advantageous to future applications of chromatography.

To fully understand UTLC, the properties of planar chromatography and how it works must be explored. The basic setup of any planar chromatographic system is given in Figure 1.1. In planar chromatography, the interaction between the mobile phase (or solvent) and the analyte (or solute) is described by the retardation factor (R_f). The retardation factor is similar to the retention factor in column chromatography and is a unitless measurement of retention. The value of R_f always falls between 0 to 1 and is given in equation (1.1).

$$R_f = \frac{d_a}{d_{mp}} \tag{1.1}$$

In this equation, d_a is the distance traveled by the analyte in a given amount of time, and d_{mp} is the distance traveled by the mobile phase or solvent front in the same amount of

time, with both measurements beginning at the initial spotting point.[4,5] Although the value of the retardation factor does not give any definitive qualitative information that can be used to describe the efficiency of planar separations, this value does help with identifying analytes if their separation is taking place under identical experimental conditions to those used to obtain a reference value.

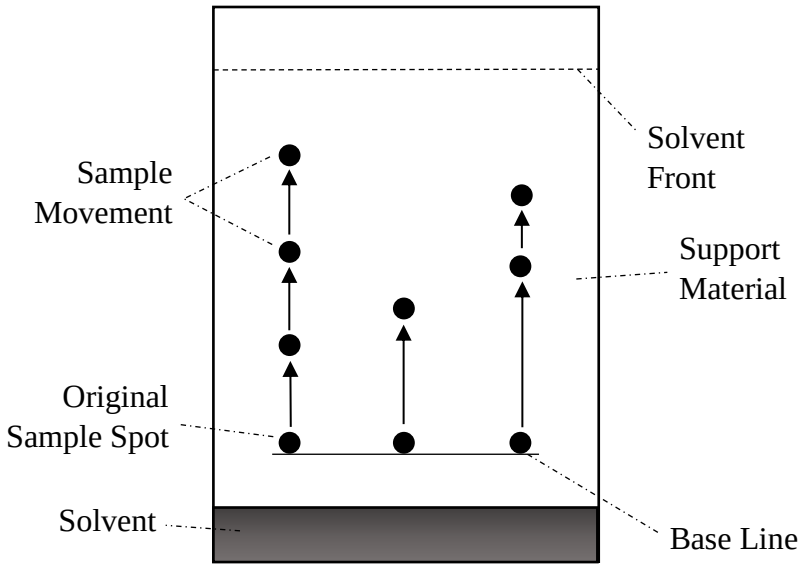

Figure 1.1. The theory of planar chromatography. A sample is applied in a spot, and the mobile phase is wicked onto the system via capillary forces. The sample will separate into its components based on how strongly each analyte interacts with the stationary phase. The retardation factor of an analyte is measured in relation to the base line, or point of sample application, on the support material.

Other parameters that can help describe separation efficiency in planar chromatography are the plate height (H) and the plate number (n), which are given by equations (1.2) and (1.3). As the plate number increases and the plate height decreases, the overall efficiency of the system improves and results in sharper peaks or bands.

$$n = 16\left(\frac{d_a}{W_{b,a}}\right)^2 \tag{1.2}$$

$$H = \frac{d_a}{n} = \frac{{W_{b,a}}^2}{16 d_a} \tag{1.3}$$

In these equations, d_a is the distance traveled by the analyte from the initial spotting point and $W_{b,a}$ is the baseline width of the analyte peak.[1]

Another way of describing the plate height in planar chromatography is by using a modified form of the van Deemter equation, known as the Knox equation, as shown in equation (1.4).[6]

$$H = A\sqrt[3]{u} + \frac{B}{u} + Cu \tag{1.4}$$

The A term in the equation corresponds to how eddy diffusion affects the separation. The effect of A can be decreased by using smaller particles for the support and making sure it is well packed. The B term corresponds to how longitudinal diffusion affects the separation. The effect of the B term on the separation can be decreased by increasing the flow rate, which allows the analyte to pass through the system quicker, leading to less longitudinal diffusion. The C term corresponds to how the resistance to mass transfer affects the separation. The effect of the C term can be decreased by minimizing the time

it takes for partitioning to occur for a solute between the mobile phase and stationary phase.[2,7]

Like TLC and high-performance TLC (HPTLC), the separations that occur on an UTLC plate can be characterized by these various parameters. UTLC was formally introduced in 2002 in studies by Hauck and Schultz.[3,8,9] UTLC is unique compared to other types of planar chromatography because of its support size, with monolithic supports of around 10 µm serving as the stationary phase/support. Separations also occur within distances of 1-3 cm in UTLC, compared to a much longer separation distance in TLC and HPTLC. Another novel feature of UTLC is that the stationary phase/support can be directly bonded onto the silicon or a glass slide, which acts as the substrate.[9] The use of a monolith allows a smaller amount of stationary phase/support to be used because of the complexity of the monolith. The monoliths are typically made up of two different sized pores: macropores, which measure greater than 50 nm, and mesopores, which measure between 2-50 nm. The small pore sizes allow for better separation in the short distances used by UTLC. Another advantage of utilizing UTLC plates is that the volume of material that is used (i.e. both the solute and solvent) is significantly less than other planar chromatographic systems.[5] The analyte can be applied in nanoliter quantities and most of the time only a few milliliters of solvent is needed to perform the separation. The application of such small quantities of liquid will be discussed in a later chapter.

There have been several recent studies that have shown how UTLC can be used in diverse applications. Most commonly, UTLC is incorporated into lab-on-a-chip devices and paired with an offline detector to provide rapid, portable screening devices.[10–12] The most frequently used offline detectors are based on surface enhanced Raman

spectroscopy. This pairing has been used in the analysis of a variety of analytes, including organic and biochemical mixtures. These separations can also be enhanced by incorporating silver nanoparticles into the stationary phase/support to provide for even more efficient separations.[13,14] Nanoparticles and other support ligands will be discussed in a later section of this chapter.

1.2 AFFINITY CHROMATOGRAPHY

Affinity chromatography is a chromatographic technique that utilizes biologically-related compounds as a stationary phase to perform selective separations.[15–20] A simple diagram of the separation process in affinity chromatography is shown in Figure 1.2. In affinity chromatography, a binding agent (or "affinity ligand") is first immobilized onto a support. A solution is then applied to the system and the desired analyte is allowed to bind to the affinity ligand while non-retained sample components are eluted from the system. The desired analyte is then eluted off the column by changing the system conditions, which is most commonly achieved by changing the composition of the mobile phase. By returning the system to the original conditions, the affinity ligand is then allowed to regenerate for future use. This format gives good reproducibility throughout the experiments and also lowers the cost of the overall method by reusing the binding agent.[15–17]

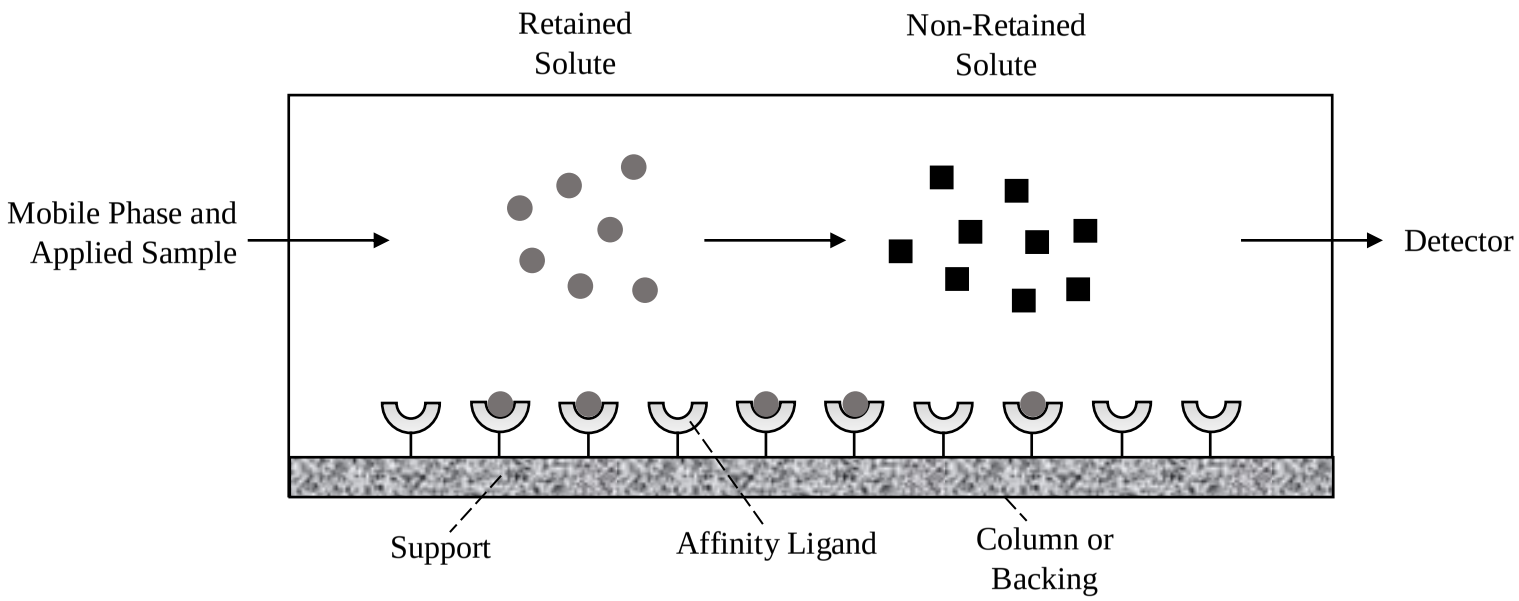

Figure 2.2. General scheme for using an immobilized binding agent, or affinity ligand, in an affinity chromatography system for the separation and recognition of a target analyte from other, non-retained sample components.

Valuable information about the analyte can be gained by using affinity chromatography, such as its retention factor (k), association constant (K_a), and dissociation constant (K_d). All of these values can be calculated by knowing the concentration of the analyte and of the solution, the amount of mobile phase that has been applied, and time the analyte has eluted off the column. The retention factor, association constant, and dissociation constant are described by equations (1.5-1.7).

$$k = \frac{t_r - t_m}{t_m} \tag{1.5}$$

$$K_a = \frac{[A-L]}{[A][L]} \tag{1.6}$$

$$K_d = \frac{1}{K_a} \tag{1.7}$$

In these equations, t_r and t_m are the retention time of the analyte and the column void time, respectively; [A] is the concentration of the analyte in the applied sample solution, and [L] is the concentration of the affinity ligand that is immobilized onto the support of the system.[17]

Although traditionally performed in the form of column chromatography, affinity chromatography can also be applied to UTLC under the right conditions.[21–23] The retention factor that is used in column chromatography will be replaced in this case by the retardation factor, as given earlier in equation (1.1). One setback in applying the principles of affinity chromatography to UTLC is that the support/stationary phase of the UTLC system should be compatible with the affinity ligand. This is also a concern with column chromatography, but UTLC tends to employ a greater variety of supports that

would not be a good host for biological ligands. For instance, silica with a hydrophilic layer is often used as a support in high performance affinity chromatography (HPAC); this means silica supports must be designed on a micro-scale for use in a comparable separation method in UTLC.[14] Some of the nanomaterials that are being used as supports in UTLC will be discussed in the next section.

1.3 NANOMATERIALS AS CHROMATOGRAPHIC SUPPORTS

Using nanomaterials as a chromatographic support is a growing field of study. Nanomaterials have several advantages compared to other chromatographic support including their ability to sometimes provide greater sensitivity, higher binding capacity, and higher enrichment efficiency. Another large advantage of using a nanomaterial as a chromatographic support is that it is highly customizable, which allows for greater selectivity in the system.[10,13,14,24,25] This also makes nanomaterials of interest as possible supports for affinity chromatography. Nanomaterials have a higher surface-to-volume ratio than traditional particle-based supports.[10,13,14] For this reason, nanomaterials are the most commonly used chromatographic support in UTLC because they can provide good separation efficiencies on a small scale.

Nanomaterials are becoming more common in affinity chromatography due to their ease of customizability. This has been particularly true in two sub-branches of affinity chromatography that have used metal nanoparticles: immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC). Metal-based nanoparticle hybrid supports have been used in several studies and have been shown to provide efficient separations of biological mixtures.[21–23,26,27] Organic-based

nanomaterials are also a viable option when performing affinity chromatographic studies. In addition, carbon nanotubes have been explored for several years as a support for biological separations.[10,12]

The most common nanomaterial supports that have been used in UTLC are inorganic polymers. The most common of these nanomaterials in UTLC are silica nanoparticles, which have been either electrospun or directly deposited onto the base of the device (i.e. a glass slide or silicon).[28,29] Vapor deposition of nanoparticles will be discussed in a later chapter. There have also been several studies performed with polymer nanofibers that have been deposited on a device by using electrospinning.[30] To enhance the separations, metal nanoparticles can also coat the support. Several studies have been performed using a thin coating of Ag, Au, Zn, or Ti on top of a base nanomaterial support. This extra coating allows the sample to be more easily detected by offline detectors or allows for more specific analytes to be targeted, as in affinity chromatography.[10,11,21–23,26,27]

1.4 OVERALL GOAL AND SUMMARY OF WORK

The overall goal of this thesis is to develop and characterize nanomaterial supports for ultra-thin layer chromatography, with possible applications in affinity chromatography. A method has already been developed for depositing a SiO_2 nanomaterials onto a UTLC device, but affinity separations had not been tested in prior work with this type of substrate. The goal of this study is to develop a separation method on a UTLC device that may allow bioaffinity separations to be performed.

Chapter 2 will focus on the separation of a lipophilic dye mixture on a SiO_2 nanopillar stationary support that is present on a UTLC device. This study will discuss

the development of a method to run samples on the UTLC device effectively and to provide quantitative data on the separation. This study will also be used to determine the optimum conditions for such separations to occur. **Chapter 3** will focus on the process of immobilizing human serum albumin (HSA) onto the SiO_2 nanopillar supports. This study will examine the optimization of an immobilization method and quantification of protein that has been immobilized onto the nanopillar supports. **Chapter 4** will give an overview of the work presented in this thesis and will discuss the future directions for this project. One such future direction would be to perform UTLC studies of a lipophilic dye mixture on HSA that has been immobilized onto SiO_2 nanopillar supports.

1.5 REFERENCES

(1) Siouffi, A. M.; Bressolle, F.; Guiochon, G. Optimization in Thin-Layer Chromatography: Some Practical Considerations. *J. Chromatogr.* **1981**, *209*, 129–147.

(2) Siouffi, A. From Paper to Planar : 60 Years of Thin Layer Chromatography. *Sep. Purif. Rev.* **2005**, *34*, 155–180.

(3) Hauck, H. E.; Schulz, M. Ultrathin-Layer Chromatography. *J. Chromatogr. Sci.* **2002**, *40*, 1–3.

(4) Poole, C. F. Thin-Layer Chromatography : Challenges and Opportunities. *J. Chromatogr. A* **2003**, *1000*, 963–984.

(5) Poole, S. K.; Poole, C. F. High Performance Stationary Phases for Planar Chromatography. *J. Chromatogr. A* **2011**, *1218*, 2648–2660.

(6) Berthod, A. On the Use of the Knox Equation. II. The Efficiency Measurement Problem. *J. Liq. Chromatogr.* **1989**, *12*, 1187–1201.

(7) van Deemter, J. J.; Zuiderweg, F. J.; Klinkenberg, A. Longitudinal Diffusion and Resistance to Mass Transfer as Causes of Nonideality in Chromatography. *Chem. Eng. Sci.* **1956**, *5*, 271–289.

(8) Hauck, H. E.; Bund, O.; Fischer, W.; Shulz, M. Ultra-Thin Layer Chromatography (UTLC)- a New Dimension in Thin Layer Chromatography. *J. Planar Chromatogr.* **2001**, *14*, 234–236.

(9) Hauck, H. .; Schulz, M. Ultra Thin-Layer Chromatography. *Chromatographia* **2003**, *57*, 313–315.

(10) Beeram, S.; Rodriguez, E.; Doddavenkatanna, S.; Li, Z.; Pekarek, A.; Peev, D.; Goerl, K.; Trovato, G.; Hofmann, T.; Hage, D. S. Nanomaterials as Stationary Phases and Supports in Liquid Chromatography. *Electrophoresis* **2017**, *38*, 2498–2512.

(11) Dai, J.; Wang, M.; Liu, H. Highly Selective Enrichment of Phosphopeptides Using Zr^{4+}-Immobilized Titania Nanoparticles. *Talanta* **2017**, *164*, 222–227.

(12) Bogdanski, A.; Wistuba, D.; Larsen, K. L.; Hartnagel, U.; Hirsch, A.; Schurig, V. Reciprocal Principle of Molecular Recognition in Supramolecular Chromatography — Highly Selective Analytical Separation of Cyclodextrin Congeners on a Silica-Bonded [60] Fullerene Stationary Phase. *New J. Chem.* **2010**, *34*, 693–698.

(13) Guihen, E. Nanoparticles in Modern Separation Science. *Trends Anal. Chem.* **2013**, *46*, 1–14.

(14) Guihen, E.; Glennon, J. D. Nanoparticles in Separation Science — Recent Developments. *Anal. Lett.* **2003**, *36* (15), 3309–3336.

(15) Hage, D. S. *Handbook of Affinity Chromatography*, 2nd ed.; CRC Press: Boca Raton, 2006.

(16) Hage, D. S.; Matsuda, R. Affinity Chromatography : A Historical Perspective. In *Affinity Chromatography: Methods and Protocols*; Reichelt, S., Ed.; Springer Science and Business Media: New York, 2015; Vol. 1286, pp 1–19.

(17) Pfaunmiller, E. L.; Bas, J.; Brooks, M.; Milanuk, M.; Rodriguez, E.; Vargas, J.; Matsuda, R.; Hage, D. S. Affinity Chromatography. *Anal. Sep. Sci.* **2015**, *2*, 461–482.

(18) Rowe, L.; Khoury, G. El; Lowe, C. R. Affinity Chromatography : Historical and Prospective Overview. In *Biopharmaceutical Production Technology*; Subramanian, G., Ed.; Wiley-VCH: Hoboken, NJ, 2012; pp 223–282.

(19) Yoo, M. J.; Hage, D. S. Evaluation of Silica Monoliths in Affinity Microcolumns for High-Throughput Analysis of Drug – Protein Interactions. *J. Sep. Sci.* **2009**, *32*, 2776–2785.

(20) Zheng, X.; Li, Z.; Beeram, S.; Podariu, M.; Matsuda, R.; Pfaunmiller, E. L.; Ii, C. J. W.; Carter, N.; Hage, D. S. Analysis of Biomolecular Interactions Using Affinity Microcolumns : A Review. *J. Chromatogr. B* **2014**, *968*, 49–63.

(21) Zou, X.; Jie, J.; Yang, B. Single-Step Enrichment of N - Glycopeptides and Phosphopeptides with Novel Multifunctional Ti^{4+} -Immobilized Dendritic Polyglycerol Coated Chitosan Nanomaterials. *Anal. Chem.* **2017**, *89*, 7520–7526.

(22) Yan, Y.; Sun, X.; Deng, C.; Li, Y.; Zhang, X. Metal Oxide Affinity Chromatography Platform − Polydopamine Coupled Functional Two-Dimensional Titania Graphene Nanohybrid for Phosphoproteome Research. *Anal. Chem.* **2014**, *86*, 4327–4332.

(23) Liu, H.; Yang, T.; Dai, J.; Zhu, J.; Li, X.; Wen, R.; Yang, X. Hydrophilic Modification of Titania Nanomaterials as a Biofunctional Adsorbent for Selective Enrichment of Phosphopeptides. *Analyst* **2015**, *140*, 6652–6659.

(24) Castillo-Garcia, M. L.; Aguilar-Caballos, M. P.; Gomez-Hens, A. Nanomaterials as Tools in Chromatographic Methods. *Trends Anal. Chem.* **2016**, *82*, 385–393.

(25) Murray, C. B.; Kagan, C. R. Synthesis and Characterization of Monodisperse Nanocrystals and Close-Packed Nanocrystal Assemblies. *Annu. Rev. Mater. Sci.* **2000**, *30*, 545–610.

(26) Liu, H.; Lian, B. A Guanidyl-Gunctionalized TiO_2 Nanoparticle-Anchored Graphene Nanohybrid for Capture of Phosphopeptides. *RSC Adv.* **2018**, *8*, 29476–29481.

(27) Wu, J.; Li, X.; Zhao, Y.; Zhang, W.; Guo, L.; Feng, Y. Application of Liquid Phase Deposited Titania Nanoparticles on Silica Spheres to Phosphopeptide Enrichment and High Performance Liquid Chromatography Packings. *J. Chromatogr. A* **2011**, *1218*, 2944–2953.

(28) Jim, S. R.; Foroughi-Abari, A.; Krause, K. M.; Li, P.; Kupsta, M.; Taschuk, M. T.; Cadien, K. C.; Brett, M. J. Ultrathin-Layer Chromatography Nanostructures Modified by Atomic Layer Deposition. *J. Chromatogr. A* **2013**, *1299*, 118–125.

(29) Peev, D.; Hofmann, T.; Kananizadeh, N.; Beeram, S.; Rodriguez, E.; Wimer, S.; Rodenhausen, K. B.; Kasputis, T.; Pfaunmiller, E.; Nguyen, A.; et al. Anisotropic Contrast Optical Microscope. *Rev. Sci. Instrum.* **2016**, *87*, 1–17.

(30) Newsome, T. E.; Olesik, S. V. Silica-Based Nanofibers for Electrospun Ultra-Thin Layer Chromatography. *J. Chromatogr. A* **2014**, *1364*, 261–270.

CHAPTER 2: SEPARATION OF LIPOPHILIC DYES UTILIZING ULTRA-THIN LAYER CHROMATOGRAPHY AND SiO_2 NANOPILLARS

2.1 INTRODUCTION

Lipophilic dyes are commonly used as tags to help aid in the several types of chromatographic studies. They can be used in their simplest form on a macroscopic scale to help track tagged compounds visually, or they can be used on an instrumental level as fluorescent tags.[1–6] Because of their structure, lipophilic dyes work well in systems which use a non-polar mobile phase.[4] The separation of lipophilic dyes on a SiO_2 nanopillar support/stationary phase is an important technique to master because lipophilic dyes can be employed as a model with this method for the chromatographic separation of biological compounds.

In the following experiments, the SiO_2 nanopillars are deposited onto a glass support using glancing angle deposition (GLAD). GLAD is a physical vapor deposition technique commonly used to deposit inorganic compounds onto a variety of substrates. GLAD takes place in a high-vacuum chamber, which allows for a controlled deposition of small pillar- or column-like structures. While depositions measuring into the micrometer range (most commonly < 10 μm) can be done, GLAD experiments tend to perform good, consistent pillars in the nanometer range (i.e., depositions of <100 nm are common).[7–11] A diagram of a GLAD unit is shown in Figure 2.1.

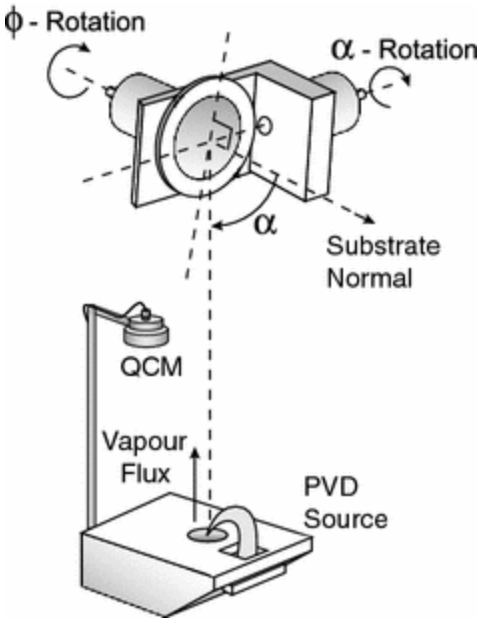

Figure 2.1. A diagram of a GLAD system. The vapor flux is deposited onto the substrate. The substrate angle can be changed to control the angle at which the deposition occurs on the substrate. This figure was reproduced with permission from [10].

GLAD operates on the principle that vapor deposition is occurring at oblique angles, which ensures that the vapor flux is not parallel to the substrate onto which it is deposited. This is what gives rise to column-like structures being deposited on the substrate. Based on the angle of the substrate during deposition, columns will form on the substrate at a desired angle to maximize the surface area of deposited pillars, as well as to determine the incident flux on the substrate. This oblique deposition, in principle, leads to the columns having anisotropic properties.[7,9]

The work in this chapter will focus on the development of a method to separate a mixture of lipophilic dyes on a SiO_2 nanopillar stationary phase on a UTLC device. Several challenges were faced during the process of trying to effectively separate the dye mixture, including optimizing the mobile phase and the concentration of the dye mixture. After optimizing these parameters, studies were performed and quantitative data were obtained from the experiments, including the retardation factor of each dye. This information could be determined visually and also confirmed the ability to use this type of material with anisotropic contrast optical microscopy (ACOM), an ellipsometric technique.[12]

2.2 EXPERIMENTAL SECTION

2.2.1 *Materials*

Dimethyl yellow indicator, Sudan Blue II (dye content 98%), hexane (for HPLC, purity > 97.0%), toluene (HPLC Plus, purity > 99.9%), 1-bromo-2-chloroethane (purity 98%), and isopropanol (HPLC grade, purity > 99.5%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 *Apparatus*

The following studies were performed using a syringe pump for dispensing a mobile phase through PEEK tubing at 0.5 µL/min. The mobile phase was comprised of a 2:1:1 mixture of toluene: hexane: 1-bromo-2-chloroethane. A Kim-wipe was cut into a strip of approximately 0.5 in × 3 in and used as a wick to transport mobile phase from the PEEK tubing onto the UTLC device. The dye mixture spot was deposited onto the UTLC plate using a GeSIM Nano-Plotter 2.1 (located in Dr. Rebecca Lai's lab in the UNL Department of chemistry) approximately 0.25 in away from the near edge of the plate, with the direction of the flux being in the same direction as the mobile phase flow. The UTLC device was contained in an evaporation dish, and once the system was set up, covered with a watchglass. A diagram of the apparatus setup is shown in Figure 2.2. Both the custom-built GLAD chamber and the Fiji200 (Cambridge Nanotech, Inc.) atomic layer deposition (ALD) systems that were used are located in the UNL Department of Electrical Engineering.

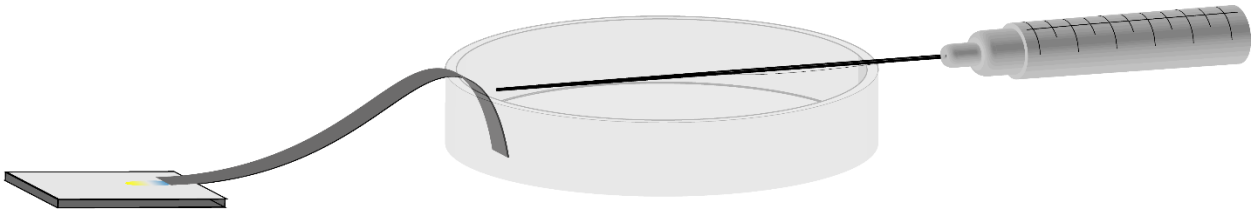

Figure 2.2. Setup used in this study for the UTLC separations. Every part of this diagram except the syringe pump is contained in a larger evaporating dish and covered with a watchglass during the separation.

2.2.3 *Preparation of UTLC plates*

The silica UTLC plate used in this study was prepared using both GLAD and atomic layer deposition (ALD). The glass substrate was cut into a 2 cm × 2 cm square, cleaned with a soap solution, rinsed with deionized water, and dried using nitrogen gas. It was then secured onto the sample holder using carbon tape and placed into a vacuum chamber; the pressure was decreased to a level of at least 10^{-9} mbar for the duration of the deposition process. The silica was deposited onto the glass substrate using a four pocket e-beam evaporation system at approximately 3 A°/s and at an 85° angle. The silica was grown to a height of 2.5 µm on the substrate. The nanopillars were then coated with a 4 nm layer of Al_2O_3 using an ALD system.

2.2.4 *Chromatographic studies*

Stock solutions of both the yellow and blue dyes were prepared at 150 mM concentrations, using toluene as the solvent. Equal parts of the yellow and blue dyes were combined to create a stock solution for the 150 mM green dye solution. More dilute solutions of green dye mixture were created with toluene for chromatographic testing, including 100 mM, 75 mM, and 50 mM solutions. These dye solutions could be used for studies performed within one week of the dye solution being made. After this point, toluene evaporation was visible in the storage vials, leading to a higher-than-desired concentration for the chromatographic studies.

A dye solution was spotted onto the UTLC plate using a nanoplotter. The dye solution could be pulled up into the nanoplotter tip and dispensed uniformly onto the surface of the UTLC plate without damaging the nanopillars. The nanoplotter produced

spots of approximately 400 to 700 pL, depending on the trial. Trials allowing for 10 droplet spots and 100 droplet spots were performed, giving an overall sample volume of approximately 5 to 50 nL in a dye spot. A picture of the size of the spot on the UTLC plate is shown in Figure 2.3.

The chromatographic studies were performed on the apparatus described and pictured in Figure 2.3. Videos were taken of the green dye solution during its separation and were referred to in the determination of quantitative measurements for each dye. Further confirmatory measurements were performed on the ACOM, which was built in the UNL Department of Electrical Engineering. A schematic of the ACOM device is provided in Figure 2.4. This technique relies heavily on anisotropic contrast to generate an image. Polarized light is generated and passes through the sample, where it then reaches a detector and is turned into an image of varying intensities using a Mueller matrices algorithm.[12]

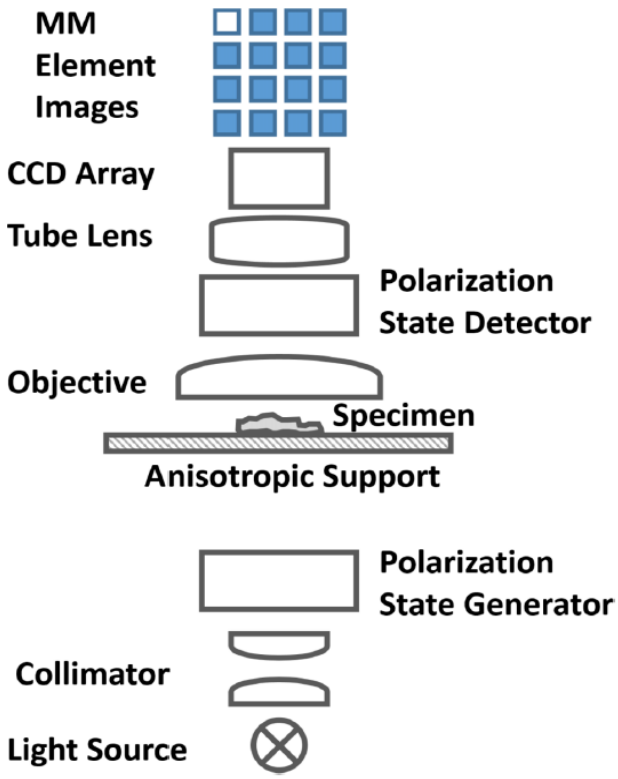

Figure 2.4. General design of the ACOM system that was used in this study. The UTLC plate was placed on top of the anisotropic support for analysis. This figure was reproduced with permission from [12].

2.3 RESULTS AND DICUSSION

2.3.1 *Determination of the mobile phase composition*

Approximately 2.5 µm tall SiO_2 nanopillars were prepared and coated with Al_2O_3, Chromatographic studies were then performed on the UTLC plate. One important step was determining the composition of mobile phase that would give the best separation for the dye mixtures. Factors that were considered during this process were the cost/availability of the mobile phase, its volatility, and its polarity. Reference tables with the characteristics of common organic solvents were consulted to help compare possible solvent combinations.[13]

Because the goal of this study was to separate a lipophilic dye mixture, a low polarity and mildly volatile solvent mixture was the best choice to ensure effective separations. Based on this criteria, toluene and hexane were chosen as the initial solvent combination to keep the mobile phase at an extremely low polarity. Different concentrations of toluene and hexane were explored, with the most positive results being seen when using an 8:7 toluene:hexane mixture. A picture of a typical separation that was observed when using an 8:7 toluene:hexane mixture as the mobile phase is shown in Figure 2.5.

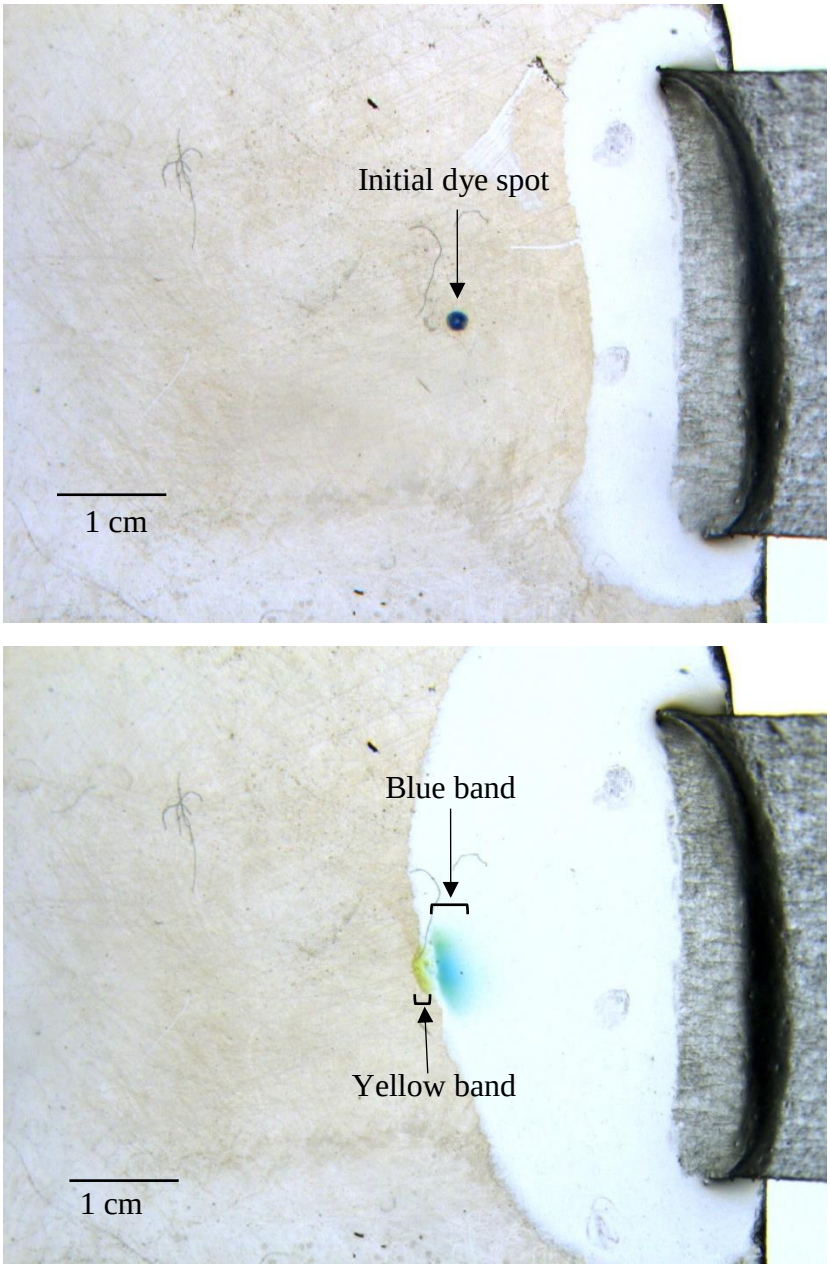

Figure 2.5. A representative picture of a separation in this study performed using an 8:7 toluene:hexane mobile phase. *(top)* Starting spot of the green dye at the beginning of the separation. *(bottom)* End of separation with the blue and yellow dye bands separated. Measurements were taken from the middle of the respective color band.

Although adequate separation was seen using this mixture, once the watchglass was removed from the system, the solvent began to evaporate quickly, and the blue dye component receded with the mobile phase. This was not ideal because the watch glass had to be taken off so that the plate could be transported to the Electrical Engineering Department for analysis on the ACOM. Because the experiment could not be run on the ACOM directly, another solvent combination was needed so that the blue dye component could be seen, both visually and through the ACOM, after the mobile phase had evaporated off the plate.

To help prevent this quick evaporation of the mobile phase once the watch glass had been taken off and the system was opened, a less volatile solvent was added to the mobile phase mixture to decrease the overall volatility of the mixture while still maintaining at least the same separation parameters. Using this logic, 1-bromo-2-chloroethane was chosen to add to the solvent mixture and different proportions of the three solvents were tested. The mixture that gave the best results was a 2:1:1 toluene:hexane:1-bromo-2-chloroethane. This mobile phase composition provided a better separation than the 8:7 toluene:hexane mixture and allowed the mobile phase to evaporate off the plate once the system was opened while still making the blue dye component visible after the separation was complete. A picture of a typical separation that was observed when using the 2:1:1 toluene:hexane:1-bromo-2-chloroethane as the mobile phase is shown in Figure 2.6.

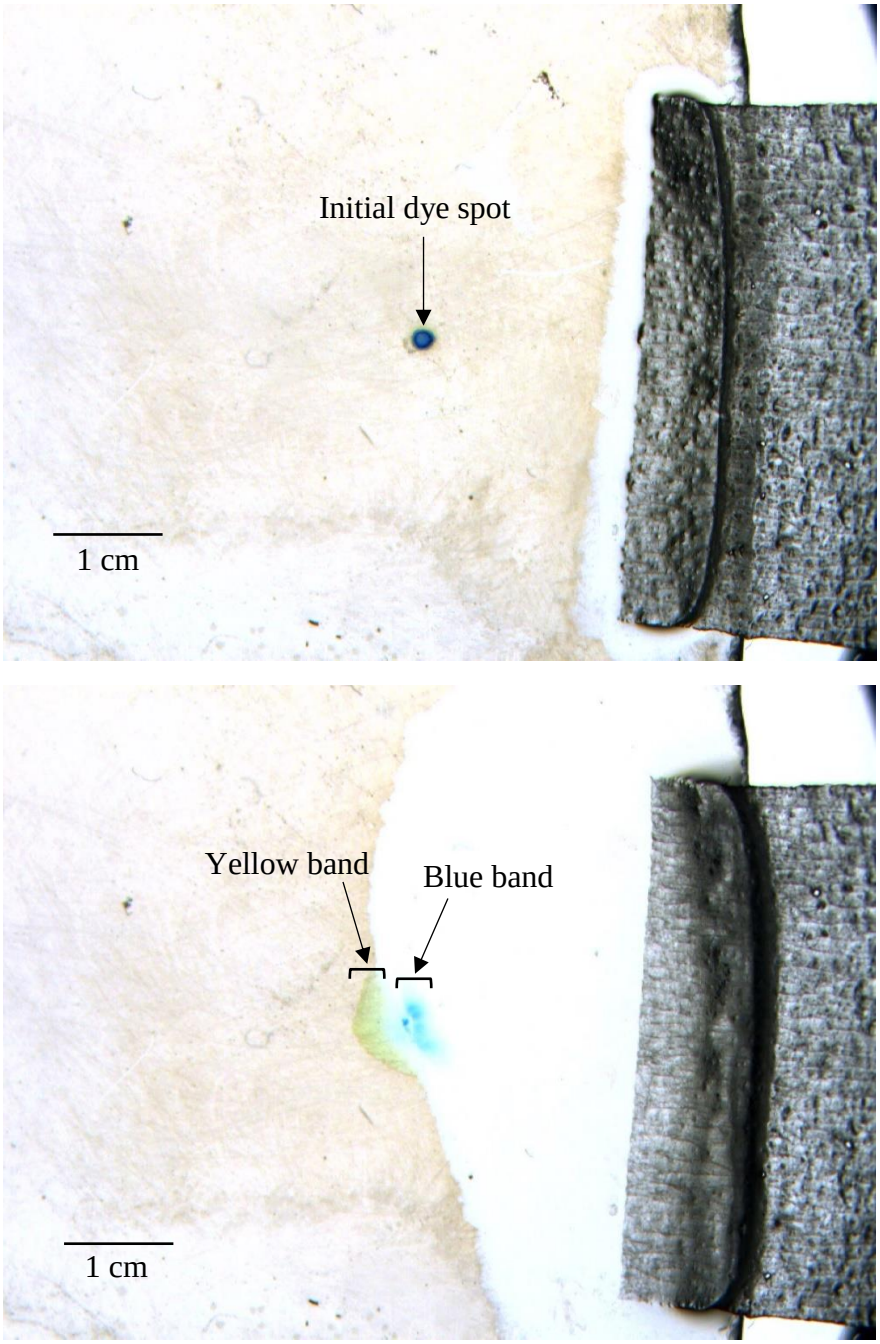

Figure 2.6. A representative picture of a separation in this study performed using a 2:1:1 toluene:hexane:1-bromo-2-chloroethane mobile phase. *(top)* Starting spot of the green dye at the beginning of the separation. *(bottom)* End of separation with the blue and yellow dye bands separated. Measurements were taken from the middle of the respective color band.

2.3.2 *Effect of analyte concentration*

Another challenge that had to be addressed was to determine the optimum settings on the nanoplotter for depositing the dye samples onto the UTLC plates. If the optimum settings were not configured for a specific dye mixture, the nanoplotter would not expel the sample from the attached tip. The three parameters that had to be optimized were the pulse width, the voltage, and the frequency. All three of these parameters were important because the tip could only dispense the chosen analyte based on the optimum electrical properties that corresponded to the analyte. For the dye mixture that was used in this study, the ideal parameters for the pulse width, voltage, and frequency were found to be 38 µs, 50 V, and 100 Hz, respectively. These values only apply to the GeSIM Nano-Plotter 2.1 found in the lab of Dr. Rebecca Lai (UNL Department of Chemistry).

Once these settings had been identified for the nanoplotter, three concentrations of the dye sample were tested to determine the lowest detectable concentration. These concentrations were 100 mM, 75 mM, and 50 mM. Because multiple droplets were used for each spot, the separation could easily be seen and measured using the 50 mM dye mixture. Separations using this concentration of dye were performed using spots formed from 10 droplets (5 nL) and spots formed from 100 droplets (50 nL). For measurements performed solely in our lab, 10 droplet spots provided more than enough analyte to follow and examine the separation. For separations that were completed and then examined using the ACOM, 100 droplet spots were used to allow for easier detection and clear data to be obtained. Overall, the measurements taken from both the 10 droplet spot separations and 100 droplet spot separation gave comparable results for the retardation factor and separation factor.

2.3.3 Determination of separation factors

After a separation had been completed on the UTLC plate, the retardation factor (R_f) and retention factor (*k*) could be calculated for each dye. The expressions used to calculate these values are given in Equations (2.1) and (2.2).[14,15]

$$R_f = \frac{\text{distance traveled by solute}}{\text{distance traveled by solvent}} \quad (2.1)$$

$$k = \frac{1\text{-}R_f}{R_f} \quad (2.2)$$

All distances for the solute bands were calculated from the middle of the band since some of the bands were spread out. These quantitative measurements were performed both visually and using the ACOM. When determining these values visually, a video of the separation was stopped, and the measurements were taken just as the system was opened and the mobile phase began to evaporate and recede. This was done so that the measurement made visually would be comparable to the measurement taken using the ACOM. The average values for the retardation factor and retention factor, in both the 10 droplet spot separations and 100 droplet spot separations, are given in Table 2.1.

	100 droplets dye	10 droplets dye
Average R_f yellow dye	0.66 (±0.04)	0.70 (±0.06)
Average R_f blue dye	0.11 (±0.07)	0.08 (±0.04)
Average k yellow dye	0.52 (±0.10)	0.43 (±0.12)
Average k blue dye	12.3 (±10.05)	14.1 (±7.47)

Table 2.1. Table comparing the retardation values and retention factors of both the yellow and blue dye components. Values are consistent, at the 95% confidence level, between the results for the 10 droplet spots and 100 droplet spots.

The ACOM measurements were performed and a picture was generated using the absorption values generated by the Mueller matrix data. These measurements were only performed using the 100 droplet spots. An image that was generated by this data is shown in Figure 2.7. In this image, the yellow dye is represented as red, the blue dye is represented as blue, and the mobile phase is represented as green.

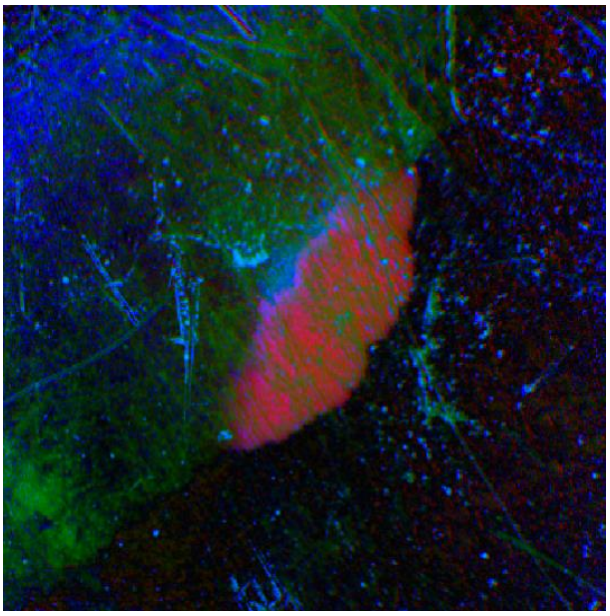

Figure 2.7. Mueller matrix image generated by the data obtained from the ACOM measurement for the separation of a blue and yellow dye mixture. Red represents the yellow dye, blue represents the blue dye, and green represents the mobile phase.

Using this image, measurements were taken to confirm that the retardation factor and retention factor were comparable to the measurements taken visually. Because the measurements for yellow were easier to take, the values were very similar, while the blue had a larger difference in the retardation factor value. A larger difference in retardation factor value lead to a larger difference in the retention factor for the blue dye when compared using the optical microscope vs. the ACOM. These values are listed and compared to the values that were determined visually in Table 2.2. No standard deviation values could be listed in the table since only one trial was measured by the ACOM.

	Optical Microscope	ACOM
Average R_f yellow dye	0.66	0.65
Average R_f blue dye	0.11	0.16
Average k yellow dye	0.52	0.54
Average k blue dye	12.3	5.25

Table 2.2. Table comparing the retardation values and retention factors from the optical microscope measurements vs. the ACOM measurement.

2.4 CONCLUSION

In this study, the formation of SiO_2 nanopillars was explored for use as a stationary phase in UTLC. Using these UTLC devices, the separation of a lipophilic dye mixture was performed, and quantitative data were obtained. It was found that the optimum mobile phase to perform these separations consisted of a 2:1:1 toluene: hexane: 1-bromo-2-chloroethane mixture. It was determined that a 50 mM dye solution could be used in these separations and that the separation could be visually quantified by using as little as a 10 droplet spot (50 nL). When performing the separation for analysis by ACOM, a 100 droplet spot was used. The measurements that were performed using the ACOM were found to be comparable to those done visually under a microscope.

Because it has been shown that lipophilic dyes can be separated on this type of UTLC plate, further exploration should be done into the possibility of using other stationary phases, such as an immobilized protein, onto the surface of the SiO_2 nanopillars. This study will be discussed in the next chapter of this thesis.

2.5 REFERENCES

(1) Anderson, M. S. Atomic Force Microscope Mediated Chromatography. *Rev. Sci. Instrum.* **2013**, *84*, 1–4.

(2) Choi, J. E.; Na, H. Y.; Yang, T. H.; Rhee, S. K.; Song, J. K. A Lipophilic Fluorescent LipidGreen1 - Based Quantification Method for High - Throughput Screening Analysis of Intracellular Poly - 3 - Hydroxybutyrate. *AMB Express* **2015**, *48*, 1–8.

(3) Howell, M.; Daniel, J. J.; Brown, P. J. B. Live Cell Fluorescence Microscopy to Observe Essential Processes During Microbial Cell Growth. *J. Vis. Exp.* **2017**, *129*, 1–10.

(4) Jensen, E. C. Use of Fluorescent Probes : Their Effect on Cell Biology and Limitations. *Anat. Rec.* **2012**, *295*, 2031–2036.

(5) Kanyal, S. S.; Häbe, T. T.; Cushman, C. V; Dhunna, M.; Roychowdhury, T.; Farnsworth, P. B.; Morlock, G. E.; Linford, M. R. Microfabrication , Separations , and Detection by Mass Spectrometry on Ultrathin-Layer Chromatography Plates Prepared via the Low-Pressure Chemical Vapor Deposition of Silicon Nitride onto Carbon Nanotube Templates. *J. Chromatogr. A* **2015**, *1404*, 115–123.

(6) Puzar, P.; Stenovec, M.; Sitar, S.; Lasic, E.; Zorec, R.; Plemenita, A.; Zagar, E.; Kreft, M.; Lenassi, M. PKH26 Labeling of Extracellular Vesicles : Characterization and Cellular Internalization of Contaminating PKH26 Nanoparticles. *BBA-Biomembranes* **2018**, *1860*, 1350–1361.

(7) Jim, S. R.; Taschuk, M. T.; Morlock, G. E.; Bezuidenhout, L. W.; Schwack, W.; Brett, M. J. Engineered Anisotropic Microstructures for Ultrathin-Layer Chromatography. *Anal. Chem.* **2010**, *82*, 5349–5356.

(8) Robbie, K.; Friedrich, L. J.; Dew, S. K.; Smy, T.; Brett, M. J. Fabrication of Thin Films with Highly Porous Microstructures. *J. Vac. Sci. Technol. A* **1995**, *13*, 1032–1035.

(9) Robbie, K.; Brett, M. J.; Lakhtakia, A. First Thin Film Realization of a Helicoidal Bianisotropic Medium. *J. Vac. Sci. Technol. A* **1995**, *13*, 2991–2993.

(10) Steele, J. J.; Brett, M. J. Nanostructure Engineering in Porous Columnar Thin Films : Recent Advances. *J. Mater. Sci. Electron* **2007**, *18*, 367–379.

(11) Vick, D.; Smy, T.; Brett, M. J. Growth Behavior of Evaporated Porous Thin Films. *J. Mater. Res.* **2002**, *17*, 2904–2911.

(12) Peev, D.; Hofmann, T.; Kananizadeh, N.; Beeram, S.; Rodriguez, E.; Wimer, S.; Rodenhausen, K. B.; Kasputis, T.; Pfaunmiller, E.; Nguyen, A.; et al. Anisotropic Contrast Optical Microscope. *Rev. Sci. Instrum.* **2016**, *87*, 1–17.

(13) Reichardt, C. *Solvents and Solvent Effects in Organic Chemistry*, 3rd ed.; Wiley-VCH: Hoboken, NJ, 2003.

(14) Poole, C. F. Thin-Layer Chromatography : Challenges and Opportunities. *J. Chromatogr. A* **2003**, *1000*, 963–984.

(15) Poole, S. K.; Poole, C. F. High Performance Stationary Phases for Planar Chromatography. *J. Chromatogr. A* **2011**, *1218*, 2648–2660.

CHAPTER 3: IMMOBILIZATION OF HUMAN SERUM ALBUMIN ON SiO_2 NANOPILLARS FOR USE IN ULTRA-THIN LAYER CHROMATOGRAPHY

3.1 INTRODUCTION

Based on the positive results obtained in the previous chapter, the possibility of immobilizing a protein onto the surface of SiO_2 nanopillars was next considered. The protein that was chosen for the following studies was human serum albumin (HSA). HSA in commonly studied in biological binding studies because it is the most abundant protein in human blood, making up approximately 50% to 60% of the total protein content that is found in the blood and with a normal concentration in the range of 35-55 g/L.[1–3] HSA is a transport protein responsible for carrying a variety of small compounds throughout the circulatory system, including fatty acids, hormones, and drugs.[4]

Studying how HSA binds to drugs in both its modified and normal forms can provide helpful information to prevent giving too much or too little of a drug in some diseases. One such area of study has been for drugs that are used to treat type-II diabetes.[5–7] Chromatographic studies of sulfonylurea drugs, which are used to treat this disease and which bind strongly to HSA, with various forms of HSA that have been modified with glucose have shown that the binding constants between these drugs and HSA can significantly vary based on the degree of HSA modification.[8–10]

HSA has been shown to bind well through covalent methods to silica supports in traditional high performance affinity chromatography (HPAC).[6,8–10] Because of this, it stands to reason that HSA could also be immobilized to the SiO_2 nanopillars that were examined in the previous chapter for use in UTLC. HSA is often immobilized onto silica

using covalent binding methods such as the epoxy method and Schiff base method.[11] However, because the UTLC plate is fragile and the SiO_2 nanopillars delicate, a much gentler, non-covalent method based on simple adsorption was used in the immobilization of HSA onto the nanopillars.

3.2 EXPERIMENTAL SECTION

3.2.1 *Reagents*

The Cibacron Blue 3GA (dye content ~55%), toluene (HPLC Plus, purity > 99.9%), isopropanol (HPLC grade, purity > 99.5%), and HSA (essentially fatty acid free, purity > 96%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The Micro BCA reagents were purchased from ThermoScientific (Rockford, IL, USA).

3.2.2 *Apparatus*

The following studies were performed using the same apparatus as described in Section 2.2.2. The mobile phase used in this study was pH 7.4, 0.067 M potassium phosphate buffer. Because this mobile phase is aqueous-based instead of organic, filter paper was used for the wick with the same dimensions as the wick described in the previous studies. In these experiments, the Cibacron blue dye solution was dispensed onto the UTLC device as a 1 µL drop from a mechanical pipet. A watchglass was not needed to cover the system in these studies because solvent evaporation during the course of the separation was not an issue when using the aqueous mobile phase.

3.2.3 *Preparation of UTLC plates*

The UTLC plates used in this study were fabricated using a custom-built GLAD chamber.[12] The glass substrates were cut into 2 cm by 2 cm pieces, washed thoroughly with isopropanol, and dried using nitrogen gas before being secured to the sample holder by using carbon tape. Three substrates could be secured onto the sample holder at the same time, which allowed for uniformity of deposition between the plates. The sample holder was placed into the GLAD chamber and maintained at a pressure of at least 10^{-7} mbar for the entire deposition. The silicon was deposited onto the substrate at a rate of 1.977 A°/s with the flux of the nanopillars being at an angle of 85° to the substrate.

The silicon UTLC plates were left out in the ambient air for one week to allow for the conversion of silicon nanopillars to silica nanopillars. Once confirmation was obtained that the nanopillars had become oxidized to silica using an X-ray photoelectron spectroscopy (XPS) measurement, stock solutions of HSA in pH 7.4, 0.067 M potassium phosphate buffer were made at concentrations of 1 mg/mL, 3 mg/mL, and 5 mg/mL. The silica UTLC plates were soaked in these HSA solutions for 72 h to allow immobilization to occur. A sample of the HSA solution that was in contact with the nanopillars was taken every 12 h to follow the rate at which HSA immobilization was occurring. These samples were analyzed using a bicinchoninic acid (BCA) assay to determine the concentration of HSA that was immobilized onto the surface of the silica nanopillars.

3.2.4 *Chromatographic studies*

A stock solution of 10 mM Cibacron blue dye was made by using pH 7.4, 0.067 M potassium phosphate buffer as the solvent. This stock solution was diluted with more

of the same buffer to create 5 mM and 1 mM working solutions to see how this dye was bound to the HSA silica UTLC plate. Because the dye solutions were made with an aqueous solvent, the shelf-life of the solutions was approximately 1 month when stored in the refrigerator. As previously stated, the dye solution was spotted onto the UTLC plate as a 1 µL drop dispensed via a mechanical pipet.

The chromatographic studies were performed to examine the behavior of the immobilized HSA on the silica nanopillars. Based on the retention of the dye solution on an HSA plate versus a control plate, it was believed that it should be possible to use this information to detect the presence of the immobilized HSA. One modification that had to be made in this experiment compared to those in the previous chapter was that because this aqueous solvent was not volatile and did not have appreciable evaporation, the flow rate of the mobile phase had to be reduced dramatically to allow for a separation to be seen.

3.3 RESULTS AND DISCUSSION

After the silicon nanopillars were deposited onto the glass substrate using GLAD, ellipsometry measurements were performed to determine the exact height of the nanopillars and the angle at which the flux was deposited. It was determined that the silicon was deposited at a thickness of 408 nm and the flux was deposited at a 62.5° angle from vertical. A scanning electron microscope (SEM) image of what the silicon nanopillars look like is shown in Figure 3.1.

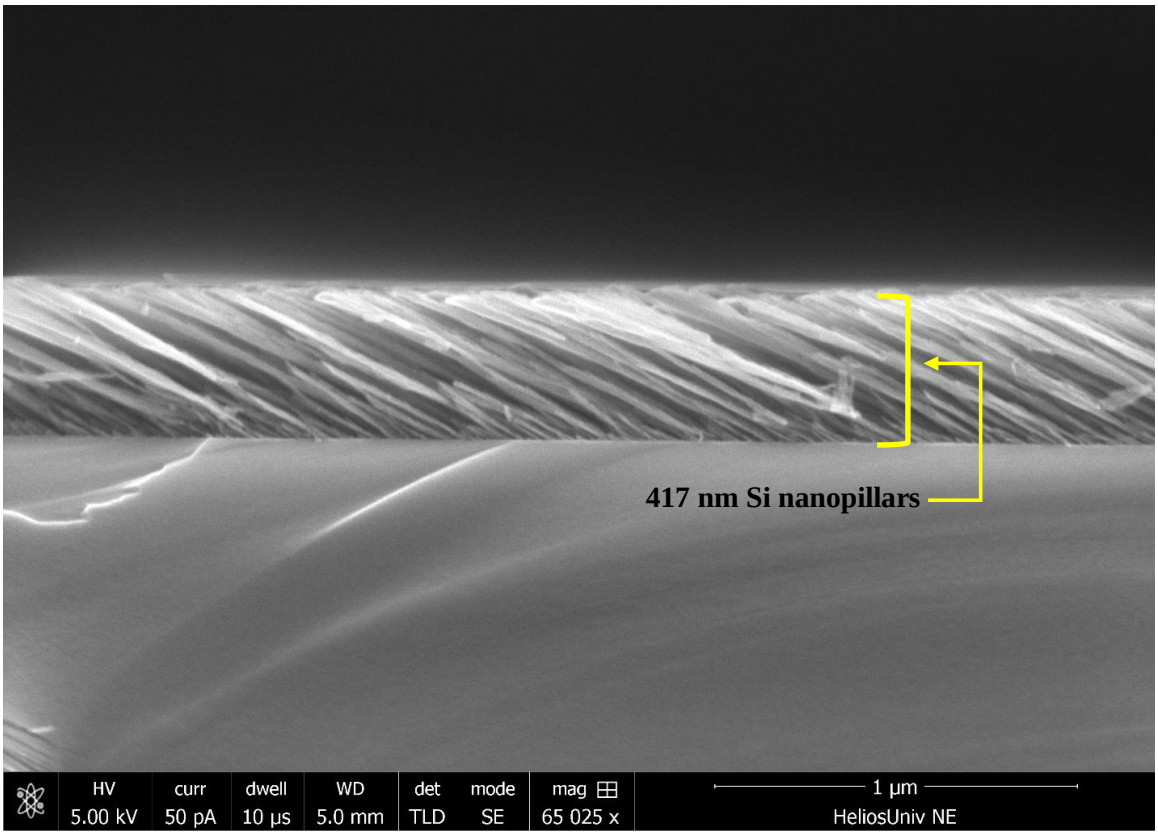

Figure 3.3. SEM image of silicon nanopillars grown using GLAD.

To confirm that the silicon had oxidized to silica by leaving it in an ambient environment, X-ray photoelectron spectroscopy (XPS) was used to examine the surface of this material. Based on the resulting spectrum, as shown in Figure 3.2, there was a silicon oxide layer present on the silicon nanopillars. Unfortunately, the depth probe on the XPS instrument was not operational at the time the measurement was done, so the exact depth of the SiO_2 layer could not be determined. Based on the properties of an XPS measurement, it was estimated that the SiO_2 layer was approximately 5 nm thick. This is known because an XPS generally measures the surface of the sample to a depth of about 10 nm.[13] Because the silicon oxide and silicon peak are approximately the same intensity, it can be inferred that the amount of silicon oxide and silicon in the sample measurement is approximately equal, leading to a SiO_2 layer of 5 nm.

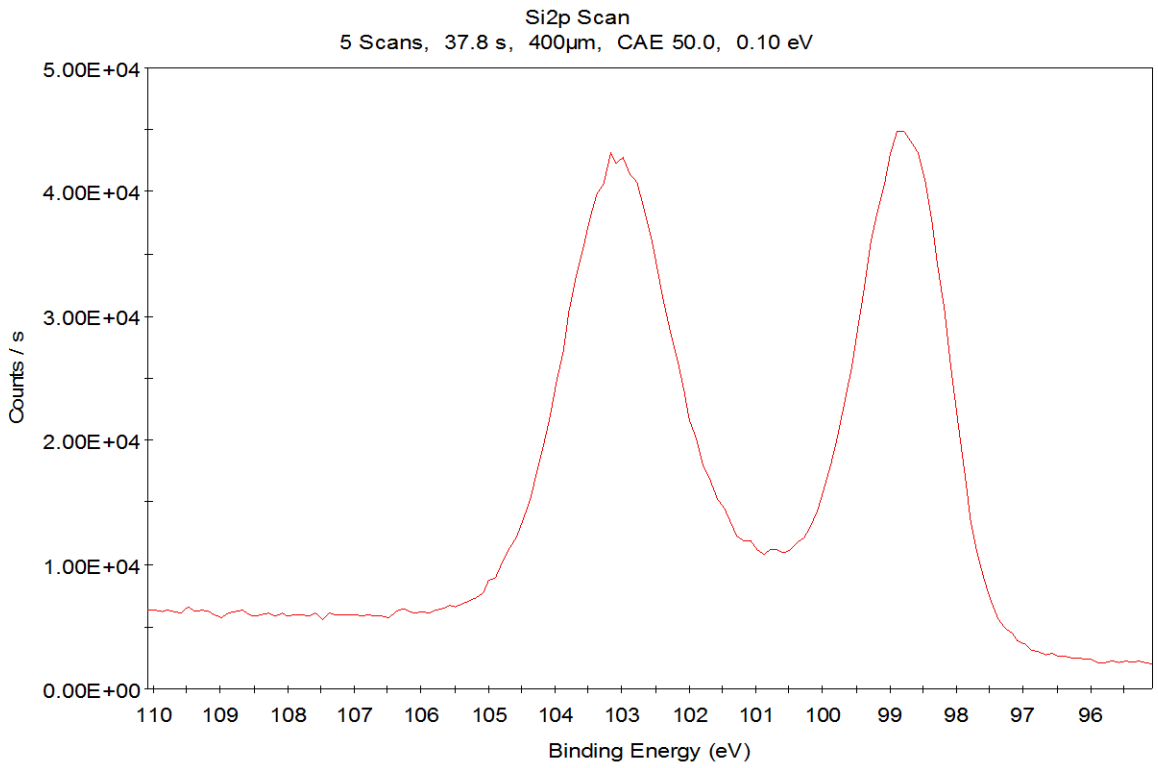

Figure 3.2. An SEM spectrum of the UTLC plate used in this study. The left peak (~103 eV) represents silicon oxide (silica) and the right peak (~98.5 eV) represents silicon. This spectrum indicates that both silicon oxide and silicon were present on the UTLC plate.

Once it was confirmed that the surface of the nanopillars had been oxidized to SiO_2, tests for immobilizing HSA onto the surface began. Because the SiO_2 nanopillars were already deposited onto the UTLC plate, complex immobilization methods were not practical. It was decided that the simplest and most effective way to immobilize HSA onto the nanopillar surface was to soak the plates in an HSA solution and allow non-covalent adsorption of HSA to occur onto the silica. The results that were obtained when soaking the plates in the three different HSA concentrations showed that using a 1 mg/mL solution was adequate because only 0.213 mg/mL of HSA was really needed to saturate the SiO_2 nanopillars. When taking into account the volume of this solution, this meant that approximately 77.1 nmol of HSA was immobilized onto the UTLC plate. Control tests were done to see if HSA would also bind to the original glass substrate. However, these experiments indicated that there was only negligible binding by HSA to the glass plate in the absence of the SiO_2 nanopillars. The data for these studies is summarized in Table 3.1. It was also determined that almost all of the protein immobilization occurred within the first 24 hours of soaking the UTLC plate in the HSA solution.

	Glass Substrate (soaked in 5 mg/mL HSA solution)	**UTLC Plate** (soaked in 3 mg/mL HSA solution)
Protein concentration at t=0 hrs	4.391 (±0.371)	1.985 (±0.153)
Protein concentration at t= 24 hrs	4.393 (±0.346)	1.772 (±0.216)
Difference in protein concentration (mg/mL)	-0.002	0.213

Table 3.1. Table comparing the protein concentration of a glass substrate and UTLC plate at 0 hours and 24 hours. These results confirm that protein is immobilized onto the UTLC plate and no protein is immobilized on the glass substrate.

Once immobilization of HSA onto the plate was achieved, chromatographic studies were performed with the plate to examine its binding properties. Cibacron blue dye was chosen for use in this study because of it has well-documented binding to HSA.[14–16] It was found that a 1 mM solution of Cibacron blue was needed to adequately see the dye moving across the UTLC plate. A pH 7.4, 0.067 M potassium phosphate buffer was used as the mobile phase in this study and dispensed via syringe pump at a rate of 0.5 µL/min. When the mobile phase was applied to the UTLC plate, the Cibacron blue was eluted off the plate. Unfortunately, as the dye was eluted off, so were the SiO_2 nanopillars. This could have occurred for several reasons. For instance, the flow rate of the mobile phase may have been too high. Another possibility is that the pH 7.4 buffer may have weakened the nanopillars and caused them to wash away. A set of images that compare an undamaged UTLC plate and one after this process are shown in Figure 3.3.

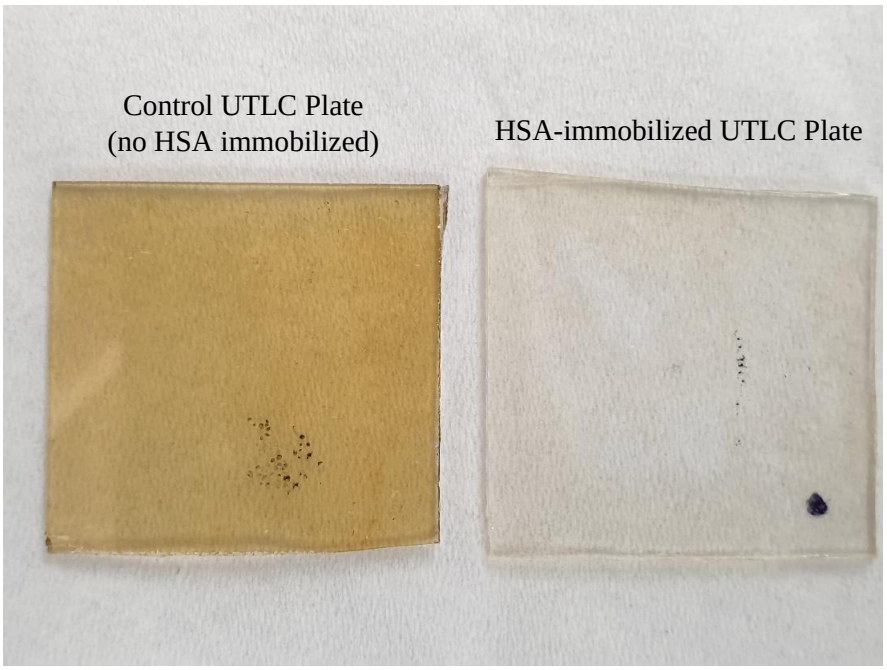

Figure 3.3. (*top image*) Image comparing an undamaged UTLC plate vs. a damaged UTLC plate. (*bottom image*) Magnified picture of damaged UTLC plate. The white streaks are where the nanopillars have been stripped off the glass substrate.

3.4 CONCLUSION

In this study, the immobilization of a protein onto the surface of SiO_2 nanopillars was performed. XPS was used to characterize the UTLC plate to confirm that the silicon had been oxidized to form silica. Next, the parameters surrounding HSA immobilization were explored and optimized so that chromatographic studies could be performed. When the chromatographic studies were performed using a Cibacron blue dye solution, it was discovered that the nanopillars were washed away with the dye solution.

It is hypothesized that the degradation of the nanopillars was caused by either the flow rate of the mobile phase or the pH/composition of the mobile phase. Ways that this can be fixed in future studies is to 1) decrease the flow rate of the mobile phase, 2) to change the composition of the mobile phase to a solution that is still at physiological pH, or 3) to change the pH of the buffer and see which of these changes does not degrade the SiO_2 nanopillars. These solutions will be discussed in the following chapter of this thesis.

3.5 REFERENCES

(1) He, X. M.; Carter, D. C. Atomic Structure and Chemistry of Human Serum Albumin. *Nature* **1992**, *358*, 209–215.

(2) Ascoli, G. A.; Domenici, E.; Bertucci, C. Drug Binding to Human Serum Albumin : Abridged Review of Results Obtained with High-Performance Liquid Chromatography and Circular Dichroism. *Chirality* **2006**, *18*, 667–679.

(3) Fanali, G.; Trezza, V.; Marino, M.; Fasano, M.; Ascenzi, P. Human Serum Albumin : From Bench to Bedside. *Mol. Aspects Med.* **2012**, *33*, 209–290.

(4) Bertucci, C.; Domenici, E. Reverisible and Covalent Binding of Drugs to Human Serum Albumin: Methodological Approaches and Physiological Relevance. *Curr. Med. Chem.* **2002**, *9*, 1463–1481.

(5) Hood, L.; Heath, J. R.; Phelps, M. E.; Lin, B. Systems Biology and New Technologies Enable Predictive and Preventative Medicine. *Science.* **2004**, *306*, 640–643.

(6) Anguizola, J.; Joseph, K. S.; Barnaby, O. S.; Matsuda, R.; Alvarado, G.; Clarke, W.; Cerny, R. L.; Hage, D. S. Development of Affinity Microcolumns for Drug − Protein Binding Studies in Personalized Medicine: Interactions of Sulfonylurea Drugs with in Vivo Glycated Human Serum Albumin. *Anal. Chem.* **2013**, *85*, 4453–4460.

(7) Hage, D. S.; Anguizola, J. A.; Jackson, A. J.; Matsuda, R.; Papastavros, E.; Pfaunmiller, E.; Tong, Z.; Vargas-badilla, J.; Yoo, M. J.; Zheng, X. Analytical Methods Chromatographic Analysis of Drug Interactions in the Serum Proteome. *Anal. Methods* **2011**, *3*, 1449–1460.

(8) Tao, P.; Li, Z.; Woolfork, A. G.; Hage, D. S. Journal of Pharmaceutical and Biomedical Analysis Characterization of Tolazamide Binding with Glycated and Normal Human Serum Albumin by Using High-Performance Affinity Chromatography. *J. Pharm. Biomed. Anal.* **2019**, *166*, 273–280.

(9) Matsuda, R.; Kye, S. H.; Anguizola, J.; Hage, D. S. Studies of Drug Interactions Wih Glycated Human Serum Albumin by High-Performance Affinity Chromatography. *Rev. Anal. Chem.* **2014**, *33*, 79–94.

(10) Jackson, A. J.; Anguizola, J.; Pfaunmiller, E. L.; Hage, D. S. Use of Entrapment and High-Performance Affinity Chromatography to Compare the Binding of Drugs and Site-Specific Probes with Normal and Glycated Human Serum Albumin. *Anal. Bioanal. Chem.* **2013**, *405*, 5833–5841.

(11) Pfaunmiller, E. L.; Hartmann, M.; Dupper, C. M.; Soman, S.; Hage, D. S. Optimization of Human Serum Albumin Monoliths for Chiral Separations and High-Performance Affinity Chromatography. *J. Chromatogr. A* **2012**, *1269*, 198–207.

(12) Sekora, D. G. Electrochromicity and Slanted Columnar Thin Films : A Combinational Studies Approach. Ph.D. dissertation, University of Nebraska-Lincoln, Lincoln, NE. **2017**.

(13) Mather, R. R. Surface Modification of Textiles by Plasma Treatments. *Surf. Modif. Text.* **2009**, *97*, 296–317.

(14) Alberghina, G.; Babulano, G.; Fisichella, S.; Renda, E.; Bianchini, R.; Forte, C. Reactive Dyes-Human Serum Albumin (HSA) Binding by Frontal Analysis Affinity Chromatography. *Gazz. Chim. Ital.* **1997**, *127*, 803–808.

(15) Leatherbarrow, R. J.; Dean, P. D. G. Studies on the Mechanism of Binding of Serum Albumins to Immobilized Cibacron Blue F3G A. *Biochem. J.* **1980**, *189*, 27–34.

(16) Tsai, J. Z.; Chen, C. J.; Liu, J. T.; Hsin, Y. M.; Chen, W. Y.; Hsueh, K. H. Development of Capacitance Measurement System for Human Serum Albumin Detection. *Electron. Lett.* **2010**, *46*, 678–680.

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

4.1 SUMMARY OF WORK

This thesis has explored the possibility of using nanomaterial supports as stationary phases for ultra-thin layer chromatography (UTLC) devices. By depositing SiO_2 nanopillars onto a glass substrate, a variety of separations could be performed on the UTLC device. Separations that were performed in this thesis included the separation of a lipophilic dye mixture and the use of UTLC in dye-protein binding studies. The lipophilic dye mixture separations could be analyzed by using a novel ellipsometry method based on anisotropic contrast optical microscope (ACOM). Because this study used SiO_2 nanopillars, dye-protein studies could also be performed by using proteins that were adsorbed onto silica. By using the data collected during these studies, quantitative parameters for these separations could be determined, such as the retention factors and retardation factors. These UTLC studies were bound to be cost-effective and relatively efficient, as well as requiring only a small amount of sample for analysis.

Chapter 1 gave an overview of the main principles that were being utilized in these studies and general scope of the studies that were to be performed. A general background of planar chromatography and UTLC, and the theory behind them, was also presented. A history and summary of affinity chromatography was provided. The use and history of nanomaterials as chromatographic supports or stationary phases was also discussed.

Chapter 2 focused on separating a lipophilic dye mixture by ULTC, which was used as a proof-of-concept study. Different mobile phases and dye separation

concentrations were tested to find the optimal parameters to perform these experiments. Based on the results of these optimization experiments, data were collected by optical microscope and used to determine the quantitative parameters for these separations, such as the retardation factor and retention. These studies also confirmed that ACOM could be used as a way to monitor the results of the UTLC separation.

Chapter 3 focused on the immobilization of a protein onto the surface of the silica nanopillars for used as a stationary phase for a UTLC device. Human serum albumin (HSA) was non-covalently bound to the silica nanopillars by soaking the UTLC plates in an HSA solution. The HSA was then used as a binding agent and stationary phase for UTLC. To confirm that the HSA had been immobilized onto the silica nanopillars, a protein assay was performed. Dye retention studies were also performed on the UTLC plates to confirm the presence of HSA on the silica nanopillars. These dye-protein studies were unsuccessful, however, because the silica nanopillars were washed away during the elution of the dye from the UTLC plate.

4.2 FUTURE DIRECTIONS OF WORK

Knowing that HSA can be bound onto silica nanoparticles opens up several possibilities for future studies. The first thing that must be studied is the reason for why the silica nanoparticles washed away during the dye-protein binding studies. While lowering the flow rate may be helpful to prevent extra pressure on the nanopillars, the solvent buffer may also need to be changed if this were the cause of the nanopillars being lost over time. This is especially important to consider in work with proteins like HSA because the UTLC plates containing these proteins would probably be stored in the same type of buffer when not in use. There are several buffer options in the literature that

should not degrade the nanopillars over time.[1,2] The most readily available of these buffers is tris(hydroxymethyl)aminomethane (Tris) buffer, which can also be used at a physiological of pH 7.4.

Once the dye-protein binding studies have been optimized, the same method could be used to perform drug-protein binding studies. To confirm that the drug-protein binding studies compare to the results for other methods in high performance affinity chromatography (HPAC), a number of previously-studied drugs could be used, such as gliclazide or glibenclamide.[3–5] If these studies produce values consistent to those in the literature, it means that ULTC may provide a cheaper method for this type of analysis. The elution runoff could also be collected and run through an off-line detector that could be hooked up to the UTLC device. This could provide real-time absorbance or fluorescence values for the separations taking place on the device.

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4.4 REFERENCES

(1) Huang, X.; Young, N. P.; Townley, H. E. Characterization and Comparison of Mesoporous Silica Particles for Optimized Drug Delivery Regular Paper. *Nanomater. Nanotechnol.* **2014**, *4*, 1–15.

(2) Yang, S.; Choi, S.; Jeon, S. M.; Yu, J. Silica Nanoparticle Stability in Biological Media Revisited. *Sci. Rep.* **2018**, *8*, 1–9.

(3) Matsuda, R.; Jobe, D.; Beyersdorf, J.; Hage, D. S. Analysis of Drug – Protein Binding Using On-Line Immunoextraction and High-Performance Affinity Microcolumns: Studies with Normal and Glycated Human Serum Albumin ଌ. *J. Chromatogr. A* **2015**, *1416*, 112–120.

(4) Tao, P.; Li, Z.; Woolfork, A. G.; Hage, D. S. Characterization of Tolazamide Binding with Glycated and Normal Human Serum Albumin by Using High-Performance Affinity Chromatography. *J. Pharm. Biomed. Anal.* **2019**, *166*, 273–280.

(5) Matsuda, R.; Li, Z.; Zheng, X.; Hage, D. S. Analysis of Multi-Site Drug – Protein Interactions by High-Performance Affinity Chromatography : Binding by Glimepiride to Normal or Glycated Human Serum Albumin. *J. Chromatogr. A* **2015**, *1408*, 133–144.