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Separation of On-Column Labeled Model Proteins with Packed Capillary Electrophoresis

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Is approved by the final examining committee:

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SEPARATION OF ON-COLUMN LABELED MODEL PROTEINS WITH PACKED

CAPILLARY ELECTROPHORESIS

A Thesis

Submitted to the Faculty

of

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by

Yingxu Hao

In Partial Fulfillment of the

Requirements of the Degree

of

Master of Science

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West Lafayette, Indiana

To My family,

For the Time being away from you,

I love you with all my heart

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAm	Acrylamide
AGET	Activator generated by electron transfer
ATRP	Atom transfer radical polymerization
BC	(Choloromethyl)phenylethyl-trichlorosilane
CE	Capillary electrophoresis
C1	Methyltrichlorosilane
C18	N-ocyadecyltrichlorosilane
DMF	N.N-Dimethylformamide
DNA	Deoxyribonucleic acid
ETOH	Ethanol
FCC	Face centered cubic
HNO_3	Nitric acid
IgG	Immunoglobulin
Me_6TREN	Tris[2-(dimethylamino)ethyl]amine
NaAs	Sodium ascorbate
NaOH	Sodium hydroxide
PAAm	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis

PDMS	Polydimethylsiloxane
SCC	Silica colloidal crystal
SDS	Sodium dodecyl sulfate
SEC	Size- exclusion chromatography
SEM	Scanning electron microscope

ABSTRACT

Yingxu Hao. M.S., Purdue University, August 2014. Separation of On-column Labeled Model Proteins with Packed Capillary Electrophoresis. Major Professor: Mary J. Wirth.

Protein drugs are increasingly developed in the pharmaceutical company. Under the regulation of FDA, high purity of therapeutic proteins needs to be maintained. Before putting those drugs in the market, fast and efficient method is in need to achieve homogeneity. Traditionally, polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), and size-exclusion chromatography (SEC) are used for the purification process. These methods have the disadvantages of low time and cost efficiency, and this quality assurance process has become the bottleneck of production. In our group, sub-micron silica colloidal particles with polyacrylamide layer on the surface are packed inside capillaries to increase the separation efficiency. In this particular project, NanoOrange dye is non-covalently associated with the protein sample to best reserve their native conformation. Proteins were separated at a distance as short as 8.2 mm in 85 seconds with extremely low plate height. The efficiency can be improved by decrease the silica particle size. This high throughput separation has a potential to be adopted in industries.

Part II of this thesis describes the cost-effective DNA microarray project. Microarray is a technology evolved from southern blotting, and it is a common tool to

measure the expression levels of the DNA samples. To quantify the DNA samples, fluorescently labeled target strands hybridize with the DNA probe which binds to a solid surface. DNA microarray is frequently utilized in clinical studies, and the efficiency is highly desired to be improved. When attach the probes on a smooth surface, the amount of DNA captured will be limited. In this research, the microarray sensitivity is increased by layering silica colloidal particles on the solid surface. Silica particles obtain face-centered cubic packing which increases the surface area to bind to the DNA probe, thus improve the microarray sensitivity. To reduce the cost of the microarray and to make it more point-of-care feasible, transparent plastic sheets is going to be researched to replace quartz silica plates. This new sensitive and cost effective microarray has a great possibility to be developed into point-of-care which detects a variety of diseases.

PART ONE:

SEPARATION OF ON-COLUMN LABELED MODEL PROTEINS WITH PACKED

CAPILLARY ELECTROPHORESIS

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Importance of Protein Homogeneity in Pharmaceutical Industry

In pharmaceutical industries, there is an increasing amount of protein drugs that are discovered and used clinically every year. Monoclonal antibodies are great treatment for serious diseases, but they are easily aggregated [1]. Thus protein drug homogeneity is essential for safety and quality purposes. An efficient protein separation tool with high resolving power is more desired now than ever[44].

1.2 Background on Silica Colloidal Crystal

Silica colloidal particles packed capillaries are wildly used in chromatography studies for protein separation. Sub-micron silica colloidal crystals can be uniformly packed into fused silica capillaries as face-centered cubic lattice, where opalescence can be observed [2]. The benefits of using sub-micron silica colloidal particles are apparent according to Van Deemter equation [3][4]:

$$H = A + \frac{B}{u} + Cu = ad_p + \frac{2\gamma D_m}{u} + \left(C_m + C_p\right)\frac{d_p^2 u}{2D_m} + C_s u \qquad \text{eq 1}$$

In this equation, dp is the particle size, Dm is the diffusion coefficient of the analyte in mobile phase, and u is the linear velocity. The A, B, and C terms contribute to

plate height, and larger numbers indicate less separation efficiency. A term is the Eddy-diffusion parameter, which is contributed by channeling through a non-ideal packing. B term is the longitudinal diffusion coefficient of the eluting particles, and it is resulted from protein spreading from the center along their migration direction [5]. Longitudinal diffusion significantly induces band broadening, and it is proportionally related with analyte diffusion coefficient D_m. The C term is a combination of adsorption kinetics and mass transfer inside the particles.

Uniformly packed sub-micron silica colloidal crystal can greatly reduce the plate height and thus achieve high efficiency[31]. Like illustrated in figure 1.1, when protein travel through the pores constructed by closely packed particles, they are separated in this sieving medium by size over time[29][30][36].
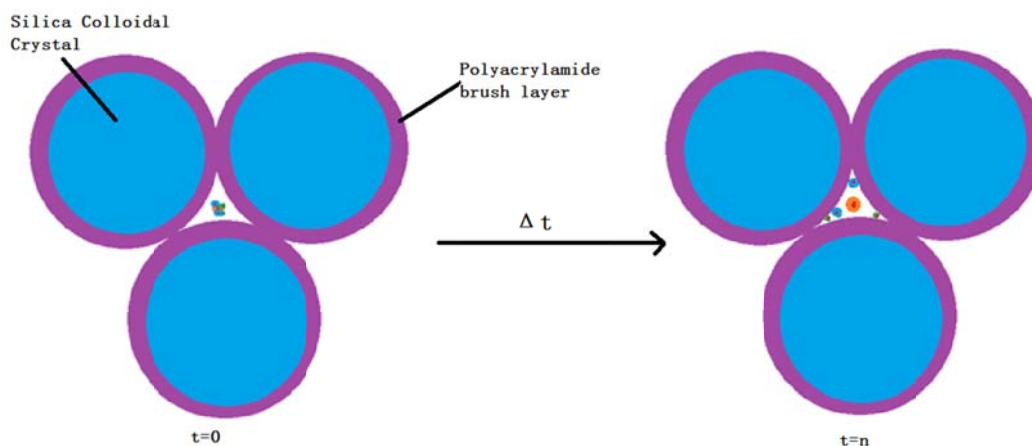
Figure 1.1 Protein travel through the pores of closely packed SCC, separating inside the sieving medium

1.3 Capillary Electrophoresis: A High Throughput Separation Method

1.3.1 Background on Capillary Electrophoresis

Capillary electrophoresis is a combination of the two powerful separation tools of slab-gel electrophoresis and chromatography. Capillary electrophoresis on fluorescent microscope is commonly used in industries, and it is used to separate protein, DNA, and other samples by size[43]. Even though it requires fluorescent labeling on the analytes, capillary electrophoresis attains the lowest detection limit among the whole field of separation[38][39]. Thus the minimum amount of sample will be consumed for each run. Sample analysis will also be more efficient with its extraordinary separation power and the speed of analysis [6]. All these advantages makes CE a fast growing tool in clinical applications, thus drives intensive research about it[37].
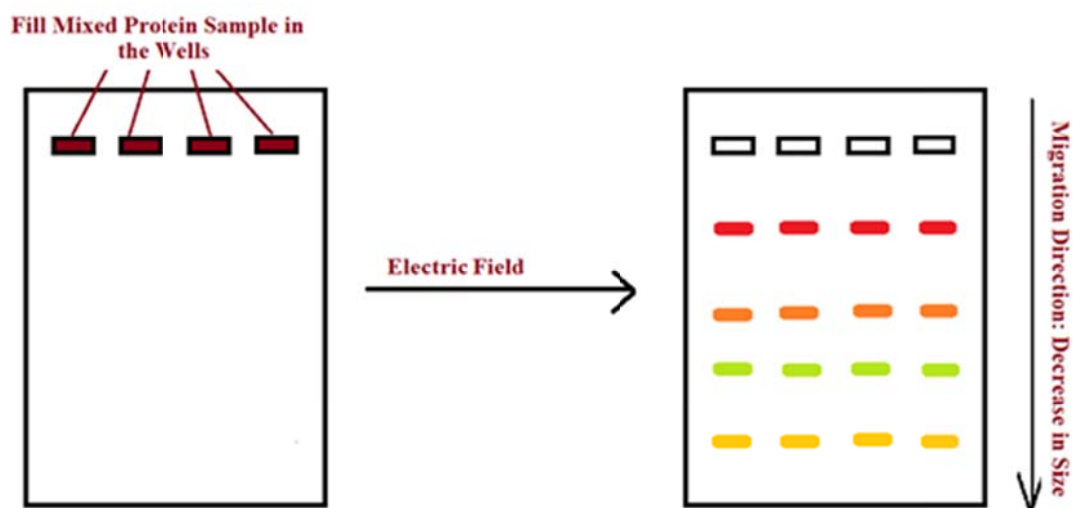
Figure 1.2 Illustration of slab-gel electrophoresis

1.3.2 Voltage Supply Affects the Separation Results

Electricity is the driving power for protein motion, and optimizing the voltage is one key factor to control the peak width. Increase the voltage results in a reduced separation time:

$$t = \frac{L}{v} = \frac{L^2}{\mu V}$$

eq 2

In the equation, L is the separation distance, v is the velocity, μ is the electrophoretic mobility, and V is the voltage. Voltage inverse relates with the separation time, but there is still an upper limit for the voltage applied.

Joule heating is one consequence from high voltage [7]:

$$Heat = VIt = I^2 Rt$$

eq 3

V is the voltage from the supplier, I is the current, t is the separation time, and R is the resistance from the capillary. Heat produced is proportional to the voltage, but reduces dynamic viscosity, which thus induces band broadening.

1.4 NanoOrange: A Dynamic Labeling Dye

Electrophoresis is commonly used and efficient to separate protein from their aggregates and other proteins with similar sizes. Unfortunately, fluorescent labeling is required to observe analyte motion using capillary electrophoresis under fluorescent microscope. Many frequently used fluorescent dyes covalently associate with protein sample, thus alter from their native conformation and increase the amount of aggregation. In this research, nanoOrange was used to form non-covalent bonds with protein samples to minimize protein conformation change[40].

NanoOrange is a type of merocyanine dye that can be used in both protein separation and quantitation [8]. Just like figure 1.2, isolated nanoOrange in solution shows neglectable amount of fluorescence, but produces high intensity fluorescence while interacting with detergent-coated proteins [42]. This property allows NanoOrange assay to obtain high sensitivity and signal to noise ratio. In SDS detergent solution, protein forms micelles to wrap nanoOrange inside it and they fluorescent as a whole system. SDS may also unwind the protein molecules, and NanoOrange associate to their surface. Both type of the protein-dye binding will result in the conformation change of the dye, thus the fluorescent gets enhanced afterwards [9].
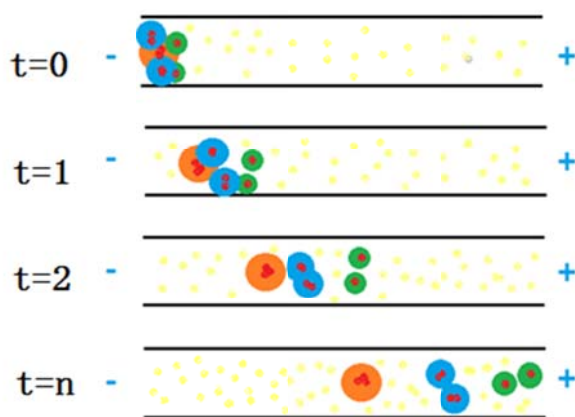
Figure 1.3 NanoOrange dye included in protein micelle in the SDS detergent solution, travel inside the capillaries over time.

The NanoOrange assay is capable to detect protein with a concentration as low as 10nm/ml, and exhibits relatively low protein- to-protein variation [10]. The limitations of nanoOrange is that the sample is only stable for 6 to 12 hours, where sample preparation is required each time[33].

1.5 Research Goals

The goal of this work is to prove the feasibility of replacing covalently labeled dyes with NanoOrange, and separate the model proteins at baseline. Partial separation of the three model proteins was described by our former group member Dr. Birdsall, and baseline separation is desired to be completed by optimizing separation conditions.

The result is approached in a variety of ways. To obtain a clean spectrum, and best observe the fluorescent complex inside the capillary, sample preparation was studied. Separation efficiency is proportional to the size of silica colloids. To reduce the plate height and improve the separation, different sized silica particles were tested. To best separate the analytes, packing conditions were verified for the optimization purpose.

1.6 Project Overview

This report is focused on utilizing nanoOrange as the on-column labeling tool for capillary electrophoresis and optimizing the separation conditions.

Chapter one reviews the background and theory behind the research. Submicron silica colloids were proved to be a feasible material to pack inside capillaries and a high throughput protein separation tool previously in the group. Sample preparation time is reduced by utilizing NanoOrange as an on-column labeling tool. Conditions for the protein separation were studied to minimize the band broadening, thus achieve baseline separation of protein.

Chapter two reviews the materials and methods used to develop the research step by step.

Chapter three stated that separation of the three protein was achieved, which proves NanoOrange as the candidate of our new labeling dye. The silica particle size is one important factor which controls the separation efficiency.

Chapter four concludes the research result and points out the future direction of this project. Silica particle size can be reduced even more to achieve higher resolution, and bare silica particles may even be a feasible separation material with small enough particle size.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Materials

Silica colloidal particles of 350 and 500nm in diameter were purchased from Fiber Optic Center (New Bedford, MA). NanoOrange protein quantification kit was purchased from Life Technologies (Carlsbad, CA). Teflon coated capillaries with 100 i.d. and 75 i.d. were both purchased from Polymicro Technologies (Phoenix, AZ). Packing parts including unions, frets, and tightening tools were ordered from Valco Instruments Co. (Houston, TX). All the silanes were ordered from Gelest Inc. (Morrisville, PA).Model proteins, solvents, running buffer, and reagents are bought from SigmaAldrich Co. (St. Louis, MO). Stacking buffer was prepared in lab with 2% SDS, 3% sucrose, 1mM EDTA, and 62 mM tris.

2.2 Protein Preparation

Carbonic anhydrase, trypsin inhibitor, and lysozyme are the three model protein used in this research. Pure protein solid were dissolved into 500ul PBS solution at a concentration of 2mg/ml. Protein samples were prepared as in figure 1.3. For each individual protein sample, 10 ul of the protein stock solution was added into 4 ul of NanoOrange dye, and 26 ul of the pre- mixed stacking buffer which contains 62mM Tris,

1mM EDTA, 3% sucrose, and 2% SDS. The sample was denatured at 96°C for 10 minutes, and cooled down before running on the microscope.
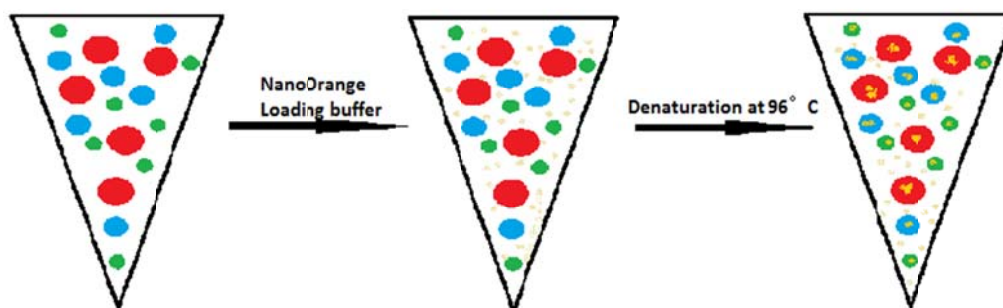
Figure 1.4 Sample preparation of protein-nanoOrange complex.

For the three protein mix sample, 12ul of NanoOrange and 78 ul of stacking buffer was added to 10 ul of each protein. The total of 120 ul analyte was treated exactly the same way with individual protein samples.

2.3 Silica Colloidal Crystal Preparation

2.3.1 Thermal Treatment of SCC

A series of calcination and annealing process were done on the packing material to achieve the best packing and modification. Nonporous sub- micron silica particles were calcined 3 times at 600°C for 12 hours, and fully suspended with 100% ethanol each time after to minimize the amount of aggregation and promote the formation of siloxane bonds[32]. Annealing was done at 1050°C for 3 hours to melt and smooth the

particle surface. The bulk material consists of more bonds than the surface, thus the high temperature offers ignorable change towards it [12].

2.3.2 Rehydroxylation

Annealed particles are cooled and sonicated in MilliQ water to achieve full suspension, and rehydroxylation was done with 50:50 mixture of HNO_3: H_2O. By heating the suspension to 270°C for 12 hours, silica particles are fully hydroxylated (Figure 2.1). The suspension was divided into 6 samples, and centrifuge with ultrapure water and ethanol for 3 times each to remove the acidic solution. After drying within the 60°C oven, the sample was re-sonicated in ethanol to undergo filtration. Two micron meshes were applied to remove the aggregates and retain the sub-micron particles that were uniform in size.
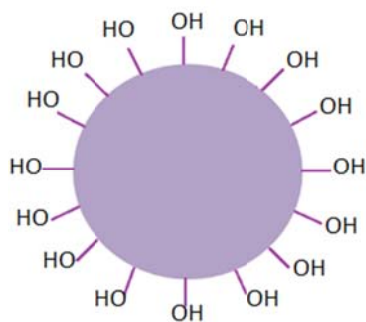
Figure 2.1 Illustration of fully hydroxylated SCC.

2.4 Capillary Preparation

2.4.1 Condition Fused Silica Capillaries

Teflon coated 100 id capillaries were conditioned with 0.1M sodium hydroxide using a syringe pump for 20 minutes. MilliQ ultrapure water and 100% ethanol were followed to remove the base. Capillaries were cut into 12cm in length and dried in 60°C vacuumed oven for 10 minutes.

2.4.2 Capillary Packing with SCC

Bare silica slurries were prepared at 35% w/w concentration in water. Slurries were pushed into capillaries with syringe after full suspension. Packing was done under 7500 psi while sonication for 20 minutes using a 0.5um frit. After depressurize the system, silica particles are uniformly packed as face-centered lattice, and the capillaries have the length in the order of 3cm. The packing apparatus is showed on figure 2.2.
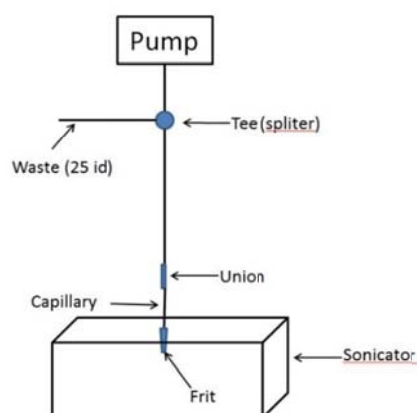
Figure 2.2 Pressure packing apparatus.

2.4.3 In Capillary Chemical Modification

Packed capillaries were modified by growing a brush layer of polyacrylamide on the silica surface [8]. As shown in Figure 2.3 A, two percent (chloromethyl)phenylethyl-trichlorosilane was mixed with methyltrichlorosilane, which is a spacer, at a 20:1 ratio in anhydrous toluene. The reaction solution wicked into the capillaries, and reacted with silica surface for 12 hours under nitrogen. An immediate rinse of the capillaries with anhydrous toluene was done at 3500 psi with pressure pump to remove the excess reagents. The capillaries were then dried inside the 120°C oven for 2 hours.

Activators generated by electron transfer (AGET) were used to replace traditional ATRP to perform the polymerization. Acrylamide, $CuCl_2$, Sodium ascorbate, and tris(2-dimethylaminoethyl)amine was mixed into a total of 6 ml, 50:50 IPA:H_2O solution as illustrated in figure 2.3 B[34]. Pressure pump was set at 3500 psi to push the reaction solution into the packed capillary, and the polymerization lasts 3 hours. Rinsing for 30 minutes is required immediately with 50:50 IPA:H_2O to remove reaction solution from the capillary[11][35]. Capillaries are fully dried in the vacuum desiccator prior to setting it inside the refrigerator for storing. The fully modified capillary is ready to be used for electrophoresis.
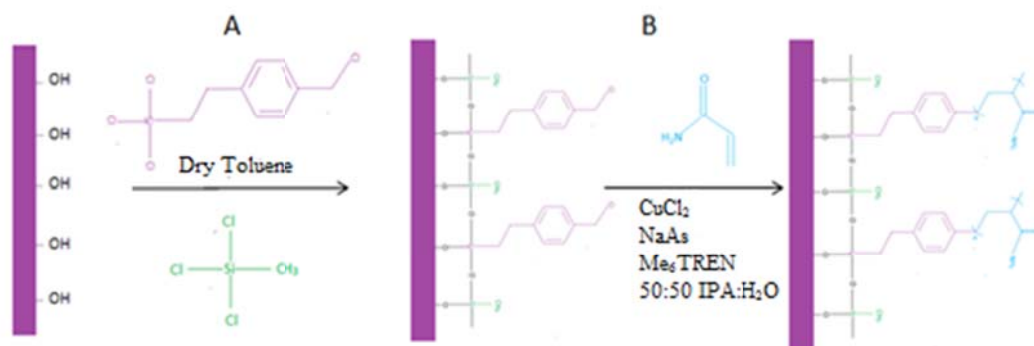
Figure 2.3 Reaction scheme of activators generated by electron transfer. Polyacrylamide brush layer growing on the silica surface

2.5 Apparatus Setup of Capillary Electrophoresis

As shown below in figure 2.4, dual PDMS reservoirs were stabilized on glass plate to keep the capillary in position on the fluorescent microscope. Two platanium electrodes were placed in the reservoirs and dipped inside the running buffer. The slightly basic running buffer was composed of 25 mM tris, 192 mM glycine and 0.1% w/v SDS. The buffer was diluted 10 times before application. Because the small proteins used were negatively charged, sample was loaded into the end of the capillary by the negative electrode.
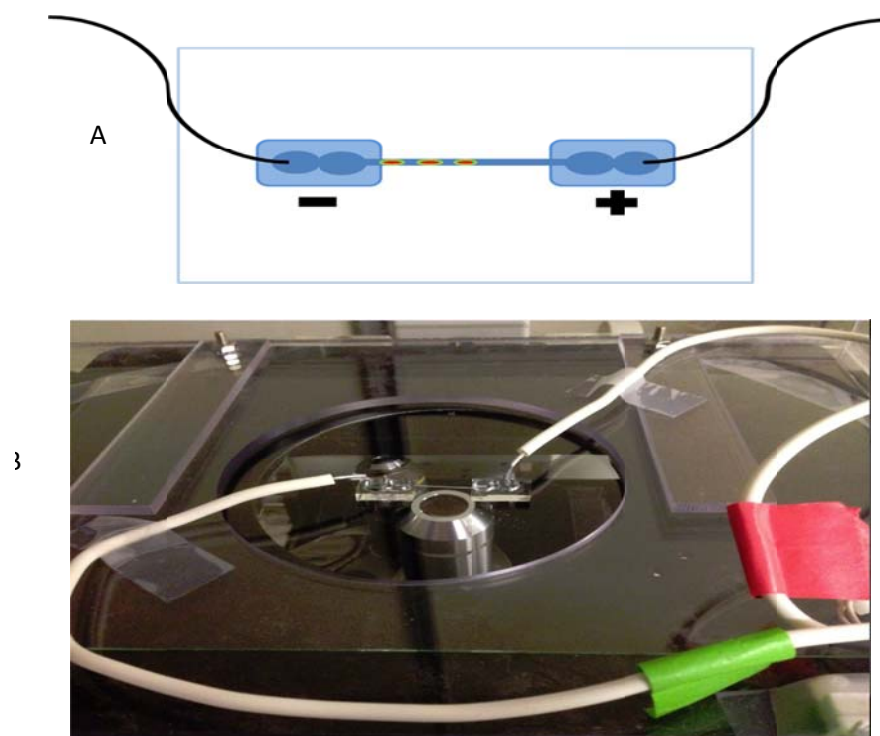
Figure 2.4 A. Illustration of Experimental set up for capillary electrophoresis. B. Capillary electrophoresis setup.

2.6 Fluorescent Microscopy Setup

In this research, blue excitation light was used because NanoOrange Dye has a broad excitation peak centered at 470nm and an emission peak centered at 570 nm [7]. Excitation wavelengths were provided by a mercury lamp from Nikon Instruments (Melville, NY), and the emission light was captured by filter cubes which were purchased from Omega Optical (Brattleboro, VT). Protein fluorescent spots were detected by the 2X objective lens of the inverted Nikon Eclipse TE2000-U (Melville, NY), and then the signal was transferred to the ProEM 512 CCD camera (Princeton Instruments, Trenton, NJ). To observe protein motion as fluorescent spots inside the capillaries on the computer, software Winview 32 (Princeton Instruments, Trenton, NJ) was used. After

protein motion was recorded as a movie, electropherograms can be conducted to visualize

the separated peaks.

CHAPTER 3: RESULTS AND DISCUSSION

3.1Uniformed Packing with All Particle Sizes

Pressure was applied to all the capillary packing, and uniformed packing was achieved with all the particle sizes. Yet, smaller particle size results in the more ordered packing. In figure 3.1, the particle sizes from above to bottom are 350 nm, 500 nm, and 750 nm, and the blue hue decreases with the increasing particle sizes. When fill liquid into the interstitial space of packed capillaries, their bragg diffraction angle of light shifts and give rise to a blue color. When observe the ethanol rinsed packed capillaries under the zoom microscope, smaller particle size results in a brighter color with opalescence, whereas the larger silica particles provide more faded colors.
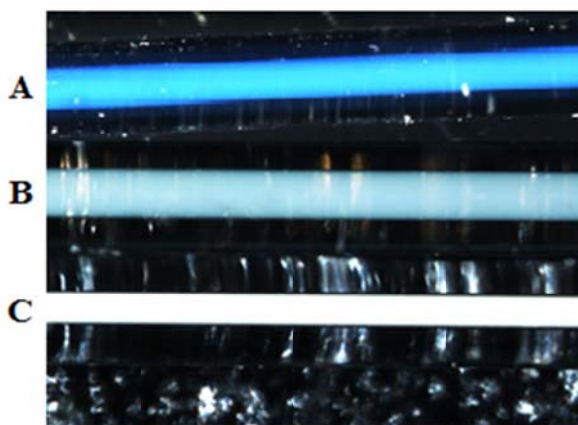
Figure 3.1 Comparison of different sized particles packed inside the capillaries. A. 350 nm SCC packed bed shows opalescence with a bright blue color. B. 500 nm SCC packed bed provides a faded blue color. C. 750 nm packed capillary shows a white color.

Figure 3.2 shows the zoom microscope image of the two capillaries both packed with 350 nm SCC inside 100 id. Capillary A is uniformly packed with no obvious crack in the middle, thus it is ready to undergo the modification process. Capillary B shows a crack in the middle of the capillary, which could be resulted from rapid depressurization. The peak shape will be altered while proteins travel through the cracked portion. Thus, capillaries like this will not be applied in the research.
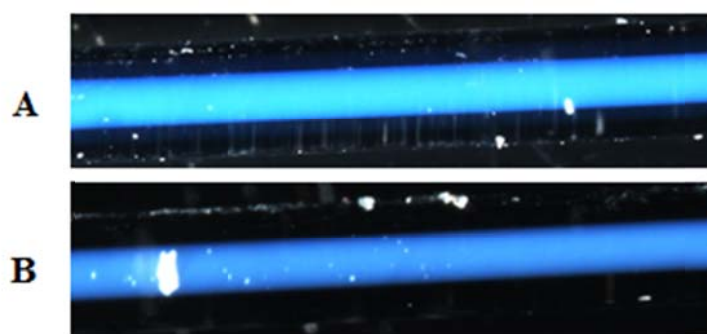
Figure 3.2 A. 350 nm packed capillary with no obvious crack. B. 350 nm packed bed with crack in the middle.

3.2 SDS Affects NanoOrange Fluorescent Intensity

Initially, NanoOrange protein samples were prepared with the dilution buffer provided by the protein quantitation kit and 1% SDS was mixed with the TG-SDS buffer. Fluorescent signal was not observed inside the capillary, even after connect the system with electricity for a long time. So the pre- mixed stacking buffer replaced the dilution buffer. Stacking buffer contains multiple ions that trap protein sample in between and obtain sharp peaks. In the stacking buffer, 2% SDS was added and heated with protein.

Protein fluorescent spot was visualized with this preparation method, thus being used for the later analysis.

SDS is required in this experiment in order to observe in- column fluorescent spots. As a surfactant, SDS binds to the protein molecules, and unfolds them into linear structures with negative charges on the surface. In this situation, NanoOrange will either bind to the evenly charge protein-SDS complex surface or the hydrophobic region inside the micelle (Figure 3.3). NanoOrange will undergo a conformation change upon binding, and get enhanced fluorescent signal.
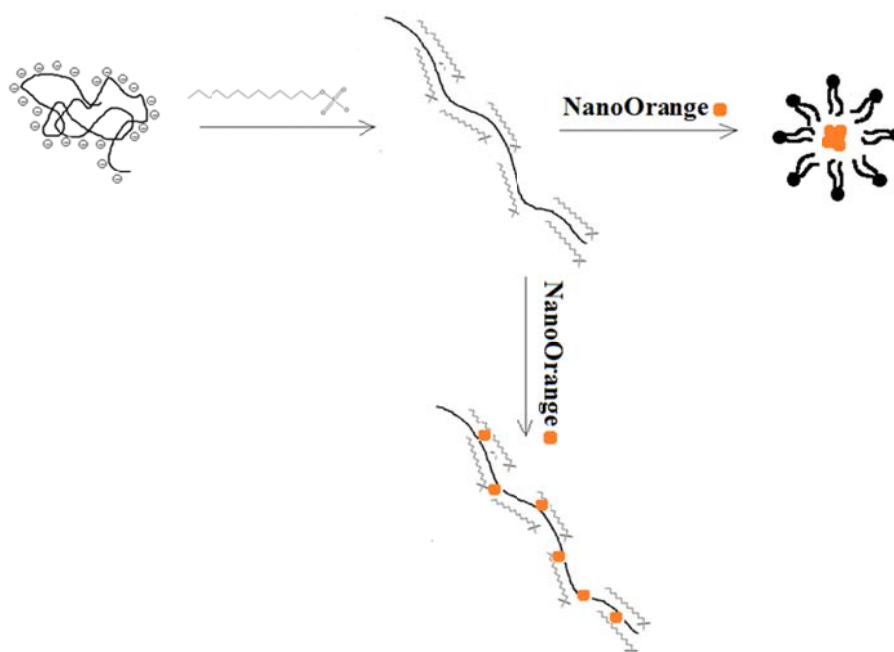
Figure 3.3 Association of NanoOrange dye with Protein-SDS complex. Two forms may be observed. NanoOrange will either be contained inside the protein micelle or attach to the linear protein surface.

<u>3.3 SCC Size Determines the Separation Efficiency</u>

Silica colloid particle size is one important factor that affects the separation efficiency. Lysozyme, trypsin inhibitor, and carbonic anhydrase are the three model proteins, and their molecular weight and radii are listed in table 3.1 below. The result of 750 nm, 500 nm, and 350 nm silica particles were compared, and theoretically, smaller particle size improves the separation [13].

Table 3.1 Size and weight of the Three Model Proteins

Protein	M.W. (kda)	Radius of gyration (nm)
Lysozyme	15	1.9
Trypsin inhibitor	20	2.3
Carbonic Anhydrase	29	2.8

The protein mix was first separated with 750 nm particle packed capillaries (Figure 3.4). They are separated with a huge cluster spot in the middle, and a slightly faded spot leading. Theoretically, the three proteins were equally mixed, and the spots should have similar brightness. There is a high possibility that the smear in the middle contains more than one protein. Thus the separation efficiency is in need to be improved, and the research was moved onto using 500nm particles packed capillaries.
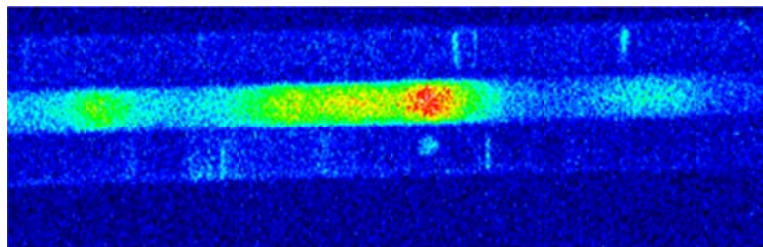
Figure 3.4 Protein was separated with 750 nm particles.

Separation with 500 nm particle packed capillaries provides more widely separated spots at 8mm, but some tailing and background noise were still observed (Figure 3.5). To observe more resolved peaks, the research moved onto using 350 nm particle packed capillaries.
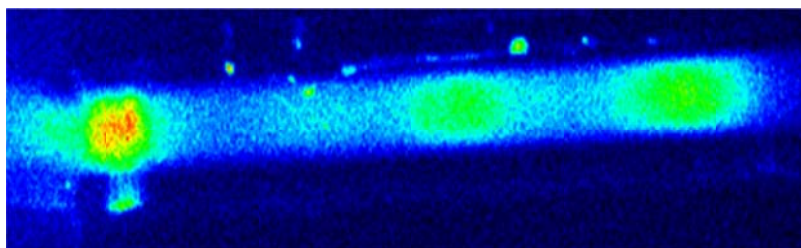
Figure 3.5 Model protein was separated with 500nm particles.

The three proteins have similar motion rate initially, but the last spot tend to stop moving very soon. While generating electropherogram, an image window needs to be selected and all three spots need to travel through it. Since the last spot was not able to go through the window, an electropherogram with all three peaks may not be generated. In order to conquer this problem, the sample preparation step may be revised by modifying

the loading buffer. Once this problem is solved, electropherograms may be conducted in the future to observe the separation efficiency.

Previously described by Birdsall et al., 350nm particles packed micro-channels were capable to partially resolve the three model proteins. But the capillaries were pressure packed and the particle arrangement was tighter than micro-channels. Protein motion was not observed in the 350 nm close-packed capillaries. To increase the pore size, the polymerization process was reduced by half time, half acrylamide concentration, and both half time and half concentration. None of the three small proteins moved forward inside the capillary.

The molecular weights of three model protein were equal or less than 29 kDa, and they should easily move inside the pores between 350 nm particles. The pore sizes were calculated with the silica particle radii as showed in figure 3.6, and the values are listed in table 3.2. Theoretically, silica colloidal particles packs into face-centered-lattice in capillaries, and the 350 nm particles should have pores with radius of 145 nm. The three small proteins have radii between 1.9 to 2.8 nm, so there should not have any problem moving inside the pores. After eliminating the cause of pore size, the lack of protein motion is thought to be from the interaction between themselves and the nanoparticles.

Table 3.2 Pore radii from closely packed particles of varies sizes

Particle Diameter (nm)	Pore radius (nm)
350 nm	145
500 nm	207
750 nm	311
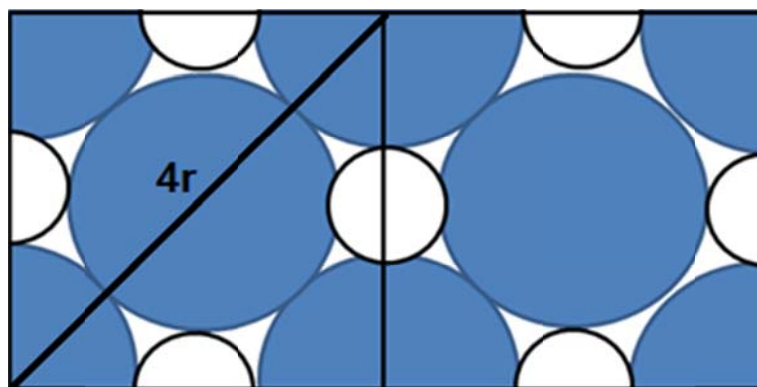
Figure 3.6 Face-centered cubic packing of silica colloidal particles

Another issue is the reproducibility of the experiment. The AGET modified packed capillaries get clogged after each run and the results were believed to be not reproducible. To remove the protein cluster, capillaries were run with in 10X concentrated TG-SDS buffer. ATRP takes place in a sealed reaction vessel and the condition may reduce the amount of protein clustering inside the capillary. ATRP shall be tried in the future to compare with the result from AGET modified capillaries.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Future Directions

In order to get the protein sample travel through 350 nm particles, other methods are designed and to be tried in the future. Because the model proteins were observed to move inside the 500nm packed capillaries, the capillary can be packed into two sections just like figure 4.1. The 10% 500 nm silica particle solution can be first wicked into a 12 cm capillary, and pressure pack at 7500 psi. After getting a short plug with 500 nm particles, a carrier capillary filled with 35% of 350 nm silica solution will be added on top of the packed one. The capillary will be pressure packed one more time and form a plug above the 500 nm portion like the figure drawn below. For a capillary like this, the protein should at least move through the 500 nm part, and have a potential to move forward inside the 350 nm plug.

Figure 4.1 Capillary packed with 500 nm particles in the front, and followed with 350 nm particles.

4.2 Conclusions

The three model preoteins, lysozyme, trypsin inhibitor, and carbonic anhydrase were separated with both 500 nm and 750 nm silica packed capillaries. As an on- column labeling method, NanoOrange non-covalently binds to the protein to keep them in their native conformation and saves the sample labeling time. To continue this work, some pharmaceutical important protein drugs like IgG and PSA have a potential to be separated with the same method. Also, combining with magnetic solid-phase extraction or other extraction methods, this separation method can be applied to real- life clinical sample analysis [14][41].

PART TWO: DEVELOP COST- EFFECTIVE DNA MICROARRAY FOR

POINT-OF-CARE APPLICATION

CHAPTER 5: INTRODUCTION AND BACKGROUND

5.1 Introduction: Imbalanced DNA Methylation, Cause and Effects

In most industrialized countries, obesity is a fast growing disease that millions of patients suffering from. Adults are trying different methods to lose weight, but limited number of people is concerned about infant patients and how DNA methylation affects baby obesity. Folic acid, S-Adenosyl methionine (SAM), and vitamin B are the key component for the methyl making pathway, and they are suggested during pregnancy to aid the methylation process of the baby's DNA. With too little methyl-donating nutrient between the time in the womb to earlier age, certain regions of the human genome can be under-methylated lifelong, and thus suffering from diseases [15]. The defects of lacking DNA methylation was previously studied in University of Utah. In addition to causing obesity, the mice with complete agouti gene demethylation also suffers from increasing risk of cancer and diabetes [16].

DNA methylation alters gene expression by adding a methyl group to the cytosine which located next to guanine. Between 60% to 90% of CpGs are methylated at the 5 position on the cytosine ring, and tipping the balance between methylation and lack is known to cause cancer, obesity and other types of serious disease [17][45][46]. Imbalanced DNA methylation may affect gene transcription by impede its binding to transcriptional proteins and the formation of compact and inactive chromatin[18][26].

A fast, cost effective, and portable diagnostic tool is desired to track the amount of methylated DNA frequently. Treatment and diet change may be applied immediately to save the child from irreversible situation.

5.2 Current Analytical Techniques for DNA Quantitation

5.2.1 DNA Microarray

Microarray technology is one important tool in life science research, and it can also be applied for clinical purposes. To analyze and quantify a DNA target, its complementary oligonucleotides are immobilized on solid substrates [19]. Then the fluorescent labeled analyte binds to the spot, and can be visualized under the detection instrument[47]. Traditionally, microarray focuses on gene expression quantitation and analysis, and it is now also increasingly used in protein and carbohydrate analysis.

For microarray clinical diagnostics, faster analysis can be obtained by increasing the sensitivity. In addition to quantify the known DNA with its comprehensive libraries of oligonucleotides, high sensitivity microarray is also capable to capture and detect genes with low expression rates[27]. As the optical detection method was greatly improved, the amount of analytes bound to each microarray spots became the bottleneck.

5.2.2 Hydrophilic Gels

Hydrophilic gels is one method to provide a large volume of analyte on the spot, but its long mass transportation time and strong background noise defeats the purpose of improving sensitivity [20]. Increasing the amount of analytes bounding concentration on

the spots can be achieved by utilizing sub-micron silica colloidal particles which enhances spot surface area. Silica colloidal particles can be uniformly packed into face-centered cubic, extend spots into three dimensions [21][28]. Advantages like neglectable mass transportation time, reduced scattering, and cost-efficiency makes silica colloidal particles to be one promising material for DNA microarray technology.

5.3 Research Goals

In this research, a cost effective DNA microarray is expected to be established to quantify DNA methylation. In order to commercialize and apply the DNA microarray in daily life, the quantitation efficiency and uniformity was in need to be studied. On one single microarray slide, multiple spots for different samples may be tested. After optimize spot size and solid surface material, the microarray is desired to be applied in point-of-care to detect obesity, cancer, and other DNA related diseases.

5.4 Project Overview

Chapter five reviews the background of DNA methylation. In human genome, the amount of DNA methylation needs to be balanced to keep the body from disease. Microarray, with the characteristics of being easy to use, is a candidate to monitor the DNA methylation as point-of-care. Previously in the Wirth group, silica colloidal crystal was proved to improve the amount of DNA bonding to the solid surface. In this research, the previous work will carry onto the DNA methylation microarray design.

Chapter six describes the detailed procedure of silica and solid substrate preparation. The hydrophilic fused silica slides were all prepared in the same fashion.

The size of the microarray spots were prepared differently to observe their influence upon the capturing efficiency.

For the result portion in chapter seven, the three batches of the microarray slides were prepared to compare with each other. To suspend silica particles, ethanol worked better while trying to remove the solvent.

Chapter eight reviews the possible directions to further develop the project. Safety and cost are the two important factors to determine if the microarray was feasible for everyday application. For the easy disposal and lower price, the project is shifting from quartz silica slides to transparent sheets. Since microarray was never developed on plastic materials, this project is obtaining a bright future for the application purposes.

CHAPTER 6: MATERIALS AND METHODS

6.1 Chemicals and Materials

Quartz silica slides were purchased from G.M. Associates, Inc. (Oakland, CA)).Silica colloidal particles of 150 nm in diameter were purchased from Fiber Optic Center (New Bedford, MA). Both the n-ocyadecyltrichlorosilane and 3-aminopropyltrimethoxysilane were purchased from Gelest Inc. (Morrisville, PA).

6.2 Silica Colloids Preparation

Silica particles were calcined three times at 600°C for 12 hours, and then annealed at 1050°C for 3 hours. Then the particles were rehydroxylated in 50% nitric acid solution. After rinsed, filtered, and dried in the vacuum oven, silica slurries were prepared. Previously described by Zheng et al., silica colloids were prepared into 10, 5, and 2.5 mg/ml slurries [22]. Two sets of a total of 6 slurries were generated: one set dissolved in water and the other in 100% ethanol. All the slurries were stored in 7 drum vials.

6.3 Quartz Silica Slide Preparation

6.3.1 Condition and Rehydroxylation

Quartz silica slides are used to carry silica colloidal particles and to develop the microarray on. The commercially bought slides are first cleaned with 0.1M NaOH base bath, and followed by rising with ultrapure water and ethanol. Rehydroxylation was done at 270°C with a 300 ml equal mixture of HNO_3: H_2O (w/w) for 12 hours to regenerate silanol groups on the slides for the following chemical modification.

6.3.2 Silane Reaction for the Hydrophobic Slide Surface

After rinsed with ultrapure water for 3 times and with ethanol for 1 time, the slides are dried to prepare for a silane reaction. To convert the whole slide into hydrophobic, a 2% C18 in toluene was reacted with the silica surface for 12 hours. After rinsed with anhydrous toluene, the rehydroxylated slides were kept in the sealed reaction vessel to maintain a contamination-free environment.

6.3.3 Microarray Spot Assembly

Three sets of slides were designed like shown in figure 6.1, and prepared in parallel to compare the results. Previously described by Zheng et al., silica colloidal crystal is capable to enhance the sensitivity of the microarray by letting the sample traveling through the pores. For the first set, Silanol groups were then regenerated to pack layers of silica colloids on the 6 DNA capturing spots. Etchant was used to cleave off the hydrophobic chain and retain the hydrophilic surface on the desired spot while

the rest of the slide remains hydrophobic [3]. Vinyl sheets with 6 holes were stick onto the slides to prevent the hydrophobic portion from the etchant. Etchant solution was then smeared on the silica surface. To observe evenly etched hydrophilic surface, etchant solution was re-layered every 10 seconds for a total of 1 minute. Q-tips are then rinsed with water and used to wipe off the etchant solution thoroughly. The slides were dipped into the previously described slurries for the hydrophilic spots to capture silica colloids. The slides were set horizontally for the solvent to dry up, and repeat the same procedure to obtain multiple layers to achieve the optimized efficiency.

The second set of slides was prepared with silica colloidal crystal packed on the whole surface. The exact rehydroxylation step described above was done on the whole slide. The whole slide was dipped into the 10mg/ml 150 nm silica suspension, and getting dried in the 60°C vacuum oven. This step repeats 4 to 10 times depend on the desired thickness of the silica layer [23].

The last batch of slides was spotted with the traditional microarray spotter and silica colloidal crystal pile up on each location. The slide modification was done like described above until after the silation step. Microarray spotter was dipped into the etchant to get it on each tip. Then the tips were stamped onto the slide surface to cleave off the hydrophobic C18 chain and regenerate the silanol groups for the micro-spots. After removing the etchant with ultrapure water, the slides were set inside the silica solution, and dried under 60°C vacuum oven. After this step, the microarray is ready for adding the DNA probe, and capturing methylated DNA.
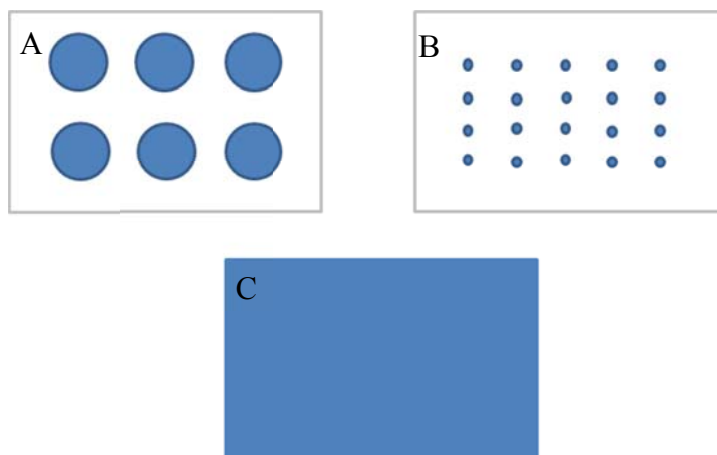
Figure 6.1 Three designs for the microarray slides. A. Six macro spots with silica colloids on the surface. B. Traditional microarray slides with multiple micro spots. C. Cover the whole slide with SCC.

CHAPTER 7: RESULTS AND DISCUSSION

7.1 Silica Slurry Solvent Optimization

Because this is the early stage of the project, the microarray is only at the first phase. Two solvents were compared by their apparent benefits.

Water was chosen because bare silica suspends the best in it, and the slurries will only stick onto the hydrophilic part of the slide. The solvent takes a long time to dry after each dip. Drying the slide vertically gave uneven layers of silica surface; drying horizontally carries a "coffee-cup ring" on the edges. In order to get rid of the ring, the slide was set inside the oven horizontally for 3 minutes to remove the solvent. This fast drying method worked on the traditional microarray slides, and it will be researched on in the future on the other two sets of microarray slides.

Slurry set two was suspended in ethanol. Ethanol was the preferred solvent by its easy drying property, but its hydrophobicity caused silica particles spread on the slide surface. The slides were dried both vertically and horizontally, but silica smear remained on the hydrophobic portion. To strengthen the hydrophobicity of the slide surface, the portion of n-octadecyltrichlorosilane will need to be increased in the future development of the project.

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7.2 Hydrophobicity of the Slides: Dipping Results

One current existing problem is the hydrophobicity of the slide surface. Previously, n-octadecyltrichlorosilane (T-C18) and trichloromethane (T-C1) were reacting with the slide surface at 2 to 1 ratio, where trichloromethane was behaving as a spacer between the hydrophobic C18 chains. This type of slide surface is not hydrophobic enough so when dipping inside the ethanol suspension, the slurry smears on the whole slide. To improve this situation, the hydrophobicity is in need to be increased. In the future, two sets of surface will be experimented to study the surface. The first batch of slides will be modified with T-C18 only. One concern about this method is that the C18 chains are too long, and without the spacer, many silanols may be exposed and make the surface hydrophilic.

The other silation method is to modify the slide surface with C18:C1 at a 20:1 ratio. The majority of the surface area will be associated with the long hydrocarbon chain and being more hydrophobic; the excess silanol group can be modified with the spacer. The overall hydrophobicity can be increased this way (Figure 7.1).
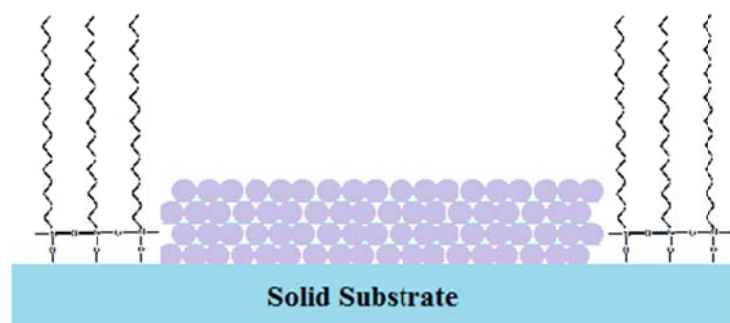

Figure 7.1 Side- view of DNA Microarray. Pack microarray spots with SCC, and grow
C18 on the other parts of the slide to maintain hydrophobicity.

CHAPTER 8: CONCLUSION AND FUTURE DIRECTIONS

8.1 Conclusions

At this early stage of the project, many factors of the silica slides are still in need to be studied prior moving towards DNA quantitation. Until this point, silica colloidal particles were proved to be able to associate with the hydrophilic silica surface. Etching the silica surface for one minute was able to cleave off the hydrophobic C18 chain.

8.2 Future Directions

One direction for the project to do is to modify the solid surface from glass to transparent plastic sheet. Hydrophobic toner can be used to print on the slide surface, and the microarray spots will be left blank and being hydrophilic[25]. Then the silica particles can be packed on those areas to achieve high fluorescent intensity just like discussed above.

After getting the optimized slide surface, the research can be continued to the next phase where DNA probe will be grown on the silica surface. According to Palanisamy et al., a molecular inversion probe was designed to incorporate inosine nucleotides in it and complement potential DNA methylation sites [24]. Target unmethylated DNA can be recognized with thus sequence and get measured. A method may be designed in the future to grow such DNA probe onto silica colloids. Then, the

methylated NDA will be labeled and captured by the probe. For this step, miniaturized cell-phone based fluorescent microscope can be used for the detection part to make the DNA microarray more cost-efficient and portable.

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