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Monolithic protein stationary phases prepared by the sol-gel method for use in affinity chromatography

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Monolithic protein stationary phases prepared by the sol-gel method for use in affinity chromatography

by

Jeffrey Aaron Crank

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
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Iowa State University
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Jeffrey Aaron Crank

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
To Wayne and Erma Crank for inspiration to succeed and to Greg and Caryl Crank for support and giving me the strength to persevere.
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ABSTRACT

Capillary monolithic silica columns used for normal and reversed phase separations offer the advantage of lower backpressure and higher efficiency compared to packed columns. However, capillary monolithic columns have not been applied to affinity chromatography. We used the sol-gel method to encapsulate proteins in a silica matrix for capillary affinity chromatography. A monolithic protein stationary phase was prepared by the tetramethyl orthosilicate procedure. The protein containing sol was injected into a 75µm i.d. capillary that had been pretreated with sodium hydroxide. The sol gelled into a rigid micro-porous and macro-porous stationary phase. The porosity of the gel was characterized by scanning electron microscopy and Brunauer-Emmett-Teller adsorption/desorption isotherms. Ubiquitin, bovine serum albumin (BSA), and catalase at different loadings were used in preliminary tests to determine the effects of protein size and concentration on the stationary phase properties. Preliminary separations were performed using the pumps from a capillary electrophoresis instrument with BSA doped monolithic columns.
CHAPTER 1. INTRODUCTION

Affinity chromatography is a liquid chromatographic technique in which a biologically active molecule is attached to a silica support to serve as the stationary phase. These biologically active molecules range from metal chelates [1] to proteins. This technique takes advantage of the natural selectivity of the immobilized molecule for particular analytes. The silica support is typically a bonded diol silica [2] that is activated with either 1,1-carbonyldiimidazole [3] or N-hydroxysuccinimide ester [4]. Activation allows for the covalent attachment of proteins or other biomolecules to the stationary phase. Once immobilized, the biomolecules separate analytes based on molecule-molecule, protein-molecule, or protein-protein interactions. Although these proteins are highly selective for analytes there is still a need to develop columns with higher efficiency.

The total performance of a column can be described by the van Deemter equation.

The van Deemter equation states $H = Au + \frac{B}{u} + Cu$ where $A$ equals eddy diffusion, $B$ equals the molecular diffusion, $C$ equals the resistance to mass transfer, and $u$ equals the linear velocity. Each of these factors accounts for a different type of band broadening. Eddy diffusion accounts for the different path lengths that an analyte adopts while traveling through the stationary phase. If the diffusion path difference is large then the band will be broadened. Molecular diffusion accounts for the diffusion of the analyte along the axial direction, however because of the low diffusion coefficient of liquids this term is typically ignored. This term is proportional to the time the analyte spends on the column and only contributes to band broadening in liquid chromatography when the flow rate is below optimal. When the mobile phase flow rate is too slow the diffusion coefficient becomes
significant and the analyte will diffuse in all directions causing the band to be broadened. The resistance to mass transfer term includes both mass transfer from the mobile phase to the stationary phase and mass transfer within the stationary phase. The mass transfer within the stationary phase is typically ignored because diffusion in a solid is very slow. Mass transfer from the mobile phase to the stationary phase is the major contributor to the resistance to mass transfer term. Significant band broadening occurs due to resistance to mass transfer when the packing material is large and the diffusion coefficient of the analyte in the mobile phase is small. Band broadening of the mass transfer term is proportional to the square of the particle size and inversely proportional to the diffusion coefficient of the analyte in the mobile phase.

Many methods have been used to achieve higher efficiency, examples include the development of ultra high pressure liquid chromatography (UHPLC) [5], open tubular columns, capillary electrochromatography (CEC) [6,7], and monolithic columns [8]. UHPLC increases efficiency by reducing the particle size of the packing material to less than 5 µm. Smaller particles result in reduced eddy diffusion and resistance to mass transfer. However, as the particles become smaller the backpressure dramatically increases. Typical pressures of UHPLC are on the order of 100,000 psi. Although UHPLC increases efficiency, it is not widely used because the instrumentation is often problematic and costly. CEC has gained wider acceptance. CEC combines the strengths of capillary electrophoresis and liquid chromatography. CEC uses small packed particles and electrophoretic flow to increase efficiency. Small particles are used to decrease eddy diffusion. This method employs electro-osmotic flow to overcome the high backpressure associated with small particle size and to decrease the width of the analyte band. Open tubular columns also increase the
column efficiency by reducing eddy diffusion and the resistance to mass transfer. Eddy diffusion is essentially zero because the stationary phase in open tubular columns coats the column wall, which makes the path length of the each analyte molecule the same. The resistance to mass transfer term is reduced due to the thin coating of the stationary phase on the column wall. The thin coating allows for fast diffusion in and out of the stationary phase. However, this method has not gained wide acceptance because of limited loading of the analyte. Monolithic columns have found the most success. Their small continuous skeleton with large through pores allows for a small particle size, decreased diffusion path length, and decreased backpressure. Monolithic columns have been used in combination with both CEC and liquid chromatography to yield the most efficient affinity chromatographic separations to date.

Monolithic stationary phases are generally prepared by the acid catalyzed hydrolysis of a tetraalkyl orthosilicate, typically tetramethyl orthosilicate. Hydrolysis of the tetraalkyl orthosilicate produces a solution of orthosilicic acid and alcohol, often referred to as the sol. The sol is then subject to a series of polycondensation and water elimination steps during which gelation occurs (Figure 1). Many different types of monoliths can be prepared by changing the starting materials or by functionalizing the silica after gelation. Normal phase columns are the simplest monoliths to prepare and require no change in starting material to produce bare silica. Reversed phase monoliths can be prepared by using either an alkyl trialkyl orthosilicate as the starting material [9,10] or by functionalization of the silica after gelation has occurred [11,12]. Affinity chromatography columns can be prepared by either adding protein or other biomolecules directly to the sol or by covalently attaching them to the silica.
Monolithic columns have many advantages over packed columns, including low pressure requirements, high efficiency, and ease of preparation. Monolithic columns have a low pressure drop that results from the numerous large through pores in the skeletal structure which decrease the flow resistance. Pressures that are typically used for monolithic liquid chromatography are less than 100 psi. Secondly, monoliths exhibit higher efficiency than packed columns. Some monoliths show as many as 100,000 theoretical plates. The higher efficiency is due to the small skeleton size and large through pore size. Monolithic columns also require no packing or frits. It is often difficult to pack the capillaries used in CEC or micro-LC with beads. Since gelation takes place inside the capillary, no packing is necessary. Frits for CEC and micro-LC are also difficult to prepare reproducibly and can cause air bubbles within the column which disrupt the flow of the mobile phase. Monolithic stationary phases covalently attach to the capillary wall. This attachment occurs through condensation reactions between orthosilicic acid and free silanol groups on the capillary wall, therefore frits are not needed. In addition, monoliths show no evidence of gel shrinkage, require relatively little protein for immobilization within the stationary phase, are
easily prepared, and proteins are stabilized by the silica matrix. This stabilization occurs due to hydrogen bonding interactions between the silanol groups and the protein and also because the protein is constricted in the pore. This constriction only allows a small degree of protein unfolding which preserves its conformation.

Until recently few improvements have been made in the area of affinity chromatography [13]. Most of the studies in this field has dealt with packed immunoaffinity columns [14-16]. Immunoaffinity columns often have sufficient selectivity but are applicable to only a small number of analytes. Related studies with monolithic columns have shown wider applicability. These studies involved the encapsulation of bovine serum albumin within a silica matrix [17,18]. Albumins have been shown to selectively bind many different molecules. The encapsulation of proteins within the matrix is preferred over covalent attachment for many reasons. Covalently attached proteins often have altered properties. Often when a tether is attached to a protein the affinity and shape of a protein are significantly altered and result in reduced binding constants. Proteins encapsulated in silica on the other hand have been shown in many cases to be stabilized by the silica [2,19-23]. Herein we report the preparation, characterization, and performance of a protein monolithic stationary phase for use in affinity chromatography.

References


CHAPTER 2. CHARACTERIZATION OF MONOLITHIC SILICA

Introduction

Morphology of silica stationary phases in liquid chromatography is an important aspect in separations. However, the characterization of monolithic silica has not been stressed in recent literature. Instead, only the performance of monolithic silica columns has been reported. This study presents the characterization of monolithic silica stationary phases under many different experimental conditions and by many different techniques. Monolithic silica has been characterized with a range of proteins and with varying concentrations of proteins. The bulk properties of monolithic silica have also been characterized. Characterization was carried out with light microscopy, scanning electron microscopy, and nitrogen adsorption-desorption analysis.

Experimental

Chemicals

Tetramethyl orthosilicate (TMOS) was obtained from Acros. Polyethylene glycol (PEG) M.W. 10,000, sodium hydroxide, acetic acid, ammonium hydroxide, ethanol, ubiquitin, bovine serum albumin, and catalase were purchased from Sigma Aldrich. Trifluoroacetic acid was obtained from Alfa Aser.

Equipment and supplies

Fused silica capillary (75 µm I.D.) was purchased from Polymicro Technologies L.L.C. TTF teflon tubing was obtained from Valco Instrument Co. Inc. Scanning electron microscopy (SEM) experiments were performed on a JEOL 840A SEM with a tungsten
filament. Calcination experiments were carried out in an Applied Test Systems Inc. series 3110 tube furnace. Nitrogen adsorption-desorption data was collected on a Micromeritics ASAP 2000 sorptometer.

**Sol-gel Procedure**

The procedure for sol preparation was adapted from Ishizuka et. al. The sol was prepared by dissolving 1.06 g PEG in 10 mL of 0.01 M acetic acid. TMOS (10 mL) was then added to the solution and stirred at 0°C for 45 min. For undoped gels, no protein was added. For doped gels various amounts of protein were dissolved in water, added to 2 mL of fully hydrolyzed sol, and stirred at room temperature. Ubiquitin and bovine serum albumin were added to the sol to produce a concentration of 1, 2 and 10 mg protein/mL of sol. Because catalase is only sparingly soluble in water, it was loaded only at 1 and 2 mg/ml of sol. It should be noted that as the protein was added to the sol the solution became turbid to different degrees depending on the identity and concentration of the protein.

**Preparation of capillary columns**

Sodium hydroxide (1.0 M) was injected into the capillary with a syringe that had been inserted into a stretched section of teflon tubing. The syringe was then removed from the teflon tubing and the ends of the capillary were sealed by inserting the free end of the capillary into the teflon tubing where the syringe had been, thereby creating a loop. The capillary was then incubated in a water bath at 40°C for 3 hrs. This pretreatment was used to produce an elevated number of silanol groups which are used to attach the gel to the capillary wall[24]. The sodium hydroxide was then expelled from the capillary with air from a
syringe. The capillary was washed with water, followed by removal of the water with air. The sol was injected into the dry capillary and placed in an oven at 40°C overnight. Parafilm was stretched over the ends of the capillary to ensure that the gel remained wet. Several centimeters were cut off each end of the capillary and air was passed through the column to remove water and methanol. The total column length was 39 cm with an effective length of 29 cm. The column was washed with 0.01 M ammonium hydroxide and incubated at room temperature for 3 hrs to increase the number of micro-pores and meso-pores [11]. The ammonium hydroxide was removed with air and the column was washed with ethanol, filled with water, and stored in the refrigerator at 4°C.

Characterization

Nitrogen adsorption-desorption analysis

For nitrogen adsorption-desorption analysis, the sol was prepared as stated above. The sol was then cast into polystyrene cuvettes. The cuvettes were covered with parafilm and incubated at 40°C overnight. The gel was then submerged in 0.01 M ammonium hydroxide for 3 hrs, washed with ethanol, and allowed to air dry. Once dry, the gel was placed in a quartz jacket and calcinated at 400°C for 24 hrs with a ramp time of 1 hr. Next, the gel was ground in an agate mortar and dried under vacuum overnight. Nitrogen adsorption-desorption analysis was subsequently carried out. Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) calculations were used to obtain surface area and pore size distribution, respectively.
SEM Analysis

For SEM analysis, the sol and capillaries were prepared as stated above and cut widthwise in several places before analysis. Each capillary was cut several times and analyzed to ensure that a representative sample was taken. An accelerating voltage of 20 kV was used for all SEM imaging.

Results and Discussion

Silica morphology

All capillaries were inspected with light microscopy to detect shrinkage of the gel. In all cases no voids or shrinkage were observed. SEM was used to monitor the morphology of the silica. It was found that the silica particles were on the order of a nanometer and the silica had large through pores. The through pore dimensions are controlled by hydrogen bond interactions between the PEG units and the polymerizing sol[25-27]. PEG units below 10,000 M.W significantly reduce the through pore dimensions, which reduces the flow of the column. The absence of shrinkage was also observed by SEM. The silica skeleton was found to be attached directly to the capillary wall and to fill the whole capillary as seen in figure 2.

Figure 2) SEM images of undoped monoliths (a) 1,000x magnification (b) 3,000x magnification
It was also observed that the silica monolith does not degrade after many chromatographic runs. After 74 runs the silica still had no appreciable morphology change (data not shown).

**Effects of protein identity**

The effects of protein identity, protein concentration, and the morphology of bulk gel were investigated by SEM. Three different proteins were selected to span a wide range of molecular weights and surface properties. Ubiquitin, bovine serum albumin, and catalase were encapsulated in silica. The molecular weights of these proteins are 8,600 Da, 66,000 Da, and 230,000 Da respectively. The surface properties of these three proteins vary in motif and isoelectric point (pI). Ubiquitin is small single chain protein that has both α-helix and β-sheets with a pI of 6.8. Bovine serum albumin is a double chain protein and has an all α helical structure with a pI of 4.7. Catalase is a 4 domain, 4 chain protein with 4 hemes that has both α-helix and β-sheets and has a pI of 5.2. Even with the many differences between these proteins, there was a negligible change in morphology of the silica due to the identity of the protein.

Figure 3) SEM image of Monolithic silica doped with 2 mg/ml of (a) ubiquitin (b) bovine serum albumin (c) catalase
Figure 3 shows monoliths doped with 2 mg/mL of ubiquitin, bovine serum albumin, and catalase at 1000x magnification. Even when the silica is examined at 3000x magnification no difference in morphology can be seen (figure 4). The silica particle and through pore dimensions stay relatively constant, but because of the amorphous nature of the silica very small changes are difficult to detect. The absence of morphology change is a great advantage to this method. The absence of morphology change allows for the encapsulation of many different proteins without the fear of changing the column morphology. This allows the method of encapsulation of proteins in silica monoliths for liquid chromatography to be applicable to many analytes without large procedure changes. Many proteins are selective for specific families of compounds (i.e. fatty acids, small organics) and can be easily encapsulated to separate compounds in these families without changing the morphology of the column. However, the protein identity did affect the gelation time with the albumin sol gelling first, ubiquitin sol gelling second, catalase sol gelling third, and the gelation of undoped sol last. Proteins have been shown to catalyze the hydrolysis and gelation of silicates [28-30]. This effect accounts for the differences in gelation time of the ubiquitin,
albumin, catalase, and undoped sols. With the fact that the identity of proteins will affect the gelation time but not the morphology of the silica, it is assumed that any number of different proteins for further separation applications could be encapsulated without silica morphology change.

**Effects of protein concentration**

Three different protein concentrations were investigated. Proteins were loaded into the sol at concentrations of 1, 2 and 10 mg protein/mL of sol. The loading of a sol with 20 mg/mL was attempted, but gelation occurred too quickly. This quick gelation did not allow the gel to attach to the walls of the capillary and the gel was ejected during washing. No appreciable difference could be seen between the monoliths containing different concentrations of protein. Figure 5 shows SEM photographs at 1,000x magnification of monoliths doped with bovine serum albumin, at different concentrations.

![SEM images](image)

Figure 5) SEM images of monolithic silica columns with albumin concentration of (a) 1 mg/ml (b) 2 mg/ml (c) 10 mg/ml

When these gels were examined at 3,000x magnification, there was still no appreciable difference in morphology. It was also observed that the concentration of protein influenced gelation time. As protein concentration increased the gelation time decreased. This
phenomenon is assumed to be closely related to gelation due to protein identity. Due to the fact that proteins cause gelation, it is logical to speculate that as the concentration of the protein increases, the amount of catalyst increases, and the number of nucleation sites increases which decreases gelation time.

**Bulk Gel**

The mass of gel in the capillaries was insufficient for nitrogen adsorption-desorption experiments so bulk gel samples were prepared. Experimental conditions were varied to ensure that the bulk silica gel conditions and the capillary silica were the same. The bulk gel conditions most similar to the capillary conditions are stated above in the experimental section. Figure 6 shows a SEM comparison between bulk gel and gel inside a capillary.

![Figure 6](image)

**Figure 6** Comparison of bulk gel (a) and gel inside capillaries (b)

A comparison was also made between bulk gel and ground gel. It is necessary to make this comparison to ensure that the physical structure of the silica did not change and nitrogen adsorption-desorption data was not biased by a physical change in the silica. Figure 7 shows that the silica gel had no change in morphology when ground. SEM was also used to ensure
that there was no morphology change in the silica after the sample was calcinated and
ground. Figure 8 shows that there is no change in the particle and through pore dimensions
of bulk silica after the silica was calcinated and ground.

![Figure 7) SEM images showing that there was no morphology difference between (a) bulk and
(b) crushed gel](image1)

Figure 8) SEM images at 1,000x magnification (a) and 3,000x magnification (b) showing no morphology
change in monolithic silica that has been crushed and calcinated

To ensure that the gel had no directionality, the gel was cut lengthwise and widthwise and
analyzed by SEM. Figure 9 shows a comparison of uncut bulk gel, gel cut widthwise, and
gel cut lengthwise. In all cases the gel did not show any directionality. This is an important
aspect when making monolithic columns because if a column was directional then separation
could be affected depending on the flow direction.
The effects of temperature on gelation and morphology of the silica was also examined. It was found that gelation is very temperature sensitive and that the morphology of the silica is affected by the gelation temperature. As the temperature of gelation is decreased, the through pores become smaller and the silica particles become larger. A comparison of silicas that have been gelled at room temperature and at 40°C shows that the room temperature gelation of the sol has increased the size of the silica particles by approximately a factor of 5 (Figure 10). The through pore dimensions have decreased in size, but because of the irregular shape of the through pores it is difficult to quantify the degree of change.

Nitrogen Adsorption-Desorption Analysis
Nitrogen adsorption-desorption isotherms showed type IV behavior which is consistent with multilayer adsorption followed by capillary condensation in micro-pores [31]. A typical isotherm is shown in figure 11.

![Figure 11](image)

Figure 11) Typical adsorption-desorption isotherm showing type 4 behavior

The hysteresis in the adsorption-desorption isotherm is due to the fact that the partial pressure for filling the pores is less than the partial pressure for removing the nitrogen. During the desorption phase the pores in the silica act as a bottleneck so that the partial pressure must be higher to fully desorb all nitrogen. BET calculations from nitrogen adsorption-desorption isotherms showed that the surface area of calcinated albumin doped silica was $746 \pm 4 \, \text{m}^2/\text{g}$ and that the surface area of calcinated undoped silica was $717 \pm 10 \, \text{m}^2/\text{g}$. The difference between the surface area of doped and undoped gel is not significant. The pore volume and average pore diameter for albumin doped gel and undoped gel were found to be $0.406 \, \text{cm}^3/\text{g}$ and less than 2nm, respectively. The average pore volume is given as less than 2nm because the lower limit of the instrument is 2nm. Silica that had not been calcinated had a surface area of less than $10 \, \text{m}^2/\text{g}$. This reduced surface area is due to the blocked adsorption of nitrogen by the organic PEG units. It is believed that the PEG forms a soft shell around the
silica particles and does not allow the nitrogen to enter the pores. This blocking occurs when either the ends of the PEG stick out of the surface of the silica, forming a layer, or by the PEG ends cross linking with one another and forming polymer chains across the pores. However, this blocking can not be total blocking of the surface because there is a definite difference in separation of the same analytes using doped and undoped columns as shown in chapter 3.

Conclusions

In this study a variety of different methods and experimental conditions have been used to characterize monolithic silica stationary phases prepared by the sol-gel method for capillary liquid chromatography. Inspection by light microscopy showed that the monolith completely filled the capillary and that there was no gel shrinkage. It was also observed through SEM that the gel did not shrink. SEM showed that the monolithic silica has small particles that are on the order of a nanometer and that there are many large through pores.

When ubiquitin, bovine serum albumin, and catalase were encapsulated in silica no morphology change was observed. However, a difference in gelation time was observed. This difference in gelation time is due to the identity of the protein. When sols were made with varying protein concentrations there was also no morphology change. Although the morphology did not change, the gelation time decreased as the protein concentration was increased. It was also shown that crushed and calcinated bulk gel has the same morphology as gel inside the capillaries and does not show directionality. BET calculations from nitrogen adsorption-desorption isotherms show that the surface area of doped and undoped calcinated silica are not significantly different and that uncalcinated silica has a greatly reduced surface
area due to nitrogen adsorption blocking. Finally because there was no morphology change
due to protein identity, no morphology change due to protein concentration, and no
significant difference in silica surface area it is assumed that any protein can be encapsulated
in silica and used as a monolithic stationary phase.

References

Chemistry 73 (2001) 420A.
[28] I. Slowing, N. Kostic, in Midwest Regional American Chemical Society Meeting,
CHAPTER 3. AFFINITY CHROMATOGRAPHY SEPARATIONS

Introduction

Affinity chromatography using proteins encapsulated in monolithic silica as a stationary phase has only recently been reported in the literature [17]. One shortcoming of these studies is the lack of comparison between doped and undoped column separation performance. This study presents a comparison between the separation performance of doped and undoped columns and the separation performance of these doped columns with small drugs and dipeptides.

Experimental

Chemicals and supplies

Chemicals and supplies are the same as outlined in chapter 2.

Equipment

All separations were carried out using a Beckman P/ACE MDQ capillary electrophoresis (CE) instrument with a single wavelength UV detector.

Sol-gel procedure and preparation of capillary columns

Sol-gel procedure and the preparation of capillary columns are the same as outlined in chapter 2.
**Affinity chromatography separations**

The flow rate of the column was controlled by air pressure (in psi) applied to the sample vial in the CE instrument. To determine a standard flow rate in units of volume per time, blanks with no injection were run for extended periods of time. The inlet vial and waste vial were weighed on an analytical balance before and after the run to determine flow rate. The pressures used in the experiments were 2 psi for the injection and 65 psi for the separation, which correlate to 3.3 nL/min (55.5 pL/s) and 270 nL/min respectively.

Analytical separation run times total 20min and 15s, 15 s for the injection and 20 min for the separation. All runs used 0.1% TFA in water as the mobile phase, held at a constant temperature of 22°C, and were monitored at either 214 nm or 280 nm. To determine whether protein was leaching from the column, comparisons were made between a series of blank runs on doped and undoped columns. The absorbance and chromatogram features of these blank runs were examined for differences and similarities. All $\alpha$ and $R_s$ values were obtained from retention times. Retention times were used because no dead volume measurement could be obtained due to the retention of all analytes by BSA.

**Results and Discussion**

**Protein Leaching**

Protein leaching, or the absence there of, was studied by comparing blank runs from doped and undoped columns on a sensitive scale. When blanks from doped columns were run a characteristic broad trailing peak was observed. (figure 12a) This was initially thought to be protein leaching. However, blanks run on undoped columns gave the same characteristic broad peak (figure 12b).
Also it was calculated with an extinction coefficient for albumin of .667 that if a capillary doped with 10 mg/ml of albumin leached all of its protein in the length of the detection window the absorbance would be .33. The broad peak had an absorbance of approximately .008 absorbance units without changing absorbance even after many runs. From this evidence it was concluded that there was no appreciable protein leaching. Also when the scale at which an analyte is typically seen is taken into account the characteristic broad tailing peak is not seen and the baseline for blank runs were flat (Figure 13).
**Analytical separations**

Separations were performed with a mobile phase of .1% TFA in water with undoped or albumin doped silica as a stationary phase. Separations with mobile phases other than .1% TFA in water were also performed, but these mobile phases did not increase separation efficiency and are not reported here. Albumins are known to bind to fatty acids, metal ions, and small drugs so initial separations were performed with acetylsalicylic acid (aspirin) and salicylic acid. The average retention time of aspirin on an undoped column was show to be 5.5 min. Salicylic acid was retained slightly more with an average retention time of 5.9 min. A mixture of aspirin and salicylic acid showed average retention times of 6.6 min and 7.4 min respectively with a separation factor (α) of 1.1 and a resolution (Rₙ) of .90. Retention times of undoped columns were variable with retention times sometimes deviating more than a minute. Baseline separation of the analytes was not achieved with undoped columns (figure14).

![Figure 14] Chromatogram showing partial separation of aspirin and salicylic acid. (α = 1.1 Rₙ = .9)

Separations performed with an albumin doped column showed constant retention times. The average retention time of aspirin on an albumin doped column was shown to be 2.5 min. Salicylic acid was again retained more and had a average retention time of 4.4 min. A mixture of aspirin and salicylic acid showed retention times of 2.2 min and 4.2 min
respectively with a $\alpha$ of 1.8 and $R_s$ of 1.5. Albumin doped columns showed shorter retention times than undoped columns and achieved baseline separation (figure 15).

![Figure 15](image.png)

Figure 15) Chromatogram showing full baseline separation of aspirin and salicylic acid ($\alpha = 1.8 R_s = 1.5$)

Shorter retention times on doped columns seem counter intuitive because better efficiency and separation is achieved on doped columns. One possible explanation is that albumin is blocking some of the pores. The average pore diameter of the silica is less than 2 Å with the dimensions of albumin being 6 x 10 x 6 Å. So it is conceivable that the albumin could be blocking the pores thereby decreasing retention, but also interacting with the analyte to increase separation.

It was observed that the salicylic acid peak was broadened both as a single analyte and as a mixture on albumin doped columns. One possible explanation for the loss in efficiency of the salicylic acid peak is hydrogen bonding. Hydrogen bonding is well known to broaden peaks due to a decrease in mass transfer. This seems to fit the data since aspirin has one H-bond donor site and salicylic acid has two. But since there is not direct evidence as to why this peak was broadened we do not offer any further speculation as to why this peak was broadened.
Dipeptides analytes were also run on albumin doped columns. The dipeptides chosen were His-Gly, His-Leu, Gly-Trp, and Trp-Ala which showed retention times of 4.4 min, 4.5 min, 5.2 min, and 5.0 min respectively. These analytes showed constant retention times and partial separation (figure 16).

Figure 16) Chromatogram showing partial separation of dipeptides

His-Gly and His-Leu could not be resolved ($R_s = 0.7$) and appear as a peak with a shoulder at 4.4 min. The His-Gly and His-Leu peak could be baseline resolved ($R_s = 1.9$) from the Gly-Trp peak. Also the Trp-Ala peak could only be partially resolved ($R_s = 0.7$) from the Gly-Trp peak. There are three possible explanations for the failure of the albumin doped column to fully separate the four dipeptides. First, the selectivity of albumin for dipeptides may not be great enough to achieve baseline separation. Second, if the concentration of albumin is too low, then even if the selectivity of albumin is great enough for baseline separation the analyte will not interact with enough albumins to be fully separated. Third, if the PEG is blocking the surface of the silica, the dipeptides approach to the albumin may be hindered because of the dipeptides size.
Conclusions

In this study a variety of different analytes were separated with albumin doped columns. It was shown that the retention times of analytes on undoped columns were variable and baseline separation could not be achieved. Albumin doped columns, on the other hand, showed constant retention times and baseline separation ($R_s = 1.5$) of aspirin and salicylic acid. Dipeptides could be partially separated and had constant retention times. The partial separation of dipeptides could be due to many factors and will be investigated further in future experiments.

References

CHAPTER 4. GENERAL CONCLUSIONS

A protein doped monolithic silica stationary phase has been prepared by the sol-gel method for use in affinity chromatography. In chapter 2 it was shown that the identity of the protein does not affect the morphology of the silica. The protein identity does, however, affect the gelation time of the sol which is caused by the interaction of the protein with orthosilicic acid. It was also shown that protein concentration does not affect the morphology of the silica, but does affect the gelation time. In chapter 3 baseline separation of aspirin and salicylic acid with albumin doped columns was achieved and partial separation of dipeptides was achieved. Future considerations include optimizing the albumin doped columns, separation of proteins with albumin doped columns, increasing protein loading, and encapsulation of different proteins to select a greater number of analytes.