I, Amina M Darwish, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Chemical Engineering.

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Silica Surface Modifications for Protein Separation

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Silica Surface Modifications for Protein Separation

by

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Abstract

Proteomics has emerged as the next phase of understanding diseases and ailments after the completion of the human genome project. There are countless examples of protein deficiencies leading to serious ailments. Therefore, proteins have been studied as biotherapeutics and have potential applications in the drug discovery process. The advancement of such biological applications demands simultaneous advancement in protein separations on both the analytical and preparative scales. Current challenges include reducing cost and increasing the speed and the yield of production while maintaining the structure of the proteins.

The current standard separation method used in the industry is liquid chromatography. It is one of the few scalable, nondestructive methods that can be used for both analytical and preparative separations. The stationary phases and proteins used in this research are based on industry and research practices. The most commonly-used stationary phase material is silica, due to its chemical and mechanical stability. Chemical functionalizations of silica surfaces, which are heavily documented, allow control of the strength and nature of interactions with the stationary phase. Thus, silica was used as a base material. Similarly, lysozyme is a commonly-used sample material due to its low cost and availability. Consequently, there is a wealth of scientific literature on the properties lysozyme, and it is used as the test protein for the majority of the research reported here.

Silica was functionalized with amino acids using a peptide synthesis method and the effect of amino acid functionalization on lysozyme adsorption was determined. The net charge difference between the surface and the adsorbed protein was the main driving force for the adsorption of lysozyme onto carboxyl- and amine-functionalized silica. Net charge difference did not affect
lysozyme adsorption onto amino acid-functionalized silica. Rather, specific interactions between lysozyme and the amino acid functionalities determined adsorption behavior.

Multicomponent multilayer adsorption has been successfully modeled. The predicted mechanism of adsorption was mathematically validated. Multicomponent chromatographic models can now utilize type II adsorption models as well as type I and linear adsorption. This model can further be tested for other proteins.

The effect of surface curvature on protein adsorption was studied. A pore size effect is observed for lysozyme adsorption onto different functionalizations. The difference in pore size changed the shape of the adsorption isotherm. Entropic considerations such as the range of motion of the protein attachment to the surface were found to the impact the energy of adsorption.

Finally, selective surfaces for metalloproteinase adsorption from venom were synthesized and tested. The aspartic acid functionalized surface was eight times more selective for metalloproteinase over other proteins in solution. The strength of adsorption onto the aspartic acid surface is consistent with disintegrins modes of attachment. This shows that adsorption to the surfaces is specific and can mimic biological interactions.
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All praise be to God, the Lord of the World, and prayers and blessings be on His beloved Prophet. This journey was full of ups and downs, but in the end the juice was worth the squeeze.

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List of Symbols

\( a_i \) Activity of component \( i \) in the liquid phase,

\( \bar{a}_i \) Activity of component \( i \) on the surface,

\( A_L \) Area covered by single protein, Å \(^2\)

\( A_S \) Specific surface area of the adsorbent, \( \text{m}^2 \text{ g}^{-1} \)

\( C \) Constant in GAB isotherm, dimensionless

\( C_j \) Constant in GAB isotherm for surface \( j \), dimensionless

\( D \) Ligand density, molecule nm \(^{-2}\)

\( e \) Charge of an electron, \(-1.60 \times 10^{-19} \ \text{C}\)

\( \varepsilon_0 \) Permittivity of free space, \(8.85 \times 10^{-12} \ \text{C}^2 \text{ m}^{-1} \text{ J}^{-1}\)

\( \varepsilon_r \) Dielectric constant of mobile phase, dimensionless

\( F \) F-statistic value

\( f \) Constant in GAB isotherm, dimensionless

\( f_j \) Constant in GAB isotherm for surface \( j \), dimensionless

\( \Delta G_j^0 \) Standard Gibbs free energy change for adsorption onto surface \( j \), kJ mol\(^{-1}\)

\( \Delta \bar{G}_j^0 \) Standard Gibbs free energy change between the adsorbent onto the surface \( j \) and the subsequent layer, kJ mol\(^{-1}\)
\[ \Delta \tilde{G}_{cfj}^0 \] Standard Gibbs free energy change for adsorption from the bulk onto surface \( j \), kJ mol\(^{-1} \)

\[ \Delta \tilde{G}_{fj}^0 \] Standard Gibbs free energy change for adsorption from the bulk onto an adsorbed layer on the surface \( j \), kJ mol\(^{-1} \)

\[ \Delta \tilde{G}_{kj}^0 \] Standard Gibbs free energy change for adsorption from the bulk onto surface \( j \), kJ mol\(^{-1} \)

\[ \Delta H^I \] Enthalpy change associated with the first exothermic peak, kJ mol\(^{-1} \)

\[ \Delta H^II \] Enthalpy change associated with the second exothermic peak, kJ mol\(^{-1} \)

\[ \Delta H^III \] Enthalpy change associated with the final endothermic peak, kJ mol\(^{-1} \)

\[ \Delta H_{Ads} \] Total change in enthalpy change for an adsorption event, kJ mol\(^{-1} \)

\[ \Delta H_{Des} \] Total change in enthalpy change for a desorption event, kJ mol\(^{-1} \)

\[ \Delta H_{Net} \] Total change in enthalpy change for an event, kJ mol\(^{-1} \)

\[ \Delta H_j^0 \] Standard enthalpy change for adsorption onto surface \( j \), kJ mol\(^{-1} \)

\[ \Delta H_{Cj}^0 \] Standard enthalpy change between the adsorbent onto the surface \( j \) and the subsequent layer, kJ mol\(^{-1} \)

\[ \Delta H_{cfj}^0 \] Standard enthalpy change for adsorption from the bulk onto surface \( j \), kJ mol\(^{-1} \)

\[ \Delta H_{fj}^0 \] Standard enthalpy change for adsorption from the bulk onto an adsorbed layer on the surface \( j \), kJ mol\(^{-1} \)
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I$</td>
<td>Ionic strength of the solution, M</td>
</tr>
<tr>
<td>$k_o^{-1}$</td>
<td>Debye length, Å</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann constant, $1.38 \times 10^{-23}$ J K$^{-1}$</td>
</tr>
<tr>
<td>$K$</td>
<td>Equilibrium constant in Type I isotherm, dimensionless</td>
</tr>
<tr>
<td>$M$</td>
<td>Mechanism equation</td>
</tr>
<tr>
<td>$M_{ligand}$</td>
<td>Molecular weight of ligand, g ligand$^{-1}$</td>
</tr>
<tr>
<td>$N_A$</td>
<td>Avogadro constant, $6.02 \times 10^{23}$ mol$^{-1}$</td>
</tr>
<tr>
<td>$n_i$</td>
<td>Number of molecules of aggregates of species $i$ on the surface</td>
</tr>
<tr>
<td>$N_s$</td>
<td>Ligand surface coverage, mmol g$^{-1}$</td>
</tr>
<tr>
<td>$q$</td>
<td>Surface coverage, μmol g$^{-1}$ adsorbent</td>
</tr>
<tr>
<td>$q_i$</td>
<td>Surface coverage of species $i$, μmol g$^{-1}$ adsorbent</td>
</tr>
<tr>
<td>$q_m$</td>
<td>Monolayer capacity in isotherm, μmol g$^{-1}$ adsorbent</td>
</tr>
<tr>
<td>$q_{Lim}$</td>
<td>Monolayer coverage of adsorbed lysosyme complexes, μmol g$^{-1}$ adsorbent</td>
</tr>
<tr>
<td>$q_{Mm}$</td>
<td>Monolayer coverage of adsorbed myoglobin and myoglobin-lysosyme complexes, μmol g$^{-1}$ adsorbent</td>
</tr>
<tr>
<td>$Q$</td>
<td>Heat, kJ</td>
</tr>
<tr>
<td>$R$</td>
<td>Gas constant, $8.314$ J mol$^{-1}$ k$^{-1}$</td>
</tr>
<tr>
<td>$S_{BET}$</td>
<td>BET surface area, m$^2$ g$^{-1}$</td>
</tr>
</tbody>
</table>
\( \Delta S_j^0 \) Standard entropy change for adsorption onto surface \( j \), kJ mol\(^{-1}\)

\( \Delta S_{Cj}^0 \) Standard entropy change between the adsorbent onto the surface \( j \) and the subsequent layer, kJ mol\(^{-1}\)

\( \Delta S_{Cfj}^0 \) Standard entropy change for adsorption from the bulk onto surface \( j \), kJ mol\(^{-1}\)

\( \Delta S_{jfj}^0 \) Standard entropy change for adsorption from the bulk onto an adsorbed layer on the surface \( j \), kJ mol\(^{-1}\)

\( T \) Temperature, K

\( \theta_i \) Surface fraction of species \( i \), dimensionless

\( \mu_B^0 \) Standard-state chemical potential of the adsorbate in solution, kJ mol\(^{-1}\)

\( \mu_B^* \) Standard-state chemical potential of the adsorbate on the surface in the second layer or higher, kJ mol\(^{-1}\)

\( \hat{\mu}_i \) Chemical potential of species \( i \) in solution, kJ mol\(^{-1}\)

\( \mu_{jB}^0 \) Standard-state chemical potential of the adsorbate (B) on surface \( j \), kJ mol\(^{-1}\)

\( W_{AA-loss} \) Weight loss from TGA for amino acid functional groups, %

\( W_{APTS-loss} \) Weight loss from TGA for amine functional groups, %

\( W_{loss} \) Weight loss from TGA, %

\( x \) Mole fraction of the protein in the liquid phase, dimensionless

\( X \) Unoccupied surface site
$Z_i$ Charge of ion $i$, C
Abbreviations

APTES  (3-aminopropyl) triethoxysilane
BET  Brunauer Emmett Teller
CD  Circular dichroism
CPTES  3-Cyanopropyltriethoxysilane
DF  Degrees of freedom
DIC  N,N'-diisopropylcarbodiimide, 99%
DMF  N-N-Dimethylformamide
DSC  Differential scanning calorimetry
EDTA  Ethylenediaminetetraacetic acid
FMC  Flow microcalorimetry
Fmoc  Fluorenylmethyloxycarbonyl
FT-IR  Fourier transform infrared spectroscopy
LYS  Lysozyme from chicken egg white
MCF  Mesostructured cellular foam
MYO  Myoglobin from equine heart
NMR  Nuclear magnetic resonance
OtBu 5-tert-butyl ester

Oxyma Ethyl (hydroxyimino) cyanoacetate

Pbf Pentamethyldihydrobenzofuran-5-sulfonyl

RGD Arginine-glycine-aspartic acid

RV Residual variance

Si-500-AW 500Å Acid Washed silica

Si-1000-AW 1000Å Acid Washed silica

Si-500-NH2 500Å Amine functionalized silica

Si-1000-NH2 1000Å Amine functionalized silica

Si-500-G 500Å Glycine functionalized silica

Si-1000-G 1000Å Glycine functionalized silica

Si-500-D 500Å Aspartic acid functionalized silica

Si-1000-D 1000Å Aspartic acid functionalized silica

Si-500-K 500Å Lysine functionalized silica

Si-1000-R 1000Å Arginine functionalized silica

Si-1000-RGD 1000Å Arginine, Glycine, Aspartic acid functionalized silica

TFA Trifluoroacetic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet Spectroscopy</td>
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</table>
Chapter 1 Introduction and Objectives
The study of the human body has fascinated scientists for centuries. The ability to treat disease has changed the course of history. As science has progressed, the segregation lines between the different disciplines of science have faded. The complexity of the universe has exposed an infinite number of marvels to be explored. Such is the example of engineers, who were formerly known for building bridges and buildings are now involved in the purification of proteins for medical and pharmaceutical needs.

After the completion of the Human Genome Project[1, 2] in 2000, proteomics, the study of protein structure and function, became a new frontier in advancing the understanding of human biology.[1-3] The study of proteins opens the door to understanding countless diseases and ailments.[2, 4] The Protein Databank has published research on almost 100,000 proteins to date.[5] Proteins have been studied for use as biotherapeutics and have potential applications in all stages of the drug discovery process. The role of proteomics in drug discovery and development process is shown in Figure 1-1.

There are numerous studies of proteins for each stage of the drug discovery and development process. The drug development process begins with a broad understanding a disease for diagnostics and target identification. Next is target validation and prioritization, which depend on a thorough understanding of disease pathways. Finally the lead target is identified and drugs are identified based on the target structure. Finally, the drug is optimized for the specific target and tested in preclinical and clinical trials for approval.
As an example of proteins being used in diagnostics and target identification, Celis et al. studied the proteins in tumor interstitial fluid collected from invasive breast carcinomas for tumor biomarkers that could be used for early detection and prediction.[6] As an example of target validation and prioritization, Hawkins et al. studied the function and interaction of proteins in the blood-brain barrier and their impact in the prevention and treatment of neurological diseases.[7] As an example of lead identification, Ferguson conducted an extensive review on the crystal structure of a human integral membrane protein, which can be used for structure-based drug design for inflammation, respiratory and cardiovascular disease.[8] As an example of specific protein selectivity, Ruoslahti compiled data on the selectivity of the arginine-glycine-
aspartic acid (RGD) sequence and its role in cell adhesion. Drugs designed on the RGD sequence can provide new treatments for diseases such as thrombosis, osteoporosis, and cancer.[9] All such examples require the purification of individual proteins for further study.

The advancement of such biological applications demands simultaneous advancement in protein separations on both the analytical and preparative scales.[3] Many analytical tools have been employed for protein separation, such as gas chromatography, liquid chromatography, capillary electrophoresis, and microchip electrophoresis.[10] Of these separation methods, the only scalable, nondestructive method is liquid chromatography; therefore, it is the dominant method used for large-scale preparative separations.[11] The current challenges include reducing cost and increasing the speed and the yield while maintaining the structure of the proteins.[12] To achieve such developments, the current understanding of separations must be supplemented with a deeper understanding of equilibrium thermodynamics for the interactions among the different components in involved in the separation.[13]

The goal of the research presented in this dissertation is to study to the equilibrium thermodynamics of specific protein and stationary phase interactions. This introduction will include an overview of proteins in general and will introduce the proteins that were used in the research reported in this dissertation. Then chromatography and its role in the pharmaceutical industry will be discussed. The stationary phases that were used in this research will be presented as well as the advantages of the materials and conditions that are chosen. Next, the equilibrium thermodynamics of the adsorption of the protein from the liquid to the stationary phase will be discussed. An introduction to each system that was studied will be presented. Finally, the specific objectives of the research will be presented with an overview of the remaining chapters.
1.1. Proteins

A thorough understanding of proteins is essential to understand biological functions, biological abnormalities that lead to disease, and ultimately to cures for the diseases.[14, 14-20] A common example of a protein that is used as a pharmaceutical agent is insulin, a protein and hormone that regulates blood sugar levels. Diabetic medications can target insulin release in the body or supplement low levels of insulin.[21] Similarly, many newly developed drugs targets are proteins; therefore, understanding the target proteins can substantially accelerate drug development.[4, 14, 15, 17, 22-27] However, the complexity of proteins presents a number of challenges. Proteins have highly specific structures needed to fulfill their required biological functions. Many proteins attach to target molecules in a lock and key method that can only be accessed by the specific protein and the specific target molecule.[28-31] Disruptions to the normal function of the protein can lead to disease. For example, a genetic deficiency in producing rhodopsin or any other retinal protein can lead to blindness.[32] Deficiency in lysozyme M leads to an increased susceptibility to middle ear infections that are common in children.[33] These ailments vary in severity and economic impact, but can be traced back to deformity or a deficiency of a particular protein. After the cause of the disease is identified, the protein in question must be purified to be studied as a target protein or mass-produced as a drug. However, as was previously discussed, proteins have a complex structure that makes them sensitive to changes in the surrounding environment.[34] For the protein structure to be maintained, the surrounding solutions used in the separation must not denature the protein beyond repair at any point during the separation.

Due to the wide range of proteins and their sensitivity to solution conditions, research studies typically focus on one type of protein. Proteins are divided into three classes based on their
structure, globular, fibrous and membrane proteins. Globular proteins are “globe-like” and roughly have a spherical to ellipsoidal shape. They are not involved in the structural binding of cells like the fibrous proteins, and are not bound to a lipid bilayer like the membrane proteins. Enzymes are globular proteins that act as biological catalysts. Cell metabolic processes are largely driven by enzymes that function as highly efficient molecular machines.[35]

Lysozyme is commonly used a model protein due to its low cost and high availability.[36] Consequently, there is a wealth of scientific literature on the properties of lysozyme, and lysozyme is therefore used as the test protein for the majority of the research reported here, particularly in Chapters 3-5. Myoglobin is another widely available, low-cost globular protein that has been thoroughly studied; myoglobin is used as a test protein in Chapter 4. Finally, Chapter 6 presents the separation of metalloproteinase from snake venom, which is a natural proteinaceous mixture. Lysozyme, myoglobin, and metalloproteinase are introduced in greater detail below.

1.1.1 Lysozyme

Lysozyme’s function as an enzyme with ability to lyse bacteria was first published by Alexander Fleming in 1922.[37] Lysozyme is characterized by its ability to lyse the β-(1,4)-glycosidic bond between N-acetylMuramic acid and N-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer.[37] Lysozyme is found in many animal species and is used to cleave bacterial cell walls as a part of the immune system’s antibacterial defense.[37] Its presence in human mucous membranes served as part of the body’s immunological response system.[38] Within the last decade, lysozyme has been heavily studied due to its application as a preservative in food and pharmaceuticals.[39, 40] Lysozyme is present in all major taxa of living organisms. The three main types of lysozyme present in the animal kingdom are the c-type
(chicken or conventional type), the g-type (goose type) and the i-type (invertebrate type) lysozyme. The most commonly studied lysozyme is the c-type, which includes hen egg white lysozyme and human lysozyme.[37] The amino acid sequences for chicken egg white lysozyme [41] and human lysozyme [42] have been published. Due to the lower cost of the material, chicken egg white lysozyme is used in most of the research reported here. The properties of lysozyme properties are discussed in detail in Chapter 2.

1.1.2 Myoglobin

The three-dimensional structure of myoglobin was determined in 1958 through X-ray analysis.[43] distinguishing it as the first protein to have its three-dimensional structure determined.[44] It is part of the globin family of super proteins that dates back to early microorganisms.[45] It is a physiologically important oxygen carrier and oxygen store in prokaryotes, unicellular eukaryotes, plants and animals.[46] In vertebrates, including mammals, it is found in cardiac myocytes and oxidative skeletal muscle fibers.[44] As well as carrying and storing oxygen, it was recently found to scavenge nitric oxide and reactive O₂ species, which makes it of great interest for environmental applications.[47] Equine heart myoglobin is used as a test protein in this research. Its structure and amino acid sequence is available in published literature. [48] The properties of myoglobin are discussed in detail in Chapter 2.

1.1.3 Metalloproteinase

Metalloproteinases are protein-cleaving enzymes with a central metal ion. Metalloproteinases that contain zinc, or metazincs, are grouped into four subfamilies based on their structure and function: the astacins, the matrixins (matrix metalloproteinases), the adamalysins (reprolysins or snake venom metalloproteinases), and the serralysins (large bacterial proteinases).[49] Snake venoms are protein and peptide mixtures. Viper venoms often include metalloproteinases, which
increases vonom potency.[50] Snake venom metalloproteinases can be clinically useful.[51] Similar to other protein-based pharmaceuticals, the biologically activity of metalloproteinases can make them more effective than standard medications. They are biologically structured to bypass immune responses and are therefore the subject of research.[52] As of the end of 2011, there were six FDA approved drugs from venomous sources.[53] Vipers are part of a large variety of different species that use venom for protection from predators or to attack their prey. *Crotalus ruber ruber*, a species of viper commonly found in Southern California and in Mexico,[54] is the source of the venom used in this study. The metalloproteinase of this species has yet to be sequenced, as it must first be isolated from the remaining proteins in the venom to be thoroughly studied. Strategies to accomplish such a separation will be discussed later in the introduction.

### 1.1.4 Protein mixtures

Venom is an example of a naturally occurring proteinaceous mixture from which metalloproteinases can be separated. This is a common situation for protein separations: proteins must be separated from complex mixtures that typically include other proteins. Examples include recovery of lysozyme from hen egg white,[55] recovery of α-Lactalbumin and β-Lactoglobulin from whey,[56] recovery of stroke[57] and Alzheimer’s[58] biomarkers from blood plasma, and recovery of intracellular recombinant cutinase from *E. coli* cell homogenate.[59] The competing proteins can affect the protein separation. The interactions of a two-protein system are studied in Chapter 4, and the separation of a specific protein from a proteinaceous mixture is reported in Chapter 6.
1.2 Chromatography

Chromatographic protein separations are used in all the stages of drug discovery, from target identification and validation and to toxicological profiling in preclinical and clinical settings. The need for high-purity proteins creates a demand for cost-effective, high-efficiency chromatographic separations. This demand is expected to continue to increase with further development of proteomics.[2] The pharmaceutical industry’s demand for efficient, high-resolution separations has cemented chromatography in the market due to a lack of competing technologies.[60] Chromatography can be used as a fast, high sensitivity analytical tool. Extensive research has been conducted using liquid chromatography with mass spectroscopy allowing the analysis and identification of various proteins.[2] Liquid chromatography is one of the few analytical lab techniques that can be expanded to large scale separations. Due to its scalability and the wealth of available research, liquid chromatography is the standard industrial separation method for proteins.[61] The estimated value of the liquid chromatography industry was $7.6bn in 2013, and is estimated to reach $10bn by 2018.[62]

Liquid chromatography involves the partitioning of different components of a liquid sample or feed between a solid stationary phase and a liquid mobile phase. The stationary phase is a solid adsorbent that is tightly packed into a column. The mobile phase flows through the column at a set flow rate. At time zero, a sample dissolved in the mobile phase is introduced into the column. The different components of the sample separate as they travel through the column. Partitioning between the mobile and stationary phase occurs based on the equilibrium thermodynamics of the sample and the stationary phase. Components of the sample with an affinity for the stationary phase stay in the column longer. This difference results in different retention times in the column causing the components of the sample to separate based on their
affinity for the stationary phase. The type of chromatography is defined through nature of the interactions with the stationary phase. The different modes of separation are shown in Figure 1-2.

![Figure 1-2: Types of Chromatography](image)

The action principle for each type of chromatography is the driving force that causes sample components to adsorb or not adsorb to the stationary phase. The separation mechanism is the characteristic on which the sample components are separated. The type of chromatography, the action principle, and the separation mechanism are shown in Table 1-1. Factors that determine which method is used include solubility, hydrophobicity, iso-electric point, separation scale, efficiency and cost.[63]

### 1.3 Stationary phase

The desired characteristics of chromatographic stationary phases are high selectivity, high capacity, low non-specific adsorption, incompressibility, minimal mass transfer limitations, non-toxicity, long term column performance (reusability), and low cost.[12] Silica is often used as a base material for chromatographic separations due to its mechanical stability and controllable surface chemistry and morphology.[60, 64-68] The morphology of silica can affect adsorption;
key morphological properties include pore size, particle size, surface area, and pore volume.[69-76]

Table 1-1: Chromatography action principles [64]

<table>
<thead>
<tr>
<th>Chromatography type</th>
<th>Action principle</th>
<th>Separation mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>Surface binding</td>
<td>Molecular structure</td>
</tr>
<tr>
<td>Ion-exchange</td>
<td>Ionic binding</td>
<td>Surface charge</td>
</tr>
<tr>
<td>Size-exclusion</td>
<td>Size exclusion</td>
<td>Molecular shape and size</td>
</tr>
<tr>
<td>Affinity</td>
<td>Bio specific</td>
<td>Molecular structure</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>Hydrophobic complex</td>
<td>Hydrophobicity</td>
</tr>
<tr>
<td>Normal-phase</td>
<td>Hydrophobic complex</td>
<td>Hydrophobicity</td>
</tr>
<tr>
<td>Reverse-phase</td>
<td>Hydrophobic complex</td>
<td>Hydrophobicity</td>
</tr>
</tbody>
</table>

The modification, or functionalization, of silica surfaces is utilized for different types of liquid chromatography.[60, 64-68] Surface functionalization can add chemical or biological specificity to silica surfaces, allowing control of the strength of interaction with specific proteins over others with the surface.[77] The application of such surface functionalizations on chromatographic media is well-documented.[78-85] Increased complexity of the surface functionalization can produce higher specificity,[22] but also results in higher stationary phase production costs.[86] Amino acid functionalized silica surfaces are studied in more detail in Chapter 3. The background and motivation of the studying pore size and amino acid functionalized silica must first be discussed.
1.3.1 Silica pore size

The main driving force for selectivity is an obvious main focus of study. For size exclusion chromatography, that main driving force is pore size;[87-89] the effect of pore size, and in effect pore curvature, is often ignored for other types of chromatography. Pore size is often considered in terms of available surface area but not in terms of its impact on protein adsorption.[69-76] The impact of physical characteristics of the chromatographic materials on protein adsorption should be considered. The availability and distribution of surface functionalizations can be affected by pore curvature. Due to the complexity of proteins and their specificity of interaction with other surfaces, changes in surface curvature may impact adsorption. Surface curvature was in fact found to impact the biological activity of cells[90] and proteins[91] on normal[92] and reverse phase[93] adsorption media.

The size of the pore can limit the volume available for the protein to stay in solution without being affected by the surface. For example, the Debye length, which is a measure of the distance a charge propagates through liquid medium, can be calculated for interaction between the surface and the protein.[94] A pore size that is double the Debye length forces interaction between the protein and the surface and limits liquid phase protein-protein interactions inside the pores. Protein - protein interactions are difficult to measure, but can strongly influence adsorption behavior. Depending on solution conditions, proteins can form dimers or larger aggregates that can result in multilayer adsorption.[95] The number of possible layers of adsorbed protein in the pores can be limited by controlling the pore size as well as other factors such as solution conditions,[96] which will be discussed in more detail in Chapter 2.

In Chapter 5, the pore size of silica is varied to study its effect on the adsorption system, and an analysis of the thermodynamics of adsorption to each surface is conducted. The
underlying equilibrium thermodynamics should be studied to effectively understand adsorption mechanism. The derivation of popular adsorption models, such as the Guggenheim-Anderson-de Boer (GAB), based on thermodynamic considerations has previously been demonstrated.[95] Analysis of the isotherms through the GAB model can produce reliable estimations of the Gibbs free energy. The enthalpy of adsorption can be directly measured using flow microcalorimetry (FMC). Accurate enthalpic measurements can help ground theoretical modeling of these stationary phases and guide their most efficient use.

The effect of pore size on protein adsorption is important for size selective chromatography, for controlling the free pore space in which protein-protein interactions can occur, and for bimodal chromatography with a number of pore sizes. The present work studied the adsorption of lysozyme on acid-washed silica with three different pore sizes. The thermodynamics of the adsorption process were studied using batch adsorption to measure changes in chemical potential and flow microcalorimetry (FMC) to measure changes in enthalpy. The changes in entropy were calculated from the chemical potential and enthalpy. The pore size is found to affect the spontaneity, enthalpy, entropy, and reversibility of adsorption.

1.3.2 Surface chemistry

Silica and functionalized silica are widely used as stationary phases for high performance liquid chromatography (HPLC) columns in the pharmaceutical industry.[60,64-68] Chromatographic protein separations are used in all the stages of drug discovery, from target identification and validation and ultimately to toxicological profiling in preclinical and clinical settings. The need for high purity proteins creates a demand for cost-effective, high-efficiency chromatographic separations. This demand is expected to continue to increase with further development of proteomics.[2] The desired characteristics of chromatographic stationary phases
are high selectivity, high capacity, low non-specific adsorption, incompressibility, minimum mass transfer limitation, non-toxic, long term column performance (reusability), and low cost. These characteristics can be obtained by modifying the surface functionality of the silica.

Surface functionalizations can add chemical or biological specificity to silica surfaces, allowing control of the strength of the interaction with specific proteins over others with the silica surface. The application of such surface functionalizations on chromatographic media is well-documented. Depending on the functionalization chosen, proteins can be adsorbed with varying degrees of reversibility or can be excluded from the surface. Increased complexity of the surface functionalization can produce higher specificity, but also results in higher costs. Affinity chromatography, which uses a biologically related agent, or affinity ligand, as a stationary phase to selectively retain analytes or to study biological interactions, is extremely specific. However, it requires a complex procedure of generating and purifying antibodies for a lock and key approach to protein purification. The simpler surface functionalities are far lower in cost than affinity chromatography, but can provide only simple separations based on charge, hydrophobicity, or size. New surface functionalities, such as amino acid functionalities, must be considered to achieve higher selectivity at lower costs.

Amino acids are the building blocks of proteins. They are made of a central carbon atom attached to an amine group, a hydrogen atom, a carboxyl group, and a defining side, or R, group. Amino acids are a less expensive alternative to antibodies, but can provide a more specific biological interaction than the simpler surfaces. The binding mechanism used to anchor the amino acids to the surface must also be considered as it will undoubtedly have an effect on the surface chemistry. This study uses peptide synthesis technique to attach the amino acids in an
attempt to get the most biologically compatible surface. Peptide synthesis methods have seen recent advancements towards large scale commercialization.[98] The successful commercialization of these techniques can also be beneficial for silica surface modifications.

Few studies have considered amino acid functionalized surfaces as stationary phases for chromatographic applications. The surface functionalizations have been achieved using a number of different methods. For example, a capillary electrophoresis surface was developed by grafting proline to a fused silica support through a 3-trimethoxysilylpropyl chloride connector.[99] Similarly, glutamic acid was bound to silica gel (30 nm pore diameter) using 3-glycidyloxypropyltrimethoxysilane to silica and used as a cation-exchange surface.[100] Amino acid functionalizations have the additional benefit of providing biologically compatible surfaces. For example, leucine-functionalized SBA-15 was used as a drug carrier.[101] Single amino acids and amino acid chains were added to silica surfaces using connectors as mentioned above and have been bound to PEG,[102, 103] dextran[104] and silica using click chemistry.[105] The study reported here considered the use of amino acid-functionalized silica surfaces as a stationary phase for protein separations; the amino acids were attached to amine functionalized silica through the creation of a peptide bond. Lysozyme was chosen as a test protein because of its relatively small size and stability and because lysozyme adsorption has been studied extensively. Adsorption studies were conducted on carboxyl- and amine- functionalized silica, and on silica functionalized with glycine (R = H), aspartic acid (R = CH2-COOH), and lysine (R = (CH2)4-NH2). These amino acids were chosen to provide side groups that correspond to the surface chemistry of the amine and carboxyl ligands. The ligands were found to affect the electrostatics of the adsorption, while the amino acid functionalizations were found to be
unaffected by the electrostatics of the R group. The lysozyme was instead more affected by the specific amino acid on the surface.

1.4 Adsorption

Chromatographic separations are governed by the equilibrium thermodynamics of a sample between the mobile phase and the stationary phase. Different components of the sample equilibrate at different strengths, which results in a separation. Changes in the liquid or stationary phases can have an effect on the thermodynamics of adsorption.[106] Due to the complexity of proteins, qualitative descriptions of protein interactions are often far easier to realize than quantitative descriptions. However, the ability to provide quantitative energies of interaction- for example as a support to overarching qualitative conclusions- can help shed more light on a complex system. It also helps the researcher make more concrete decisions about the modes of separation.

A number of mathematical models have been previously developed to study the equilibrium between an adsorbate and a solid adsorbent.[107] The five types of adsorption isotherms are shown in Figure 1-3.

![Figure 1-3: The five types of adsorption isotherms [107]](image-url)
Models are used based on the general shape of the isotherm. The Langmuir isotherm, for example, is often used for Type I isotherms, where the amount of material adsorbed to the solid remains constant past a certain liquid concentration; assuming that adsorption to a single surface layer. The Brunauer, Emmett and Teller (BET) model and the Guggenheim–Anderson–deBoer (GAB) model are often used for Type II isotherms, where the amount of material adsorbed increases with increasing concentration, remains constant, then continues to rise again.[108] Both BET and GAB allow for multilayer adsorption to the surface, and are based on different thermodynamic models.[95]

Equilibrium constants can be measured experimentally and then translated into chromatographic column design. Thermodynamic phenomenon and adsorption models can then be incorporated into liquid chromatography design models.[109, 110] Collecting reliable thermodynamic data about an adsorption system can be challenging. Gibbs free energy can be calculated from measured equilibrium constants to describe the spontaneity of adsorption. The thermodynamic derivations of equilibrium constants from popular Type I[111] and Type II[95] adsorption models are available in scientific literature. The Gibbs free energy was calculated from the results most of the systems studied in this research.

1.5 Thermodynamics of Adsorption

Adsorption models should predict the amount of adsorbate attaching to the surface based on the amount of the adsorbate in the liquid phase. The Langmuir isotherm includes a single interaction energy between the adsorbate and adsorbent and assumes that adsorption is limited to a single layer on the surface. The BET isotherm assumes that infinitely deep multilayers can form, with the assumption that the energy of adsorption to the multilayer is the same as that of adsorption to the adsorbent surface. The GAB isotherm allows for formation of multilayers, but
unlike the BET equation, the energy of adsorption to the multilayer can differ from the energy of adsorption directly to the surface.[95] Each interaction energy is represented by a parameter in the equation that represents that change in chemical potential that occurs as the adsorption occurs.

Thermodynamic derivations of commonly-used adsorption isotherms has been published. The Langmuir adsorption model is shown in Eq. (1-1).[94]

\[ q = q_m \frac{Kx}{1 + Kx} \]  

(1-1)

In this isotherm, \( x \) is the mole fraction of the protein in the liquid phase, \( q \) is the protein surface coverage (\( \mu \text{mol g}^{-1} \)), \( q_m \) is the monolayer surface coverage (\( \mu \text{mol g}^{-1} \)), and \( K \) is a dimensionless adsorption coefficient. While a more rigorous thermodynamic analysis of the adsorption process would use the protein activity rather than the protein mole fraction, under the conditions used in this research, the protein activity coefficient can be taken to be one.[112]

The adsorption constant \( K \) is related to the standard Gibbs free energy change of adsorption as shown in Eq.(1-2); this equation was thermodynamically derived based on the limiting assumptions of surface homogeneity, immobility of adsorbed species, negligible interactions between adsorbed molecules, and representation of adsorbate activity as the unoccupied fraction of adsorption sites.[94] The thermodynamic derivation of the Langmuir adsorption model has been previously described by Liu et al.[111]

\[ \Delta \tilde{G}_j^0 = \mu_{jB}^0 - \mu_B^0 = -RT \ln K_j \]  

(1-2)

In this equation, \( \Delta \tilde{G}_j^0 \) is the standard Gibbs free energy change for adsorption onto surface \( j \) (\( J \text{ mol}^{-1} \)), \( \mu_{jB}^0 \) is the standard-state chemical potential of the adsorbate (B) on surface \( j \), \( \mu_B^0 \) is the
standard-state chemical potential of the adsorbate in solution, \( R \) is the ideal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), and \( T \) is the temperature (298 K).

Other underlying assumptions of the Langmuir model must also be considered. The Langmuir isotherm assumes that molecules are adsorbed on a fixed number of well localized sites; each site may accept only one molecule in such a way that they form a monolayer.[113] Based on these assumptions, adsorption is driven by the number of available sites and not the area available for adsorption. This condition is reconsidered in Chapters 3 and 4. Other Langmuir assumptions, such as all sites being energetically equivalent and no interaction between adsorbed molecules can also be reconsidered for systems where these factors greatly affect adsorption. Despite these limitations, The Langmuir isotherm remains a widely used adsorption model.[113]

The thermodynamic derivation of BET and GAB was conducted by Pradas et al. in which the BET equation is a special case of the GAB, where \( f = 1 \). The BET and GAB isotherms are presented in Eq. (1-3) and (1-4) respectively. The BET equation assumes that the energy of adsorption to the surface is equal to the energy of adsorption to the multilayer and each subsequent layer. The GAB model differentiates the energy of adsorption to the surface from the energy of adsorption to the first layer. The energy of adsorption to subsequent layers is assumed to be equal to the adsorption to the first layer, also known as the monolayer. The Gibbs free energy is further derived from the equilibrium constants as shown in Eq. (1-5)-(1-7)

\[
\theta_j = \frac{q_j}{q_{jm}} = \frac{C_j x_j}{(1 - x_j)(1 - (1 - C_j)x_j)} \quad (1-3)
\]

\[
\theta_j = \frac{q_j}{q_{jm}} = \frac{C_j f_j x_j}{(1 - f_j x_j)(1 - (1 - C_j)f_j x_j)} \quad (1-4)
\]
\[ \Delta G_{fj}^0 = \mu_B^0 - \mu_B^* = -RT \ln f_j \]  
(1-5)

\[ \Delta G_{cj}^0 = \mu_{jB}^0 - \mu_B^* = -RT \ln C_j \]  
(1-6)

\[ \Delta G_{cfj}^0 = \mu_{jB}^0 - \mu_B^* = -RT \ln C_j f_j \]  
(1-7)

In this equation, \( \Delta G_{fj}^0 \) is the standard Gibbs free energy change for adsorption from the bulk onto an adsorbed layer on the surface \( j \) (J mol\(^{-1}\)), \( \Delta G_{cj}^0 \) is the standard Gibbs free energy change between the adsorbent B on the onto the surface \( j \) and the subsequent layer (J mol\(^{-1}\)), and \( \Delta G_{cfj}^0 \) is the standard Gibbs free energy change for adsorption from the bulk onto surface \( j \) (J mol\(^{-1}\)), \( \mu_{jB}^0 \) is the standard-state chemical potential of the adsorbate (B) on surface \( j \), \( \mu_B^* \) is the standard-state chemical potential of the adsorbate in solution, \( \mu_B^* \) is the standard-state chemical potential of the adsorbate on the surface in the second layer or higher, \( R \) is the ideal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), and \( T \) is the temperature.

Existing adsorption models for multicomponent systems are limited to Type I adsorption, which excludes multicomponent systems with multilayer adsorption. This research takes these calculations a step further through the thermodynamic derivation of a Type II adsorption isotherm for multicomponent systems. The results of a multilayer competitive adsorption study using lysozyme and myoglobin are presented in Chapter 4.

The enthalpy of adsorption can be directly calculated using calorimetry. When combined with the Gibbs free energy of adsorption, the entropy of adsorption can also be calculated. The Gibbs free energy, enthalpy, and entropy changes of the system provide detailed thermodynamic descriptions of the system and explain the underlying action principles of adsorption. Such detailed analyses are presented in Chapters 4 and 5.
1.6 Research Objectives

The goal of this research was to study the separation materials and the surrounding competing proteins in order to provide information needed to optimize the protein purification process. The specific objectives were:

I. Study of lysozyme adsorption on amino acid functionalized silica

II. Mathematical modeling of competitive adsorption between lysozyme and myoglobin on silica

III. Study the thermodynamic effect of varying silica surface curvature on lysozyme adsorption

IV. Design selective silica surfaces for the separation of metalloproteinase from snake venom

1.7 Research Overview

1.7.1 Amino Acid Functionalizations

Surface functionalization adds additional specificity to silica surfaces that allows the control of the interaction of the proteins with the silica surfaces. The application of such surface functionalizations on chromatographic media has been heavily documented.[78-85] Different types of liquid chromatography rely on differential affinities within a liquid sample to a solid stationary phase. Depending on the resulting surface chemistry after functionalization, proteins can be adsorbed with varying degrees of reversibility or can be excluded from the surface. Increased complexity of the surface functionalization results higher specificity, but also results in higher costs. Affinity chromatography is the most specific type of chromatography, but requires a complex procedure of generating and purifying antibodies for a lock and key approach to
protein purification. The simpler the surface functionalities are far lower in cost, but can only provide basic separation metrics such as charge, hydrophobicity, or size. The current study considers the use of amino acid functionalized surfaces. Amino acids are an inexpensive alternative to antibodies, but can provide a more specific biological interaction than the simpler surfaces. With advancements in peptide synthesis,[98] simple chain of a few amino acids has the potential to provide an efficient and cost effective method for the separation of biomolecules.

Chapter 3 presents the results of a study of amino acid functionalized surfaces as potential stationary phases. The hypothesis of the study is that the amino acid surfaces would provide more specific interactions than traditional chromatographic media. Silica was used as the base material for all of the functionalizations, and lysozyme was used as a test protein for all adsorption experiments. To directly compare the amino acid effect, the surfaces were functionalized chemical groups similar to the R- group of each amino acid. The functionalized surfaces and the corresponding amino acids are shown in the Table 1-2.

Table 1-2. Surface functionalities and identification of adsorbents used in the current study.

<table>
<thead>
<tr>
<th>Simple Functionalization</th>
<th>Amino Acid Functionalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>None: (Acid-washed)</td>
<td>Glycine:</td>
</tr>
<tr>
<td></td>
<td>–(CH$_2$)$_3$-[NH-CO-CH-NH$_2$]-H</td>
</tr>
<tr>
<td>Si-500-AW</td>
<td>Si-500-G</td>
</tr>
<tr>
<td>Carboxypropyl:</td>
<td>Aspartic Acid:</td>
</tr>
<tr>
<td>-(CH$_2$)$_3$-COOH</td>
<td>–(CH$_2$)$_3$-[NH-CO-CH-NH$_2$]-CH$_2$-COOH</td>
</tr>
<tr>
<td>Si-500-COOH</td>
<td>Si-500-D</td>
</tr>
<tr>
<td>Aminepropyl:</td>
<td>Lysine:</td>
</tr>
<tr>
<td>-(CH$_2$)$_3$-NH$_2$</td>
<td>–(CH$_2$)$_3$-[NH-CO-CH-NH$_2$]-(CH$_2$)$_4$-NH$_2$</td>
</tr>
<tr>
<td>Si-500-NH2</td>
<td>Si-500-K</td>
</tr>
</tbody>
</table>
1.7.2 Competitive Adsorption of Lysozyme and Myoglobin

To move from theoretical bench scale adsorbents to an analytical or preparative packed column, various models have been employed to translate the adsorption models to the chromatographic models to predict complex protein separation in the column.[109, 110] Thermodynamic parameters that are derived from the adsorption models can be directly translated into chromatographic design models.[109, 110] Therefore, adsorption models that can mathematically predict the interactions between proteins and adsorbents are essential to proteomics research and the industry. The biggest departure between the models and industrial chromatographic separation is that they describe adsorption of single components to the surface. These models are greatly deficient for use with complex protein solutions, and therefore must be modified to accommodate the adsorption of multiple components.

In a system involving more than one component, the standard adsorption models must be modified to accommodate multiple adsorbates. Such expansions have been developed for Langmuir and Freundlich adsorption models, but not for more complex multilayer adsorption models[114-117]. This study focused on expanding the GAB model to a multicomponent system. The GAB model is chosen because it assumes different interaction energies for the adsorption directly to the surface and adsorption onto the first adsorbed layer. This separation is important because protein adsorption to silica is vastly different from its adsorption to other proteins. The mathematical derivation for the multicomponent system is based on same assumptions used by Pradas et al.[95]

In Chapter 4, thermodynamic insights from modeling equilibrium isotherm are coupled with the direct measurement of enthalpy during protein adsorption, measured with flow microcalorimetry. This study used the adsorption of lysozyme (14.3 kDa, 1.9×2.5×4.3nm) and
myoglobin (17.6 kDa, 2.8×6.3×3.5 nm) as adsorbates. A multicomponent isotherm was derived based on the approach used to derive the GAB isotherm. Lysozyme and myoglobin were chosen based on their comparable size and charge at adsorption conditions. Mesostructured cellular foam silica was used as the adsorbent for all experiments.

1.7.3 Influence of Surface Curvature

Pore size is an important characteristic of the stationary phase.[87, 94, 118-126] Different pore sizes of a stationary phase are used to optimize surface area and pressure drop through a chromatography column. Furthermore, silica with uniformly sized pores is used for size selective chromatography creating the potential for molecular sieves.[89] However, even in cases that do not involve size selective chromatography, adsorption is affected by the relative distance between the protein and the surface and the proteins and each other. Previous research has applied the Debye length to estimate the effective distance of electrostatic interactions between the protein and the surface.[94] Protein-protein interactions are difficult to measure, but can have large impact on adsorption. Proteins can often form dimers or larger aggregates depending on the solution condition, which can create a multilayer adsorption onto the surface.[127, 128] By limiting the pore size, the number of possible layers in the pores can be controlled. Moreover, depending on the solution conditions, the proteins can also repel each other based on charge.[96] This would affect adsorption to the surface if the repulsion to other proteins is greater than the proteins affinity for the surface. This can be overcome using very large pore sizes. To study the impact of the pore size, the overall equilibrium of the system and the specific modes of adsorption must be studied.

Chapter 5 presents the results of a study of the thermodynamics of lysozyme adsorption on silica at different surface curvatures. Acid washed, amine functionalized and glycine
functionalized commercial silica with 500Å and 1000Å pores were used as the adsorbents. Lysozyme isotherms were measured for each silica. Flow microcalorimetry was used to determine the enthalpy of adsorption of lysozyme on the acid washed adsorbent. The isotherms provide a molar value of Gibbs free energy, $\Delta G$, and the calorimetry data provides a molar enthalpy, $\Delta H$. From both these terms the molar entropy, $\Delta S$, can also be calculated provided a complete thermodynamic analysis of the system.

1.7.4 Venom Separation

Vipers belong to a large group of venomous snakes called viperidae. These snakes produce venom, primarily to procure prey animals and also to defend themselves from predators. Most venoms consist of protein and peptide mixtures.[50] The proteins present in venom target biological structures with high specificity and are resistant to the body’s immune responses. These unique properties make them valuable pharmacological therapeutic agents. As of the end of 2011, there were six FDA approved drugs derived from venomous sources.[53] Chapter 6 studies the venom of a North American viper.

The rattlesnake, *C. ruber ruber*, a species of viper commonly found in Southern California and in Mexico,[54] is the source of the venom to be used in this study. The dry venom consists of 76% protein.[129] A more thorough analysis of the *C. ruber ruber* venom showed the presence of a metalloproteinase with disintegrin properties.[130] These metalloproteinases, present in viper venom, attack cell adhesion complexes,[131] decreasing the viscosity of the tissue thereby enhancing the penetration of the venom through tissue. Due to its ability to penetrate tissues, metalloproteinases have many potential pharmaceutical applications.[132-134] Metalloproteinases, are characterized by the presence of a metal ion in the center of the protein
molecule as shown in Figure 1-4.[135] The goal of this study is to test amino acid functionalized adsorbents for the selective adsorption of the metalloproteinase from viper venom.

Figure 1-4: Metalloproteinase

Integrins are membrane proteins facilitate the attachment of cells and proteins. Binding depends on the RGD (arginine-glycine-aspartic acid) sequence present in the collagen or other structural proteins which are found in the extra cellular matrix. Such complexes bind cells together in tissues increasing their viscosity.[136-145] Hence, RGD was used as a biological binding basis and was added to a commercially-available mesoporous silica surface to adsorb selectively the metalloproteinase from the venom mixture. *C. ruber ruber* venom was used to test the selectivity of adsorption of the metalloproteinase to the functionalized adsorbents. The total protein and metalloproteinase adsorptions were tested and compared for single amino acid functionalized surfaces and an RGD functionalized surface to determine the specificity of each attachment. The glycine surface was found to have the highest selectivity for the metalloproteinase.
Chapter 2 Materials and Methods
2.1. Chemicals

All chemicals used in this investigation are summarized in Table 2-1.

Table 2-1: Summary of chemicals and material used, and suppliers

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme from chicken egg white (LYS, 90%)</td>
<td>Sigma-Aldrich (St.Louis, MO, USA)</td>
</tr>
<tr>
<td>(3-aminoproyl)triethoxysilane (APTES)</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>3-Cyanopropyltriethoxysilane (CPTES),</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Ethyl (hydroxyimino)cyanoacetate (oxyma),</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Piperidine (ReagentPlus®, 99%)</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Spherical silica gel 60-80 Å</td>
<td>Sigma-Aldrich, Steinheim, Switzerland</td>
</tr>
<tr>
<td>Matrex normal phase liquid chromatography media Si-500A-10µm</td>
<td>Amicon, Billerica, MA, USA</td>
</tr>
<tr>
<td>Matrex normal phase liquid chromatography media Si-1000A-10µm</td>
<td>Amicon, Billerica, MA, USA</td>
</tr>
<tr>
<td>Potassium phosphate monobasic</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Sulfuric acid, extra pure, 96% solution in water</td>
<td>Acros Organics, New Jersey, USA</td>
</tr>
<tr>
<td>Dichloromethane (DCM)</td>
<td>Sigma-Aldrich (St.Louis, MO, USA)</td>
</tr>
<tr>
<td>Trifluoroacetic acid (TFA)</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Protected glycine (Fmoc-Gly-OH, ≥98% by TLC and HPLC)</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Protected lysine (Fmoc-Lys(Fmoc)-OH, ≥98% by HPLC),</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Protected aspartic acid (Fmoc-Asp(otBu)-OH, Anaspec)</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>N,N'-diisopropylcarbodiimide, 99% (DIC)</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>N,N-Dimethylformamide (DMF)</td>
<td>Tedia Company, Fairfield, OH</td>
</tr>
<tr>
<td>Nitric acid 50-70%</td>
<td>Fisher Scientific, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Pluronic® P123 surfactant</td>
<td>BASF (Mt. Olive, NJ, USA)</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Pharmco-Aaper (Brookfield, CT, USA)</td>
</tr>
<tr>
<td>Tetraethyl orthosilicate (TEOS),</td>
<td>Sigma–Aldrich (Pittsburg, PA, USA)</td>
</tr>
<tr>
<td>1,3,5-trimethyl benzene (TMB)</td>
<td>Sigma–Aldrich (Pittsburg, PA, USA)</td>
</tr>
<tr>
<td>Myoglobin ≤99.0%</td>
<td>Sigma–Aldrich (St. Louis, PA, USA)</td>
</tr>
<tr>
<td>Thioanisole purum, ≥99.0%</td>
<td>Sigma–Aldrich (St. Louis, PA, USA)</td>
</tr>
<tr>
<td>1,2-ethanediol ≥98.0%</td>
<td>Sigma–Aldrich (St. Louis, PA, USA)</td>
</tr>
<tr>
<td>Casein from bovine milk</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>2N Folin &amp; Ciocalteu’s phenol reagent</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Sodium carbonate anhydrous</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>L-tyrosine ≥98%</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>protected arginine (Fmoc-Arg(Pbf)-OH, ≥98% by TLC and HPLC)</td>
<td>Fisher Scientific (Pittsburg, PA)</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA) ≥99%</td>
<td>Sigma–Aldrich (St. Louis, MO, USA)</td>
</tr>
</tbody>
</table>
2.1.1. Solution conditions

Protein structure and interaction with other surfaces are affected by its surrounding solution. For example, lysozyme can form dimers and aggregates based on the solution conditions.[146, 147] For all adsorption experiments with lysozyme and myoglobin, the proteins were rehydrated in 0.02M phosphate buffer at pH 7 and 25⁰C. At these conditions, electrostatic interactions are still possible,[148] and lysozyme dimerization is low.[147] Previous studies have shown that lower ionic strengths, below 0.1 M, allow for electrostatic interactions, while higher ionic strengths decrease the Debye length and strongly attenuate electrostatic interactions; the calculated Debye length decreases from 30 Å to 3 Å when 1 M NaCl is added to 10 mM pH 7 phosphate buffer.[149] Higher ionic strengths can also result in irreversible lysozyme adsorption, which would not be ideal for chromatographic systems.[148]

2.1.2. Lysozyme

Lysozyme is made up of 129 residues (or amino acids). Its molecular weight is 14.3 kDa, its dimensions are 1.9×2.5×4.3 nm, and its isoelectric point is 10.8.[150] Lysozyme is a hard protein that undergoes minimal conformational changes on adsorption.[148] Lysozyme begins to form dimers in solution then crystals at high protein concentrations and high ionic strengths.[147] Like most proteins it has a UV absorbance peak at 280 nm.

2.1.3. Myoglobin

Myoglobin is made up of 153 residues. Its molecular weight is 17.6 kDa, its dimensions are 2.8×6.3×3.5nm, and its isoelectric point is 7.2. It contains iron, giving it its red color. It has UV absorbance peaks at 280 nm and 400 nm.
2.1.4. Venom

Venom was collected from *C. ruber ruber*, a viper rattlesnake commonly found in Southern California and Mexico,[54] by Dr. Sharath Krishna (Central State University, Wilberforce, Ohio). The venom was provided in lyophilized form, then rehydrated in 50 mM potassium phosphate buffer at pH 7.5 and 37° C for optimum metalloproteinase activity. Venom is a proteinaceous mixture, therefore is has a UV absorbance at 280 nm. The activity of proteinases in the venom can be detected using a casein assay. The metalloproteinases in the venom can be deactivated with addition of Ethylenediaminetetraacetic acid (EDTA).[151]

2.2. Methods

2.2.1. Surface Functionalizations

*Acid-washed silica* (Si-500-AW, Si-1000-AW) was prepared by acid washing silica overnight at 80° C under reflux using a mixture of sulfuric and nitric acids in a ratio of 1 g silica : 5 mL 5 M H₂SO₄ : 5 mL 5 M HNO₃. The silica was then recovered by filtration and rinsed with deionized water until the filtrate was neutral. The material was then dried for 24 h at 25° C. Si-500-AW/ Si-1000-AW was then washed with DMF and left to dry for 24 h at 25° C before use in adsorption experiments to maintain consistency with the amino acid functionalizations described below.

*Aminopropyl-functionalized silica* (Si-500-NH2, Si-1000-NH2) was synthesized by the reaction of acid washed silica (Si-500-AW/ Si-1000-AW) with APTES. In this synthesis, 1 g Si-500-AW/ Si-1000-AW was reacted with 5 mL APTES in 100 mL of toluene at 80° C with reflux under nitrogen. The aminopropyl-functionalized silica Si-500-NH2/ Si-1000-NH2 was recovered by filtration, washed with 100 mL of toluene, then washed with ethanol until there was no
toluene visible in the filtrate. Finally the silica was washed with water to remove the ethanol and left to air dry for 24 h at 25°C.

*Carboxypropyl-functionalized silica* (Si-500-COOH) was synthesized in a two-step process. First, 1 g Si-500-AW was reacted with 5 mL CPTES in 100 mL of toluene at 80°C with reflux under nitrogen, creating cyanopropyl-functionalized silica. The cyanopropyl-functionalized silica was then oxidized in 100mL of 50% (v/v) H2SO4 acid at 150°C for 3 h to convert the cyano group to a carboxyl group as previously described.[152]

*Glycine-functionalized silica* (Si-500-G, Si-1000-G) was synthesized by the reaction of aminopropyl-functionalized silica (Si-500-NH2/Si-1000-NH2) with Fmoc-Gly-OH in DMF solution in the presence of DIC and oxyma.[98, 153] The molar surface coverage of amines was determined to be 0.16 mmol g⁻¹ using methods described in Section 2.3 below. DIC, oxyma, and the protected amino acid were used in a minimum of three times excess the stoichiometric requirement. 0.25 mL DIC, 0.2 g oxyma, and 0.45 g Fmoc-Gly-OH were used per gram of Si-500-NH2/ Si-1000-NH2. The DIC, oxyma and the protected amino acid were preactivated in the DMF solution at 25°C for 4 min before the Si-500-NH2/ Si-1000-NH2 is added. The Si-500-NH2/ Si-1000-NH2, DIC, oxyma, and the protected amino acid are left stirring for 1 h. 100 mL of DMF is used for every gram of silica. The mixture was then filtered with DMF solvent to remove any excess amino acids. The Fmoc protective group was removed using 20% piperidine in DMF for 10 min as previously described.[154] 100 mL of the 20 vol% piperidine in DMF was used for every gram of Si-500-NH2/ Si-1000-NH2. The removal of Fmoc was verified using UV spectroscopy of the liquid solution at 289 nm. The Si-500-G/ Si-1000-G was washed with 100 mL DMF and left to dry for 24 h at 25°C.
**Lysine-functionalized** silica (Si-500-K) was similarly synthesized by the reaction of Si-500-NH2 with Fmoc-Lys(Fmoc)-OH in DMF solution in the presence of DIC and oxyma. Protective Fmoc groups were removed from both the amine group and side group using 20 vol% piperidine in DMF, then washed with 100 mL DMF and left to dry for 24 h at 25°C as previously described for Si-500-G.

**Aspartic Acid-functionalized silica** (Si-500-D, Si-1000-D) was similarly synthesized by the reaction of aminopropyl-functionalized silica (Si-500-NH2/Si-1000-NH2) with Fmoc-Asp(OtBu)-OH in DMF solution in the presence of DIC and oxyma. After verifying the removal of the Fmoc from the amine group, the tert-butyl (OtBu) protective group was removed from the side group using 50% TFA in DCM for 90 min at 25°C as previously described.[154] The Si-500-D/ Si-1000-D was washed with DMF and left to dry for 24 h at 25°C.

**Arginine-functionalized** silica (Si-1000-R) was similarly synthesized by the reaction of Si-1000-NH2 with Fmoc-Arg(Pbf)-OH in DMF solution in the presence of DIC and oxyma. After the removal and verification of the fmoc from the amine group, the pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) was removed from the side group using 20mL Reagent R (TFA/thioanisole/1,2-ethanediethiol/anisole (90/5/3/2)) for 2 hours as previously described.[155] The Si-1000-R was washed with DMF and left to dry for 24 h at 25°C.

**Arginine-glycine-aspartic acid-functionalized** silica (Si-1000-RGD) through the reaction of Si-1000-NH2 with Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, and Fmoc-Arg(Pbf)-OH in DMF solution in the presence of DIC and oxyma. The protected amino acids were attached to the surface with random positioning (Si-1000-RGD) by mixing of Si-1000-NH2 with Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH, and Fmoc-Arg(Pbf)-OH in DMF solution in the presence of DIC and oxyma using the previously described procedure. Fmoc was removed and verified for
all three amino acids simultaneously as described for Si-1000-D. After the removal and verification of the fmoc from the amine group of the arginine, OtBu and Pbf were simultaneously removed from the side group using 20mL Reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole (90/5/3/2)) for 2 hours. The Si-1000-RGD was washed with DMF and left to dry for 24 h at 25°C.

2.2.2. Mesostructured cellular foam silica (MCF)

Acid washed MCF silica was obtained from Dr. Jungseung Kim. The MCF silica had a pore volume of 2.4 cm³ g⁻¹, a BET (Brunauer–Emmett–Teller) surface area of 634.3 m² g⁻¹, and cell and window diameters of 33 nm and 16.6 nm, respectively.[148]

2.2.3. Surface Characterization

2.2.3.1. BET

Nitrogen adsorption and desorption measurements were performed on Si-500-AW and Si-1000-AW at 77 K using a Micromeritics ASAP 2010 volumetric adsorption analyzer (Norcross, GA, USA). 50 mg of the silica adsorbent was degassed at 573 K for at least 5 h prior to starting the nitrogen adsorption measurements. The Brunauer–Emmett–Teller (BET) method was used to determine the surface area at a P/P₀ range of 0.05 - 0.25; the diameter of the adsorptive molecule (N₂) was taken to be 3Å.[108]

2.2.3.2. FTIR

The functionalizations were verified using Fourier Transform Infrared Spectroscopy (FTIR) using a Nicolet Magna-IR-760 spectrometer manufactured by Thermo Scientific (Madison, WI, USA). The FTIR used a Smart Orbit Diamond ATR module with a KBr beam splitter DTGS detector with a resolution of 1.928 cm⁻¹. The wavelength range was 4000 to 400 nm.
2.2.3.3. TGA/ DSC

Thermogravimetric Analysis (TGA) was used to determine the surface coverage of the functional groups, and Differential Scanning Calorimetry (DSC) was used to determine the decomposition rate of the functional groups. TGA and DSC were conducted under argon gas with a flow rate of 20 mL min\(^{-1}\) starting at room temperature and heating to 800°C at a rate of 20°C min\(^{-1}\) using a NETZSCH STA 409 PC Luxx TGA/DSC instrument. Surface coverage was calculated from the TGA weight loss between 250°C and 800°C, using Eq. (2-1), (2-2) and (2-3) as described by Kim et al.\[106]\]

\[
N_s = \frac{W_{loss}}{100 \ g \ adsorbent} \cdot \frac{mol_{ligand}}{mol_{molecule}} \cdot \frac{1000 \ mmol}{mol_{ligand}} \tag{2-1}
\]

\[
D = \frac{N_A \times N_s}{S_{BET}} \tag{2-2}
\]

\[
W_{AA-loss} = W_{loss} - \frac{M_{NH2-ligand}}{M_{AA-ligand}} W_{APTS-loss} \tag{2-3}
\]

\(N_s\) is the surface coverage (mmol g\(^{-1}\)). \(W_{loss}\) is the percent weight loss from TGA between 250\(^0\)C and 800\(^0\)C; \(M_{ligand}\) is the molecular weight of the ligand. \(D\) is the functional group density (molecule nm\(^{-2}\)). \(N_A\) is Avogadro’s number (6.02\(\times\)10\(^{23}\) mol\(^{-1}\)); and \(S_{BET}\) is the BET surface area of the silica (m\(^2\) g\(^{-1}\)). The total weight loss percentage measured for the amino acid functionalized silicas (Si-500-G, Si-500-D, Si-500-K, Si-1000-G, Si-1000-D, Si-1000-R, and Si-1000-RGD) include the weight loss from the amino acid ligands and aminopropyl ligands. \(W_{AA-loss}\) for the amino acid-functionalized silica was calculated using Eq. (2-3) based on the total weight loss, \(W_{loss}\), the Si-500-NH2/ Si-1000-NH2 weight loss, \(W_{APTS-loss}\), and the molecular weight of the ligands.
2.2.4. Adsorption

2.2.4.1. Batch Adsorption

Adsorption isotherms for lysozyme onto each material studied were determined as follows. Batch adsorption measurements were completed using lysozyme from chicken egg white dissolved completely in 0.02 M potassium phosphate buffer at pH 7 at 23°C. In each adsorption experiment, 1 mL of protein solution was mixed with 10 mg silica in a low binding microcentrifuge tube (Eppendorf North America, Hauppaug, NY, USA). The initial lysozyme concentration ranged from 2 mg mL\(^{-1}\) (0.14 µmol mL\(^{-1}\)) to 40 mg mL\(^{-1}\) (2.8 µmol mL\(^{-1}\)) for all silica adsorbents; eight to ten different initial concentrations were used for each isotherm. The mixture was agitated on a shaker table for 24 h at 23°C and then centrifuged to remove the silica particles from solution at 5,000 rpm for 5 min. Positive controls (protein solution, but no silica) and negative controls (buffer with silica, but no protein) were included in each experimental set. The protein concentration in the supernatant solution was determined by measuring UV absorption at 280 nm with a UV-Visible spectrophotometer (Cary 50, Varian Inc., Palo Alto, CA, USA); the amount of protein adsorbed was determined by material balance. The final solution and adsorbed concentrations were determined by averaging three replicates of each experimental condition. A schematic of the experimental procedure is shown in Figure 2-1.

Figure 2-1: Procedure for batch adsorption experiments
The supernatant from each adsorption experiment was diluted and measured three times. This resulted in six or nine data points for every initial protein concentration. The resulting adsorption data were fit to a Type I (Langmuir (2-4)) or Type II (Guggenheim, Anderson and De Boer (GAB (2-5)) isotherms using the NonLinearModelFit function in the Wolfram Mathematica 9 software package (Wolfram Research Inc., Champaign, IL, USA). Several regression forms were used are presented in the results chapters.

\[
\theta = \frac{q}{q_m} = \frac{kx}{1 + kx} \quad \text{(2-4)}
\]

\[
\theta = \frac{q}{q_m} = \frac{cfx}{(1 - fx)(1 - (1 - c)fx)} \quad \text{(2-5)}
\]

### 2.2.4.2. Competitive Adsorption

LYS and MYO were dissolved completely in 0.02 M potassium phosphate buffer at pH 7 at 23°C. In each adsorption experiment, 1 mL of protein solution was mixed with 5 mg silica in a low binding microcentrifuge tube (Eppendorf North America, Hauppauge, NY, USA). The initial protein concentration ranged from 0 to 10 mg mL\(^{-1}\) (0.70 \(\mu\)mol mL\(^{-1}\)) for LYS and from 0 to 10 mg mL\(^{-1}\) (0.57 \(\mu\)mol mL\(^{-1}\)) for MYO resulting in a six by six matrix of LYS and MYO concentrations. The mixture was mixed on a shaker table for 24 h at 23°C and then centrifuged at 5,000 rpm for 5 min. Positive (protein solution, no silica) and negative (buffer with silica) controls were included in each experimental set with protein without adsorbent and adsorbent with buffer and no protein respectively.

The supernatant solution was analyzed with a UV-Visible spectrophotometer (Cary 50, Varian, Palo Alto, CA, USA) at a wavelength of 280 and 400 nm. Figure 2-2 shows the UV absorbance spectrum for both proteins. The concentration of MYO in the supernatant is

\[
\theta = \frac{q}{q_m} = \frac{kx}{1 + kx} \quad \text{(2-4)}
\]

\[
\theta = \frac{q}{q_m} = \frac{cfx}{(1 - fx)(1 - (1 - c)fx)} \quad \text{(2-5)}
\]
determined from its extinction coefficient at 400nm; then the concentration of LYS is determined from the extinction coefficients of both proteins at 280nm and the already determined concentration of MYO. The amount of protein adsorbed was determined by material balance. The final solution and adsorbed concentrations were determined by averaging two - three replicates of each experimental condition. The resulting adsorption isotherms were fit to an expansion of the Guggenheim-Anderson-de Boer (GAB) adsorption model using the Matlab software package (Mathworks, Natick, MA, USA).

Figure 2-2: Lysozyme (blue) and myoglobin (red) UV absorbance in in 0.02 M phosphate buffer at pH 7 and 23°C

2.2.5. Enthalpy

The heat of adsorption was measured with flow microcalorimetry (Gilson Instruments, Westerville, OH, USA).[94, 106, 156] A small column (0.17 mL) was packed with the adsorbents; Si-60-AW (67.3 – 82.5 mg), Si-500-AW (38.9 – 48.5 mg), Si-1000-AW (35.8 – 43.2 mg) for chapter 3 and MCF silica (14.1-16.7 mg) for chapter 5. The column was then equilibrated with flowing buffer (0.02 M potassium phosphate pH 7, 23°C, 1.85 mL h⁻¹) for at
least 16 hours. A sample loop (1.36 mL) was filled with a solution of protein dissolved in mobile phase; when the protein solution flowed to the packed bed two thermistors in the column wall output the heat of protein adsorption as a thermogram. Thermistors were calibrated with an electrical signal. The protein solution consisted of 2 mg mL\(^{-1}\) (0.14 µmol mL\(^{-1}\)), 8 mg mL\(^{-1}\) (0.56 µmol mL\(^{-1}\)), or 20 mg mL\(^{-1}\) (1.4 µmol mL\(^{-1}\)) in 0.02 M potassium phosphate buffer pH 7 for chapter 3 and 2 mg mL\(^{-1}\) lysozyme (0.14 µmol mL\(^{-1}\)), 2 mg mL\(^{-1}\) myoglobin (0.11 µmol mL\(^{-1}\)), or 2 mg mL\(^{-1}\) lysozyme with 2 mg mL\(^{-1}\) myoglobin in 0.02 M potassium phosphate buffer pH 7 for chapter 5. Experiments were conducted in duplicate and averaged; peak areas were reproducible with a standard error of 17%. In chapter 5 to determine the heat of adsorption of protein on a silica bed with adsorbed protein, a second 2 mg mL\(^{-1}\) protein solution was injected onto the same packed bed 30 min after termination of the first experiment; sequential adsorption experiments were not replicated. To calculate the heat of adsorption, thermogram peak areas were normalized by the calibration signal, adsorbent bed mass, and the protein loading determined from batch adsorption experiments. Thermograms were deconvoluted into Gaussian peaks with MagicPlot software (Magicplot Systems LLC, Saint Petersburg, Russia); 2-4 Gaussian peaks were required to get good fits to each thermogram.

2.2.6. Venom

2.2.6.1. Surface Saturation

An isotherm was generated to determine the amount of venom required to cover a single layer of the adsorbent surface. Solid lyophilized venom was dissolved completely in 50mM potassium phosphate buffer pH 7.5 and 37 °C. In each experiment 1 mL of different concentrations of venom solution up to 2 mg mL\(^{-1}\) was mixed with 2 mg silica in a low binding centrifuge tube (Eppendorf North America, Hauppauge, NY, USA). The mixture was mixed on a
shaker table for 24 h and then centrifuged at 5,000 rpm for 5 min. The supernatant solution was collected. The initial and final protein concentrations in the supernatant solution were determined using a UV-Visible spectrophotometer (Cary 50, Varian, Palo Alto, CA, USA) at a wavelength of 280 nm; the amount of protein adsorbed was determined by material balance. A schematic of the procedure is shown in Figure 2-3.

Figure 2-3: Procedure for metalloproteinase selectivity experiment

### 2.2.6.2. Venom Adsorption

Solid lyophilized venom was dissolved in 50mM potassium phosphate buffer pH 7.5 and 37 °C and mixed with each adsorbent. In each experiment, 1 mL of 2 mg mL^{-1} venom solution was mixed with 2 mg of adsorbent and left stirring on a shaking table for 24 h. The mixture was similarly centrifuged and supernatant was tested with UV at 280 nm to determine total protein adsorption and with a casein assay to determine the metalloproteinase activity. A minimum of two replicates was used for each experiment. Positive (venom solution, no silica) and negative (buffer with silica) controls were included in each experimental set.
2.2.6.3. Casein Assay

The metalloproteinase activity was determined before and after adsorption using a casein assay as previously described in the Sigma Aldrich written protocol and by Rodrigues et al. [151] The casein assay measures the amount of tyrosine that is produced as a result of casein digestion by an enzyme. Briefly, a 0.65% w/v casein solution 50 mM potassium phosphate buffer was prepared. The solution temperature is gradually increased with gentle stirring to 80-85 °C without boiling for about 10 minutes until a homogenous dispersion is achieved. The casein solution is cooled to 37 °C.

For each venom/supernatant solution, four vials were used with 0, 0.05mL, 0.1mL and 0.2 ml of the sample solution. They were each incubated with 1 ml casein solution for 10 min at 37°C. The reaction was stopped by the addition of 1 ml of 5% (v/v) trichloroacetic acid (TCA) and the mixture was left standing for 30 min at room temperature before centrifugation at 1500 rpm for 5 min. The volume of the blank, 0.05mL and 0.1mL were adjusted with more of the sample solution to be the same as the 0.2 mL vial to ensure equal protein content is equal in all the vials.

The test solutions were filtered using a 0.2 µm polyethersulfone syringe filter. 0.4 mL of the filtrate was then added to 1 mL of 500 mM sodium carbonate dissolved in water and 0.2 mL of Folin’s reagent and is mixed by swirling and incubated at 37°C for 30 min. The test solutions are then filtered again 0.2 µm polyethersulfone syringe filter. The solution absorbance is measured at 660 nm and compared to a known tyrosine calibration curve. The activity was determined using Eq. (2-6).

\[
Activity = \frac{Tyrosine\ Released \ast Assay\ total\ volume}{Enzyme\ volume \ast Reaction\ time \ast Cuvette\ volume}\tag{2-6}
\]
The activity is defined in units mL⁻¹; tyrosine released is in µmol equivalents; the total assay volume is 2.2 mL; the reaction time is 10 min and the cuvette volume is 0.4 mL. The initial and final activities were used to determine the amount of metalloproteinase removed from the venom solution. The selectivity of each adsorbent was determined as the percentage metalloproteinase removed divided by the percentage of total protein removed.

### 2.2.6.4. EDTA

The activity of the metalloproteinase was distinguished from other possible proteinases through the addition of EDTA to the venom as previously described by Rodrigues et al.[151] Briefly, a 10 mM EDTA solution was prepared and heated to 37°C. Then the ETDA solution was added to the venom in a 1:1 ratio. 0.2 mL of 2 mg mL⁻¹ venom was mixed with 0.2 mL of 10 mM ETDA and left stirring on a shaking table for 1 h. The casein assay is repeated on the venom EDTA mixture. The calculated activity is the activity of other proteinases besides the metalloproteinase.
Chapter 3 Lysozyme Adsorption on Amino Acid-Functionalized Silica

3.1. Introduction

Silica and functionalized silica are widely used as stationary phases for high performance liquid chromatography (HPLC) columns in the pharmaceutical industry.[60, 64-68] Surface functionalization can add chemical or biological specificity to silica surfaces, allowing control of the selectivity of the adsorption.[77] The application of such surface functionalizations on chromatographic media is well-documented.[78-85] For example, Takahashi et al. showed that SBA-15 synthesized using a nonionic surfactant had a much lower protein immobilization capacity than that of MCM-41 and FSM-16 synthesized using cationic surfactants.[157] The difference in charge between the surfaces greatly affected the capacity despite their similar pore sizes, thus showing the importance of the surface chemistry. Yiu et al. showed that cytochrome C, lysozyme, myoglobin, b-lactoglobulin, ovalbumin, BSA, and conalbumin could reversibly adsorb onto the native SBA-15, but would irreversibly adsorb onto thiol-functionalized SBA-15.[158, 159] Kim et al compared to adsorption of lysozyme onto MCF and amine-functionalized MCF and measured the effect of changing the surface chemistry on the thermodynamics of adsorption.[106] Depending on the functionalization chosen, proteins can be adsorbed with varying degrees of reversibility or can be excluded from the surface. Increased complexity of the surface functionalization can produce higher specificity,[22] but also results in higher costs.[86] Affinity chromatography, which uses a biologically related agent, or “affinity ligand”, as a stationary phase to selectively retain analytes or to study biological interactions,[22] is extremely specific. However, it requires a complex procedure of generating and purifying antibodies for a lock and key approach to protein purification. The simpler surface functionalities are far lower in cost than affinity chromatography, but can provide only simple separations based
on charge, hydrophobicity, or size. New surface functionalities, such as amino acid functionalities, promise to achieve higher selectivity at lower cost.

Amino acids, the building blocks of proteins, consist of a central carbon atom bonded to an amine group, a hydrogen atom, a carboxyl group, and a defining side, or R, group. Amino acids are a less expensive alternative to antibodies, but can provide a more specific biological interaction than the simpler surfaces. The method used to bind the amino acids to the surface must also be considered, as it might influence the surface chemistry. In the study reported here, a peptide synthesis technique was used to attach the amino acids to silica in an attempt to get a high-capacity surface for adsorption. Peptide synthesis methods have seen recent advancements towards large scale commercialization; the successful commercialization of these methods can also be beneficial for silica surface modifications. A schematic of the functionalization procedure is shown in Figure 3-1.

![Figure 3-1: Schematic representation of glycine functionalization of Si-500-NH2 using DIC and oxyma in DMF and Fmoc removal in 20 vol% piperidine in DMF](image)

The study reported here considered the use of amino acid-functionalized silica surfaces as a stationary phase for protein separations; the amino acids were attached to amine functionalized silica through the creation of a peptide bond. Lysozyme was chosen as a test protein because of its relatively small size and stability and because lysozyme adsorption has been studied
extensively. Adsorption studies were conducted on carboxyl- and amine- functionalized silica, and on silica functionalized, through peptide bond formation, with glycine (R = H), aspartic acid (R = CH₂-COOH), and lysine (R = (CH₂)₄-NH₂). These amino acids were chosen based on side groups that correspond to the surface chemistry of the amine- and carboxyl-functionalized silica. The adsorption studies were conducted at a low ionic strength to allow for possible electrostatic interactions that would be attenuated at high ionic strength.

3.2. Materials

(3-Aminopropyl) triethoxysilane (APTES, 99%), 3-cyanopropyltriethoxysilane (CPTES, 98% purity), ethyl (hydroxyimino)cyanoacetate (oxyma), piperidine (ReagentPlus®, 99%), dichloromethane (DCM), and lysozyme from chicken egg white (LYS, 90%) were obtained from Sigma–Aldrich (St. Louis, MO). Potassium phosphate monobasic and dibasic potassium phosphate powder, trifluoroacetic acid (TFA), protected glycine (Fmoc-Gly-OH, ≥98% by TLC and HPLC), protected lysine (Fmoc-Lys(Fmoc)-OH, ≥98% by HPLC), protected aspartic acid (Fmoc-Asp(OtBu)-OH, Anaspec), and N,N’-diisopropylcarbodiimide, 99% (DIC) were purchased from Fisher Scientific. N,N-Dimethylformamide (DMF) was purchased from the Tedia Company. Matrex normal phase liquid chromatography media Si-500A-10μm (#87076508), was purchased from Amicon, Billerica, MA, USA.

3.3. Methods

The methods used for surface functionalization of Acid-washed silica (Si-500-AW) Aminopropyl-functionalized silica (Si-500-NH2) Carboxypropyl-functionalized silica (Si-500-COOH) Glycine-functionalized silica (Si-500-G) Lysine -functionalized silica (Si-500-K) and Aspartic Acid -functionalized silica (Si-500-D) are presented in chapter 2.
Nitrogen adsorption and desorption measurements were performed on Si-500-AW. The Brunauer–Emmett–Teller (BET) method was used to determine the surface area as discussed. The functionalizations were verified using Fourier Transform Infrared Spectroscopy (FTIR). Thermogravimetric Analysis (TGA) was used to determine the surface coverage of the functional groups, and Differential Scanning Calorimetry (DSC) was used to determine the decomposition rate of the functional groups. Details of these methods and the instruments used have been previously presented in chapter 2.

Batch adsorption measurements were completed using lysozyme from chicken egg white dissolved completely in 0.02 M potassium phosphate buffer at pH 7 at 23°C. Details of the adsorption method are presented in chapter 2. The resulting adsorption data were fit to a Type I (Langmuir) isotherm using the NonLinearModelFit function in the Wolfram Mathematica 9 software package (Wolfram Research Inc., Champaign, IL, USA). Several regression forms were used; details are presented in the results section below.

3.4. Results and Discussion

3.4.1. Surface Functionalization

FTIR was used to confirm that amino acids were added to the surface through the formation of peptide bonds. FTIR spectra for Si-500-AW, Si-500-NH2, and Si-500-G are shown in Figure 3-2. Distinct peaks associated with silica were observed for all three materials around 1110 nm. The silanol peak at 966 nm disappears with the addition of the amine group for Si-500-NH2.[160] Amide peaks appear at 1674 nm and 1502 nm and a peak corresponding to a carbon–oxygen bond appears at 660 nm for Si-500-G, verifying the addition of the glycine.[161] FTIR spectra for the intermediate material Si-500-G-Fmoc, where the protective Fmoc group is still present, does not show the peptide bond as the Fmoc group is much larger than the peptide bond. The peaks corresponding to the peptide bond appear in
Si-500-G after the protective group (Fmoc) is removed. Similarly, the peptide bond is not visible for Si-500-D and Si-500-K, presumably because it is shielded by the side groups.

Figure 3-2: FTIR results for (1) Si-500-AW, (2) Si-500-COOH, (3) Si-500-NH2, (4) Si-500-G-Fmoc, (5) Si-500-G-, (6) Si-500-D, and (7) Si-500-K. Spectra are vertically shifted for better visibility.

DSC was used to determine that the amino acids attached to the aminopropyl group rather than to the surface. The DSC data shown in Figure 3-3 show the same trends for Si-500-NH2, Si-500-G, Si-500-D, and Si-500-K. Inflection points occur in the DSC curves for all four materials at approximately 100°C and 550°C. This similarity indicates that the amino acid ligands decompose at the same temperature as the aminopropyl ligands.[162] This result indicates that
the amino acids attached to the amine through a peptide bond without further interaction between the amino acids and uncovered surface silanols.

Figure 3-3: DSC data for (1) Si-500-AW, (2) Si-500-G, (3) Si-500-K, (4) Si-500-D, (5) Si-500-NH₂, and (6) Si-500-COOH under 20 mL min⁻¹ argon gas with 20°C min⁻¹ heating.

TGA was used to determine the surface coverage, ligand density and fraction of surface amines bonded with amino acids. The weight loss curves obtained in the TGA characterizations are shown in Figure 3-4.
Figure 3-4: TGA results for (1) Si-500-AW, (2) Si-500-NH₂, (3) Si-500-G, (4) Si-500-COOH, (5) Si-500-K, (6) Si-500-D under argon gas with 20°C min⁻¹ heating.

Again, Si-500-NH₂, Si-500-G, Si-500-D and Si-500-K all show similar curve shapes and decomposition temperatures consistent with attachment to the amine group through a peptide bond. Surface coverages and ligand densities determined from the TGA analysis are shown in Table 3-1. Surface coverages for Si-500-NH₂, Si-500-G, Si-500-D, and Si-500-K ranged from 132 to 155 μmol g⁻¹; the surface coverage for Si-500-COOH was 214 μmol g⁻¹. The corresponding ligand densities ranged from 0.84 to 1.36 ligands nm⁻²; assuming uniform, regular spacing on the adsorbent surface, these densities correspond to a ligand spacing of 0.5 – 0.7 nm. The amino acid coverage, defined as the percentage of surface amines bound to an amino acid, was 90% for Si-500-G, 85% for Si-500-K, and 98% for Si-500-D. Ligand densities were based on the BET surface area determined from the nitrogen adsorption/desorption measurements, 94.4 m² g⁻¹.
Table 3-1. Surface coverage, $N_s$, and ligand density, $D$, for surface amine-, carboxyl-, and amino acid-functionalized silica. Surface coverage calculated from TGA weight loss between 250°C and 800°C; ligand densities based on the BET surface area of 94.4 m$^2$ g$^{-1}$; ligand spacings estimated assuming uniform spacing on surface; charge estimated based on ligand density and degree of dissociation of terminal and side groups at pH 7.

<table>
<thead>
<tr>
<th></th>
<th>TGA Weight Loss</th>
<th>$M_{ligand}$</th>
<th>$N_s$</th>
<th>$D$</th>
<th>Ligand pacing,</th>
<th>pKa</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt %</td>
<td>mg mol$^{-1}$</td>
<td>µmol g$^{-1}$</td>
<td>ligand nm$^2$</td>
<td>nm</td>
<td></td>
<td>C m$^{-2}$</td>
</tr>
<tr>
<td>Si-500-NH$_2$</td>
<td>1.81</td>
<td>58</td>
<td>155</td>
<td>0.99</td>
<td>0.62</td>
<td>10.53 [163]</td>
<td>+0.159</td>
</tr>
<tr>
<td>Si-500-COOH</td>
<td>2.77</td>
<td>87</td>
<td>214</td>
<td>1.36</td>
<td>0.53</td>
<td>4.88 [164]</td>
<td>-0.219</td>
</tr>
<tr>
<td>Si-500-G</td>
<td>2.52</td>
<td>117</td>
<td>139</td>
<td>0.89</td>
<td>0.66</td>
<td>2.34 (-COOH)</td>
<td>+0.145</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.6 (-NH$_2$) [165]</td>
<td></td>
</tr>
<tr>
<td>Si-500-D</td>
<td>3.26</td>
<td>175</td>
<td>152</td>
<td>0.97</td>
<td>0.63</td>
<td>2.09 (-COOH), 3.86 (-R), 9.82 (-NH$_2$) [165]</td>
<td>+0.005</td>
</tr>
<tr>
<td>Si-500-K</td>
<td>3.04</td>
<td>188</td>
<td>132</td>
<td>0.84</td>
<td>0.68</td>
<td>2.18 (-COOH)</td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.95 (-NH$_2$)</td>
<td>+0.254</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.53 (-R) [165]</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-2: Type I isotherm parameters (Eqs. 6 and 7) and statistical error analysis for batch adsorption of lysozyme to functionalized silicas Si-500-NH₂, Si-500-COOH, Si-500-G, Si-500-D, and Si-500-K from 0.02 M potassium phosphate buffer at pH 7 and 25°C. All parameters are statistically significant at the 99% level (p < 0.01).

<table>
<thead>
<tr>
<th></th>
<th>Eq. (6)</th>
<th>Eq. (7) constant</th>
<th>Eq. (7) constant, Δ(\tilde{G}_D^0 = \tilde{G}_K^0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(q_m), (\mu)mol g(^{-1})</td>
<td>(\Delta\tilde{G}_j^0), kJ mol(^{-1})</td>
<td>(q_m), (\mu)mol g(^{-1})</td>
</tr>
<tr>
<td>Si-500-NH₂</td>
<td>5.2 ± 1.5</td>
<td>-27.3 ± 1.8</td>
<td>-22.59 ± 0.70</td>
</tr>
<tr>
<td>Si-500-COOH</td>
<td>15.1 ± 1.0</td>
<td>-31.04 ± 0.70</td>
<td>-31.20 ± 0.69</td>
</tr>
<tr>
<td>Si-500-G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Side Group: -H</td>
<td>16.2 ± 1.7</td>
<td>-26.99 ± 0.73</td>
<td>14.64 ± 0.82</td>
</tr>
<tr>
<td>Si-500-D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Side Group: -COOH</td>
<td>10.4 ± 4.2</td>
<td>-26.6 ± 2.1</td>
<td>-25.07 ± 0.46</td>
</tr>
<tr>
<td>Si-500-K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Side Group: -(CH(_2))(_4)-NH(_2)</td>
<td>10.4 ± 3.3</td>
<td>-25.6 ± 1.3</td>
<td>-24.56 ± 0.46</td>
</tr>
<tr>
<td>Data Points</td>
<td>44</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Parameters</td>
<td>10</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td>34</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>Residual Sum-of-Squares Error</td>
<td>64.68</td>
<td>73.27</td>
<td>74.83</td>
</tr>
<tr>
<td>Residual Variance</td>
<td>1.90</td>
<td>1.93</td>
<td>1.92</td>
</tr>
</tbody>
</table>
3.4.2. Batch Adsorption

Lysozyme was adsorbed from solution in 0.02 M potassium phosphate buffer at pH 7 and room temperature. At these conditions, electrostatic interactions are still possible,[148] and lysozyme dimerization is low.[147] Previous studies have shown that lower ionic strengths, below 0.1 M, allow for electrostatic interactions, while higher ionic strengths decrease the Debye length and strongly attenuate electrostatic interactions; the calculated Debye length decreases from 30 Å to 3 Å when 1 M NaCl is added to 10 mM pH 7 phosphate buffer.[149] Higher ionic strengths can also result in irreversible lysozyme adsorption, which would not be ideal for chromatographic systems.[148] Isotherms for lysozyme adsorption onto Si-500-NH2, Si-500-COOH, Si-500-G, Si-500-D, and Si-500-K are shown in Figure 3-5.

![Figure 3-5: Lysozyme adsorption Si-500-COOH, Si-500-G, Si-500-D, Si-500-K and Si-500-NH2 in 0.02M phosphate buffer at pH 7 and 25°C, Points represent experimental results; curves were generated using parameter values in Table 3-2 assuming the monolayer coverage \( q_m \) is the same for all surfaces and that the standard Gibbs free energy of adsorption for Si-500-D and Si-500-K are equal (\( \Delta G^0_D = \Delta G^0_K \)). Error bars represent a single standard deviation around replicate measurements.](image-url)
The data in Figure 3-4 are shown as average results with standard deviations. The standard deviation increases at higher lysozyme concentrations because only a small fraction of the lysozyme is adsorbed at the higher initial concentrations. Most published papers do not use such high concentrations due to this problem.[166, 167] For example, Yiu et al., Vinu et al., and Qiao et al. report their liquid equilibrium concentration in µmol L⁻¹, while this study reports concentrations in µmol mL⁻¹.[158, 168, 169]

Other studies that report concentrations in similar units such as Katiyar et al. do not exceed 20 mg mL⁻¹, which approximately half our highest liquid concentration.[88] The higher concentrations were important in this study to show the applicability of the model even at such high concentrations. Regardless of the precision of the data, the qualitative trends in the results are clear, and the regression results are consistent with those qualitative trends.

Isotherms similar to those in Figure 3-5 have been reported for carboxyl and amine functionalizations. He et al. used the same carboxyl and amine functionalizations on SBA-15 and reported comparable monolayer concentrations of 0.6 g m⁻² and 0.4 g m⁻² on carboxyl and amine functionalized SBA-15, corresponding to 19 µmol g⁻¹ and 7 µmol g⁻¹) in 25 mM phosphate buffer at pH 6.8. The previously published SBA-15 results are slightly greater than the 15 µmol g⁻¹ and 5 µmol g⁻¹ observed here for Si-500-COOH and Si-500-NH2; the difference may be due to the higher accessible surface area of SBA-15. The isotherms reported by He et al. are similar to those reported here.[171] Similar adsorption data for the remaining surfaces are not available in the scientific literature.
For a fixed lysozyme concentration in the liquid phase, the amount of lysozyme adsorbed followed the general trend Si-500-COOH > Si-500-G > Si-500-D ≈ Si-500-K > Si-500-NH2. The data were fit to a type I adsorption isotherm:[94]

\[
q_j = q_{jm} \frac{K_jx}{1 + K_jx}
\]  

(3-1)

In this isotherm, \(x\) is the equilibrium mole fraction of the protein in the liquid phase, \(q_j\) is the equilibrium protein surface coverage (\(\mu\)mol g\(^{-1}\)) on surface \(j\), \(q_{jm}\) is the monolayer surface coverage (\(\mu\)mol g\(^{-1}\)) for surface \(j\), and \(K_j\) is a dimensionless adsorption coefficient for surface \(j\). While a more rigorous thermodynamic analysis of the adsorption process would use the protein activity rather than the protein mole fraction, under the conditions used in this study, the protein activity coefficient can be taken to be one.[112]

The adsorption constant \(K_j\) is related to the standard Gibbs free energy change of adsorption as shown in Eq. (3-2); this equation was thermodynamically derived based on the limiting assumptions of surface homogeneity, immobility of adsorbed species, negligible interactions between adsorbed molecules, and representation of adsorbate activity as the unoccupied fraction of adsorption sites.[94] Note that the \(K_j\) used in Eq. (3-1) and (3-2) is purely thermodynamic since it based on expressing compositions as mole fractions rather than molar concentration; consequently, it does not incorporate the phase ratio.

\[
\Delta\bar{G}_j^0 = \mu_{jB}^0 - \mu_B^0 = -RT \ln K_j
\]  

(3-2)

In this equation, \(\Delta\bar{G}_j^0\) is the standard Gibbs free energy change for adsorption onto surface \(j\) (J mol\(^{-1}\)), \(\mu_{jB}^0\) is the standard-state chemical potential of the adsorbate (B) on surface \(j\), \(\mu_B^0\) is the standard-state chemical potential of the adsorbate in solution, \(R\) is the ideal gas constant (8.314 J
mol$^{-1}$ K$^{-1}$), and $T$ is the temperature (298 K). To obtain rigorous standard errors for both $q_{jm}$ and $\Delta \bar{G}_j^0$, Eqs. (4) and (5) were combined to provide the regression form shown in Eq. (3-3).

$$q = q_{jm} \frac{\exp(-\frac{\Delta \bar{G}_j^0}{RT})x}{1 + \exp(-\frac{\Delta \bar{G}_j^0}{RT})x}$$  

(3-3)

The model parameters $q_{jm}$ and $\Delta \bar{G}_j^0$ were estimated using a separate regression for each surface; the results of these regressions are shown in Table 3-2.

The isotherm expressed in Eqs. (3-1) and (3-3) is typically derived on the assumption that each adsorbate molecule binds to a single, discrete site on the adsorbent surface. In the thermodynamic development cited above, the activity of adsorbed molecules is expressed as the fraction of the adsorption sites that are occupied by the adsorbate, while the activity of the unused adsorbent is expressed as the fraction of the adsorption sites that are not occupied. This approach does not accurately describe the adsorption of a large molecule to a surface with a high density of ligands. If a lysozyme molecule is taken to be an ellipsoid with axis lengths of 1.9 x 2.5 x 4.3 nm, the projected area of lysozyme onto the adsorbent surface is on the order of 0.12 – 0.27 molecules nm$^{-2}$, depending on orientation. Since the surface ligand density is 0.8 – 1.4 ligands nm$^{-2}$ as shown in Table 3-1, the area associated with each molecule of adsorbed lysozyme includes several ligands. For each attachment to a ligand, other ligands are rendered inaccessible due to the adsorbed lysozyme, thus decreasing the activity of adsorption per ligand. Consequently, it is appropriate to treat consider an adsorption site to be a patch of the adsorbent surface rather than a single ligand. By this reasoning, the adsorbents used in this study would have the same number of lysozyme adsorption sites, and thus the same monolayer coverage for lysozyme. If this is correct, a single value of $q_m$ should adequately describe the complete data set. To test the null hypothesis that $q_m$ is constant against the alternative hypothesis that $q_m$ depends on the ligand, the data were correlated using Eq.(3-4):
Regression results are presented in Table 3-2. An F-test was used to compare the residual variance of the 10-parameter regression expressed by Eq. (3-3) and to that of the 6-parameter regression expressed by Eq. (3-4). The resulting value of the $F$ statistic is 1.01, well below the critical value of 1.74 for 5% significance with 38 and 34 degrees of freedom. Consequently, the null hypothesis cannot be rejected; the data are well described using a single value of $q_m = 14.64 \pm 0.82 \, \mu\text{mol g}^{-1}$. This monolayer coverage corresponds to a lysozyme surface density of 0.094 nm$^{-2}$, which is reasonable when compared to the dimensions of the lysozyme molecule.

Inspection of the isotherms presented in Figure 3-5 and the results for the 6-parameter regression presented in Table 3-2 suggests that the isotherms for Si-500-D and Si-500-K are not significantly different. This hypothesis was tested by fitting the data set with the constraint that $\Delta \tilde{G}^0_D = \Delta \tilde{G}^0_K$ (a 5-parameter regression); results of this regression are also shown in Table 3-2. The total residual variance for Si-500-D and Si-500-K only was then calculated and compared with the corresponding total residual variance obtained for these surfaces using the 6-parameter regression. The resulting $F$ statistic was 1.006, much less than the critical value at a 5% significance level and 17 and 18 degrees of freedom. Consequently, the null hypothesis that $\Delta \tilde{G}^0_D = \Delta \tilde{G}^0_K$ cannot be rejected.

The results of this statistical analysis of the lysozyme data indicate that for the surfaces considered in this report, differences in the adsorption isotherms are caused by differences in the chemical potential of adsorbed lysozyme and not by differences in monolayer coverage.
3.4.3. Adsorption Energetics

The carboxyl-functionalized surface (Si-500-COOH) showed the strongest lysozyme adsorption isotherm, with $\Delta \bar{G}_{\text{COOH}}^0 = -31.20 \pm 0.69 \text{ kJ mol}^{-1}$. This strong adsorption is due to the large electrostatic driving force between the negatively-charged carboxyl surface (with a surface charge density of $\sigma = -0.219 \text{ C m}^{-2}$) and the positively-charged lysozyme ($pI = 11.35$),\cite{172} which has an estimated net surface charge density at pH 7 of $\sigma = 0.037 \text{ C m}^{-2}$ (based on a net charge of $4.5 \text{ e} = 8.5 \times 10^{-19} \text{ C}$\cite{173} and a radius of gyration of 1.35 nm.\cite{147} The high lysozyme loading observed for the carboxyl-functionalized surface (Si-500-COOH) is consistent with previously reported data. For example, Fu et al. showed that the addition of carboxyl group to a silica functionalized with 3-methacryloxypropyl trimethoxysilane more than doubled lysozyme adsorption.\cite{174} The amine-functionalized surface (Si-500-NH2) had a net positive charge ($\sigma = +0.159 \text{ C m}^{-2}$), resulting in the weakest lysozyme adsorption ($\Delta \bar{G}_{\text{NH2}}^0 = 22.59 \pm 0.70 \text{ kJ mol}^{-1}$). This is due to electrostatic repulsion and is consistent with previously reported data; for example, Kim et al. found that adsorption on lysozyme onto amine functionalized mesostructured cellular foam silica was determined by a balance between electrostatic interactions and van der Waals forces. At lower ionic strengths, similar to the solution conditions used in this study, electrostatic interactions were predominant.\cite{106} These results suggest that differences in lysozyme adsorption between Si-500-COOH and Si-500-NH2 result from differences in net surface charge.

Results for lysozyme adsorption onto the amino acid-functionalized surfaces are more difficult to interpret. The net surface charge densities of the amino acid-functionalized materials were estimated from the pKa of the side group, the pKa of the terminal amino acid amine group, and the pKa of the amine groups of the unreacted aminopropyl ligands. Si-500-G has an estimated surface charge density of $\sigma = +0.145 \text{ C m}^{-2}$; Si-500-D has an estimated surface charge
density of $\sigma = +0.005 \text{ C m}^{-2}$; and Si-500-K has an estimated surface charge density of $\sigma = +0.254 \text{ C m}^{-2}$. Based on surface charge density alone, $\Delta \tilde{G}^0$ would be expected to follow the trend $\Delta \tilde{G}^0_{\text{COOH}} > \Delta \tilde{G}^0_D > \Delta \tilde{G}^0_G > \Delta \tilde{G}^0_{\text{NH2}} > \Delta \tilde{G}^0_K$. However, lysozyme adsorption onto Si-500-G ($\Delta \tilde{G}^0_G = -28.28 \pm 0.59 \text{ kJ mol}^{-1}$) is stronger than adsorption onto both Si-500-D ($\Delta \tilde{G}^0_D = -24.80 \pm 0.36 \text{ kJ mol}^{-1}$) and Si-500-K ($\Delta \tilde{G}^0_K = -24.80 \pm 0.36 \text{ kJ mol}^{-1}$) despite these surface charge densities. Remarkably, lysozyme adsorption onto Si-500-K was greater than adsorption onto Si-500-NH2 despite the more positive surface charge of Si-500-K. These observations rule out net charge difference as the predominant factor determining lysozyme adsorption onto the amino acid-functionalized surfaces studied here; the interaction between lysozyme and each amino acid-functionalized surface must be considered separately based on specific interactions between the lysozyme and the surface.

Si-500-G had the highest lysozyme adsorption of the surfaces studied; this result is consistent with the work of Lee et al., who observed stronger lysozyme adsorption on glycine-functionalized hydroxyapatite than on lysine-functionalized hydroxyapatite at pH 7.4.[175] Molecular simulation indicates that free glycine can hydrogen bond with the LYS96, ARG14, and ASP87 residues of lysozyme,[176] consistent with the observed strong adsorption of lysozyme on Si-500-G. The side group of glycine is a hydrogen atom, which should have negligible influence compared to the influences of the terminal amine group and the peptide bond. Both the terminal amine group and the peptide bond are common to all three amino acid-functionalized surfaces, but the peptide bond is more accessible on Si-500-G due to the absence of a large side group. Consequently, lysozyme interaction with glycine amide may more accurately represent the interaction between lysozyme and Si-500-G. High resolution X-ray crystallography showed that glycine amide interacts with the lysozyme surface near aromatic
residues. The strength of this interaction is confirmed by the effectiveness with which glycine amide inhibits lysozyme aggregation;[177] glycine amide more effectively prevents lysozyme aggregation than arginine, glycine, lysine and aspartic acid.[177-179]

As discussed above, net charge difference is not the primary factor determining lysozyme adsorption on the amino acid-functionalized silica surfaces used in the study reported here. This conclusion appears to be in contrast to results reported by Lee et al. in which lysozyme was adsorbed onto amino acid-functionalized hydroxyapatite; in that study, both net charge differences and the length of the side group were found to be significant factors affecting adsorption.[175] However, the hydroxyapatite surfaces used by Lee et al. were synthesized using a chemical precipitation method rather than by direct grafting of amino acid ligands to the silica surfaces via peptide bonds as was done in the study reported here. This synthesis method results in incorporation of the charged groups from the amino acid at the adsorbent surface rather than some distance away, so interactions with these charged groups would compete with nonspecific interactions with the hydroxyapatite matrix. Thus, the difference between the results reported by Lee et al. and those reported here are indicative of specific interactions between side chains in grafted amino acids and lysozyme.

Both aspartic acid and lysine residues are involved in lysozyme adsorption to other molecules. Aspartic acid residue 52 is critical for affinity binding and for lysing oligosaccharides,[180, 181] and lysine residues are found to interact with negatively-charged silica surfaces during lysozyme adsorption.[182] Therefore, both Si-500-D and Si-500-K should provide adsorption surfaces with greater lysozyme affinity than Si-500-NH2. Moreover, Ermakova et al. found that lysozyme dimerization generally occurs through the formation of salt bridges between negatively-charged residues such as aspartic acid and glutamic acid and
positively-charged residues such as lysine and arginine.[146] Although different residues can be involved in lysozyme dimerization, there are two types of lysozyme dimer complexes that can form; the free energy change of dimerization energy for the two dimer complexes are 4.75 kcal mol\(^{-1}\) (19.9 kJ mol\(^{-1}\)) and 6.3 kcal mol\(^{-1}\) (26.4 kJ mol\(^{-1}\)).[146] The free energy change of adsorption onto Si-500-K and Si-500-D (\(\Delta G^0_K = \Delta G^0_D = -24.80 \pm 0.36 \text{ kJ mol}^{-1}\)) is comparable to the two dimerization free energies. This similarity suggests that the lysozyme attaches to the Si-500-D and Si-500-K surfaces as it would another to another lysozyme molecule to form a dimer, explaining why \(\Delta G^0_K = \Delta G^0_D\).

### 3.5. Conclusion

Silica was successfully functionalized with amino acids using a peptide synthesis method and the effect of amino acid functionalization on lysozyme adsorption to silica surfaces was determined. Due to the high ligand density at the adsorbent surface, monolayer coverage is determined by the available surface area and not by the number of ligands. Lysozyme adsorption could be modeled using a single monolayer coverage for all the functionalizations synthesized in this study. The net charge difference between the surface and the adsorbed protein was the main factor influencing the adsorption of lysozyme onto carboxyl- and amine-functionalized silica. Surprisingly, net charge difference did not affect lysozyme adsorption onto amino acid-functionalized silica. Rather, specific interactions between lysozyme and the amino acid functionalities determined adsorption behavior.
Chapter 4 Competitive Adsorption of Lysozyme and Myoglobin on Mesostructured Cellular Foam Silica

A.M. Darwish, T. Robie, R.J. Desch, S.W. Thiel, “Multicomponent GAB model for lysozyme and myoglobin adsorption on silica” Microporous and Mesoporous Materials, in review 2014
4.1. Introduction

Protein capture and purification is achieved in industry using various types of chromatography. To move from theoretical bench scale adsorbents to an analytical or preparative packed column, various models have been employed to translate the adsorption models to the chromatographic models to predict complex protein separation in the column.[109, 110] Therefore, adsorption models that can mathematically predict the interactions between proteins and adsorbents are essential to proteomics research and the industry.

A number of mathematical models have been previously developed to study the interaction between an adsorbate and a solid adsorbent.[107] Models are used based on the general shape of the isotherm. Langmuir for example is often used for type I isotherms where the amount of material adsorbed to the solid remains constant past a certain liquid concentration, thus predicting a monolayer surface coverage. The Brunauer, Emmett and Teller (BET) model or the Guggenheim–Anderson–deBoer (GAB) are often used for type II isotherms, where the amount of material adsorbed increases with increases concentration, remains constant, then continues to rise again.[108] Both BET and GAB allow for multilayer adsorption to the surface, and are based on different thermodynamic models.[95] The biggest departure between the models and industrial chromatographic separation is that they describe adsorption of single components to the surface. These models are greatly deficient for use with complex protein solutions, and therefore must be modified to accommodate the adsorption of multiple components.

In a system involving more than one component, the standard adsorption models must be modified to accommodate multiple adsorbates. Such expansions have been developed for Langmuir and Freundlich adsorption models, but not for more complex multilayer adsorption models.[114-117] This study focused on expanding the GAB model to a multicomponent system.
Thermodynamic insights from modeling equilibrium isotherm are coupled with the direct measurement of enthalpy during protein adsorption, measured with flow microcalorimetry (FMC).

This study uses the adsorption of lysozyme (14.3kDa, 1.9×2.5×4.3nm) and myoglobin (17.6kDa, 2.8×6.3×3.5nm) to create a multicomponent prediction model based on the GAB equation. Lysozyme and myoglobin were chosen based on their comparable size and charge in the used solution. Both proteins are readily available for purchase and have been heavily studied by researchers. Mesostructured cellular foam silica was used as the adsorbent. Silica is an industry standard that is often used as a base material for chromatographic separations due to its mechanical stability and controllable surface chemistry and morphology.[60, 64-68]

4.2. Materials and Methods

4.2.1. Materials.

Chicken egg white lysozyme 95% (LYS) and myoglobin from equine heart ≥ 90% (MYO) were supplied by Sigma–Aldrich (Pittsburgh, PA, USA). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Fisher Scientific (Fair Lawn, NJ). Acid washed MCF silica was obtained from Dr. Jungseung Kim. The MCF silica had a pore volume of 2.4 cm$^3$ g$^{-1}$, a BET (Brunauer–Emmett–Teller) surface area of 634.3 m$^2$ g$^{-1}$, and cell and window diameters of 33 nm and 16.6 nm, respectively.[148]

4.2.2. Batch Adsorption.

Lysozyme and myoglobin were dissolved completely in 0.02 M potassium phosphate buffer at pH 7 at 25°C. At these conditions, electrostatic interactions are still possible,[148] and lysozyme dimerization is low.[147] In each adsorption experiment, 1 mL of protein solution was
mixed with 5 mg silica in a low binding microcentrifuge tube (Eppendorf North America, Hauppauge, NY, USA). The initial protein concentration ranged from 0 to 10 mg mL\(^{-1}\) (0.70 \(\mu\)mol mL\(^{-1}\)) for LYS and from 0 to 10 mg mL\(^{-1}\) (0.57 \(\mu\)mol mL\(^{-1}\)) for MYO resulting in a six by six matrix of LYS and MYO concentrations. The mixture was agitated on a shaker table for 24 h at 25°C and then centrifuged at 5,000 rpm for 5 min. Positive controls (protein solution, no silica) and negative controls (buffer with silica) were included in each experimental set.

The supernatant solution was analyzed with a UV-Visible spectrophotometer (Cary 50, Varian, Palo Alto, CA, USA) at wavelengths of 280 and 400 nm. The concentration of myoglobin in the supernatant was determined from the absorbance at 400 nm; the concentration of lysozyme was determined from the absorbance at 280 nm and the already-determined concentration of myoglobin. The amount of protein adsorbed was determined by material balance. The equilibrium protein concentrations in the liquid and adsorbent phases were determined by averaging two or three replicates of each experimental condition. The resulting adsorption isotherms were fit to an extension of the Guggenheim-Anderson-de Boer (GAB) isotherm using MATLAB (Mathworks, Natick, MA, USA). The extended isotherm and regression methods are discussed in detail in Section 3 below.

4.2.3. Flow Microcalorimetry.

The enthalpy of adsorption was measured with a flow microcalorimeter (Gilson Instruments, Westerville, OH, USA).[94, 106, 156] A small column (0.17 mL) was packed with MCF silica (14.1-16.7 mg) and equilibrated with flowing buffer (0.02 M potassium phosphate pH 7, 23°C, 1.85 mL h\(^{-1}\)) for 16 hours. A sample loop (1.36 mL) was filled with a solution of protein dissolved in mobile phase; when the protein solution flowed to the packed bed two thermistors in the column wall output the heat of protein adsorption as a thermogram. Thermistors were
calibrated with an electrical signal. The protein solution consisted of 2 mg mL\(^{-1}\) lysozyme (0.14 µmol mL\(^{-1}\)), 2 mg mL\(^{-1}\) myoglobin (0.11 µmol mL\(^{-1}\)), or 2 mg mL\(^{-1}\) lysozyme with 2 mg mL\(^{-1}\) myoglobin in 0.02 M potassium phosphate buffer pH 7. Experiments were conducted in duplicate and averaged; peak areas were reproducible with a standard error of 17%. To determine the heat of adsorption of protein on a silica bed with adsorbed protein, a second 2 mg mL\(^{-1}\) protein solution was injected onto the same packed bed 30 min after termination of the first experiment; sequential adsorption experiments were not replicated. To calculate the enthalpy of adsorption, thermogram peak areas were normalized by the calibration signal, adsorbent bed mass, and the protein loading determined from batch adsorption experiments. Thermograms were deconvoluted into Gaussian peaks with MagicPlot software (Magicplot Systems LLC, Saint Petersburg, Russia); 2-4 Gaussian peaks were required to get good fits to each thermogram.

4.3. Results and Discussion

4.3.1. Isotherms.

Equilibrium lysozyme adsorption in the presence of different initial concentrations of myoglobin is shown in Figure 4-1. The single-component adsorption of lysozyme is consistent with previously reported values, on MCF in range of 15 to 30 µmol g\(^{-1}\),[121] and within the range of other mesoporous silica with 18 µmol g\(^{-1}\) on MSE, an organosilicate, and 45 µmol g\(^{-1}\) on SBA-15.[183] These values are comparable to the 25 µmol g\(^{-1}\) reported here. The presence of myoglobin in solution appears to have little or no impact on the equilibrium lysozyme adsorption. However, the equilibrium adsorption of myoglobin decreases as the initial lysozyme concentration increases, as shown in Figure 4-2.
Figure 4-1: Lysozyme adsorption on silica in the presence of myoglobin in 0.02M potassium phosphate buffer at pH 7 and 25°C.

Figure 4-2: Myoglobin adsorption on silica in the presence of myoglobin in 0.02M potassium phosphate buffer at pH 7 and 25°C.
The single component adsorption of myoglobin is consistent with previously reported values on mesoporous silica with 10 µmol g\(^{-1}\) for MCM-41 and 30 µmol g\(^{-1}\) for SBA-15, which are comparable to the 18 µmol g\(^{-1}\) reported here.[87] The observed lysozyme adsorption behavior in the presence of myoglobin adsorption is inconsistent with simple site competition models such as the single- and multiple-component Langmuir isotherms. Instead, the equilibrium lysozyme adsorption data suggest either that the adsorption of lysozyme is independent of the adsorption of myoglobin or that there is some cooperative binding mechanism that offsets the sites occupied by adsorbed myoglobin. Since the equilibrium myoglobin adsorption decreases as the initial lysozyme loading increases, independent adsorption is ruled out. In addition, the lysozyme isotherms show some Type II character, suggesting possible multilayer formation.

The following mechanism is proposed to explain the observed competitive adsorption behavior. Steps M1 and M2 account for the direct adsorption of lysozyme, \(L\), and myoglobin, \(M\), on unoccupied surface, \(X\):

\[
X + L \rightleftharpoons XL \quad (M1)
\]

\[
X + M \rightleftharpoons XM \quad (M2)
\]

Step M3 accounts for possible formation of lysozyme multilayers as in the GAB isotherm:

\[
XL + iL \rightleftharpoons XL_{i+1} \quad (M3)
\]

Step M4 accounts for adsorption of lysozyme on adsorbed myoglobin, and subsequent formation of a lysozyme multilayer:

\[
XM + iL \rightleftharpoons XML_{i} \quad (M4)
\]
In the proposed mechanism, the adsorbed lysozyme blocks adsorption of myoglobin, but adsorbed myoglobin might act as a binding site for lysozyme and therefore have little effect on lysosyme adsorption.

This mechanism was tested in two ways, through flow microcalorimetry and by evaluating the goodness of fit obtained using the mechanism.

4.3.2. Enthalpy of Adsorption.

The enthalpy of adsorption of individual and combined lysozyme and myoglobin solutions onto MCF silica was measured as shown in Figure 4-3. Broad adsorption exotherms were observed in all three thermograms between 15 and 70 min, but differences in peak shape were observed. The myoglobin thermogram crested at 40 min, while the lysozyme and combined thermograms crested at 25 min. Desorption signals occurred between 70-100 min; small desorption endotherms were observed for lysozyme and the combined solution while small desorption exotherms were observed for myoglobin. Small desorption signals indicate that most of the protein adsorbs irreversibly. The lysozyme-silica and myoglobin-silica thermogram shapes reported here are consistent with previously-reported FMC studies.[148, 184, 185]

Interestingly, to note that while the enthalpies of lysozyme and myoglobin adsorption were comparable on a molar basis, the molar enthalpy of combined adsorption was twice as large as that for either of the individual proteins.
Figure 4-3. Averaged thermograms for the adsorption of (1) 2 mg mL$^{-1}$ lysozyme, (2) 2 mg mL$^{-1}$ myoglobin, and (3) 2 mg mL$^{-1}$ lysozyme with 2 mg mL$^{-1}$ myoglobin on fresh 14.1-16.7 mg MCF silica in 0.02 M potassium phosphate buffer at pH 7 and 25°C.

To better understand the large molar enthalpy of combined adsorption, lysozyme was adsorbed onto silica previously exposed to myoglobin and myoglobin was adsorbed onto silica previously exposed to lysozyme. The resulting thermograms are compared to thermograms for protein adsorption onto fresh silica in Figure 4-4.
Figure 4-4. Comparison between thermograms of protein adsorption on fresh silica and on silica with adsorbed protein. (1) 2 mg mL⁻¹ lysozyme on fresh silica, (2) 2 mg mL⁻¹ myoglobin on fresh silica, (3) 2 mg mL⁻¹ lysozyme on silica with adsorbed myoglobin, and (4) 2 mg mL⁻¹ myoglobin on silica with adsorbed lysozyme; 14.1-16.7 mg MCF silica; 0.02 M potassium phosphate buffer at pH 7 and 25°C.

The presence of adsorbed myoglobin had little impact on the lysozyme adsorption thermogram between 20-40 min, but significantly attenuated the thermogram between 40-70 min. This could be attributed to the saturation of the adsorptive surface, repulsion between lysozyme and adsorbed myoglobin, or myoglobin interference with a slow lysozyme adsorption process such as re-orientation on the silica surface. In contrast, adsorbed lysozyme greatly enhanced the magnitude of the myoglobin adsorption exotherm with little impact on the shape. From the
sequential adsorption experiments, it is proposed that the large adsorption exotherm observed for
the combined adsorption experiments can be attributed to the enhancement of the myoglobin
adsorption exotherm in the presence of lysozyme. All thermograms were deconvoluted into
Gaussian peaks to determine enthalpy of adsorption and desorption; the results are reported in
Table 4-1. The protein loadings shown in Table 4-1 were determined from the adsorption
isotherms.

Table 4-1. Enthalpies of adsorption and desorption of lysozyme and myoglobin on MCF silica
from 0.02 M potassium phosphate buffer at pH 7 and 25°C. Sample loop volume: 1.36 mL; flow
rate: 1.85 mL h⁻¹; adsorbent sample size: 14.1-16.7 mg.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Concentration μmol mL⁻¹</th>
<th>Protein Already Adsorbed on Surface</th>
<th>q μmol g⁻¹</th>
<th>ΔH_ads kJ mol⁻¹</th>
<th>ΔH_des kJ mol⁻¹</th>
<th>ΔH_net kJ mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYS</td>
<td>0.14</td>
<td>--</td>
<td>19.6</td>
<td>-16.4</td>
<td>0.00</td>
<td>-16.4</td>
</tr>
<tr>
<td>MYO</td>
<td>0.11</td>
<td>--</td>
<td>15.0</td>
<td>-18.4</td>
<td>-0.94</td>
<td>-19.3</td>
</tr>
<tr>
<td>LYS</td>
<td>0.14</td>
<td>--</td>
<td>23.1</td>
<td>-32.7</td>
<td>2.4</td>
<td>-30.3</td>
</tr>
<tr>
<td>MYO</td>
<td>0.11</td>
<td>15.0 μmol g⁻¹</td>
<td>17.2</td>
<td>-10.2</td>
<td>1.23</td>
<td>-9.0</td>
</tr>
<tr>
<td>LYS</td>
<td>0.14</td>
<td>19.6 μmol g⁻¹</td>
<td>5.9</td>
<td>-115.4</td>
<td>0.00</td>
<td>-115.4</td>
</tr>
</tbody>
</table>

These results are further validated by the visualization of the dynamics of myoglobin and
lysozyme competitive adsorption on SBA-15 at pH 7.1.[12] The mechanism of lysozyme
adsorption was not affected by the presence of myoglobin. However, the presence of lysozyme
caused the myoglobin uptake to change from homogenous adsorption to a shrinking core model,
which indicates the myoglobin is competing for sites and adsorbs differently with the presence of
lysozyme in solution. These findings, as well as the FMC results presented here, support
mechanism proposed here.
4.3.3. Extended GAB Model

The GAB isotherm can be extended based on the mechanism defined by Steps M1-M4 above. The development follows that of Pradas et al.[95] The variables used in the derivation are defined as follows: $a_i$ is the activity in the liquid phase, $\bar{a}_i$ is the activity of component $i$ on the surface, $n_i$ is the number of molecules of aggregates of species $i$ on the surface, $q_i$ is the surface coverage of species $i$, $X$ denotes surface, the subscript $m$ denotes monolayer, and $\theta_i$ is the surface fraction of species $i$. The chemical potentials of the species in solution, $\hat{\mu}_i$, can be written as

$$\hat{\mu}_L = \mu_L^0 + RT \ln a_L$$

$$\hat{\mu}_M = \mu_M^0 + RT \ln a_M$$

$R$ is the gas law constant and $T$ is the temperature in Kelvins.

The activity of each species on the surface is taken to be the surface fraction of that species, calculated as the coverage for that species divided by the monolayer coverage possible for that species:

$$\bar{a}_i = \frac{n_i}{n_{im}} = \frac{q_i}{q_{im}} = \theta_i$$

Note that this definition of activity does not require the assumption of discrete binding sites. Non-idealities are neglected in both the liquid and the surface phases. Based on the mechanism under consideration, at least two monolayer coverages must be considered: $q_{Lim}$, the monolayer coverage of adsorbed lysosome complexes, and $q_{Mm}$, the monolayer coverage of adsorbed myoglobin and myoglobin-lysosome complexes ($q_{MLim} = q_{Mm}$).

With these definitions in place, the chemical potentials of the surface species can be written as

$$\hat{\mu}_X = \mu_X^0 + RT \ln \theta_X$$
\[ \hat{\mu}_{XL} = \mu_{XL}^0 + RT \ln \theta_{XL} \quad (4-5) \]
\[ \hat{\mu}_{XM} = \mu_{XM}^0 + RT \ln \theta_{XM} \quad (4-6) \]
\[ \hat{\mu}_{XL_i} = \mu_{XL}^0 + (i - 1)\mu_{LL}^* + RT \ln \theta_{XL_i} \quad (4-7) \]
\[ \hat{\mu}_{XML_i} = \mu_{XM}^0 + \mu_{LM}^* + (i - 1)\mu_{LL}^* + RT \ln \theta_{XML_i} \quad (4-8) \]

Here \( \mu_{LL}^* \) is the chemical potential of lysozyme in the multilayer and \( \mu_{LM}^* \) is the chemical potential of lysozyme adsorbed to surface myoglobin.

Assuming mechanism steps M1 and M3 are in equilibrium,

\[ \hat{\mu}_{XL_i} = \hat{\mu}_X + i\hat{\mu}_L \quad (4-9) \]

Substituting Eq. (4-1), (4-4), (4-5) and (4-7) provides, after rearrangement,

\[ \frac{\mu_{XL}^0 - \mu_{LL}^*}{RT} + i \frac{\mu_{LL}^* - \mu_{LM}^0}{RT} = \ln \frac{\theta_{XL} \alpha_L^i}{\theta_{XL_i}} \quad (4-10) \]

For brevity, the chemical potential changes can be expressed using the parameters \( C_L \) and \( f_L \):

\[ \mu_{XL}^0 - \mu_{LL}^* = -RT \ln C_L \quad (4-11) \]
\[ \mu_{LL}^* - \mu_{LM}^0 = -RT \ln f_L \quad (4-12) \]

Note that \( f_L \) is a measure of the Gibbs free energy change of lysozyme for adsorption from free solution to the multilayer, while \( C_L \) is a measure of the Gibbs free energy change of lysozyme adsorption from the multilayer to the surface. It is also convenient to define the parameter \( g_L \):

\[ g_L \equiv f_L \alpha_L \quad (4-13) \]

Substituting these parameters into Eq. (4-10) yields an expression for the adsorption of species \( XL_i \):

\[ \theta_{XL_i} = C_L \ g_L^i \ \theta_X \quad (4-14) \]
A similar derivation can be applied for the adsorption of myoglobin species. Assuming mechanism steps M2 and M4 are in equilibrium,

\[ \hat{\mu}_{XR} = \hat{\mu}_X + \hat{\mu}_M \]  
(4-15)

\[ \hat{\mu}_{XR}L_i = \hat{\mu}_X + \hat{\mu}_M + i\hat{\mu}_L \]  
(4-16)

Applying Eqs. (4-2), (4-3), (4-5) and (4-7) provides the results

\[ \frac{\mu^0_{XR} - \mu^0_M}{RT} = \ln \frac{\theta_X a_M}{\theta_{XR}L_i} \]  
(4-17)

\[ \frac{\mu^0_{XR} - \mu^0_M + \mu^*_{LM} - \mu^*_{LL}}{RT} + i \frac{\mu^*_{LL} - \mu^0_L}{RT} = \ln \frac{\theta_X a_M a^i_L}{\theta_{XR}L_i} \]  
(4-18)

As before, the chemical potential changes can be expressed briefly, using the parameters \( K_M \) and \( C_{LM} \):

\[ \mu^0_{XR} - \mu^0_M = -RT \ln K_M \]  
(4-19)

\[ \mu^*_{LM} - \mu^*_{LL} = -RT \ln C_{LM} \]  
(4-20)

Note that \( K_M \) is a measure of the Gibbs free energy change of myoglobin for adsorption from free solution to the adsorbent surface, while \( C_{LM} \) is a measure of the Gibbs free energy change of lysozyme adsorption from the multilayer to adsorbed myoglobin. Applying these definitions results in the following expressions for the concentration of myoglobin species on the adsorbent surface:

\[ \theta_{XR} = K_M a_M \theta_X \]  
(4-21)

\[ \theta_{XR}L_i = K_M C_{LM} a_M g^i_L \theta_X \]  
(4-22)

Noting that the fractional surface coverage equations must sum to unity,
\[ 1 = \theta_X + \sum_j \theta_j = \theta_X + \theta_{XM} + \sum_{i=1}^{\infty} (\theta_{XL_i} + \theta_{XML_i}) \]  \hfill (4-23)

Substituting Eqs. (4-14), (4-21) and (4-22) into Eq. (4-23),

\[ 1 = \theta_X + K_M a_M \theta_X + \sum_{i=1}^{\infty} (C_L g^i_L \theta_X + K_M C_L M a_M g^i_L \theta_X) \]  \hfill (4-24)

Eq. (4-24) can be solved for \( \theta_X \):

\[ \theta_X = \left( 1 + K_M a_M + \sum_{i=1}^{\infty} (C_L g^i_L + K_M C_L M a_M g^i_L) \right)^{-1} \]  \hfill (4-25)

Since \( \sum_{i=1}^{\infty} g^i_L = g_L/(1 - g_L) \), \(|g_L| < 1\),

\[ \theta_X = \left( 1 + K_M a_M + \left( \frac{g_L}{1 - g_L} \right) C_L + K_M C_L M a_M \right)^{-1} \]  \hfill (4-26)

The total coverages for lysozyme and myoglobin adsorption can be calculated starting with the basic relationships:

\[ q_L = \sum_{i=1}^{\infty} i \left( q_{Lm} \theta_{XL_i} + q_{Mm} \theta_{XML_i} \right) \]  \hfill (4-27)

\[ q_M = q_{Mm} \theta_{XM} + \sum_{i=1}^{\infty} q_{Mm} \theta_{XML_i} \]  \hfill (4-28)

Applying Eqs. (4-14), (4-21) and (4-22) and (4-26), and further noting that \( \sum_{i=1}^{\infty} i g^i_L = g_L/(1 - g_L)^2 \),

\[ q_L = \frac{g_L (q_{Lm} C_L + q_{Mm} C_L M K_M a_M)}{(1 - g_L) \left[ (1 - (1 - C_L) g_L) + (1 - (1 - C_L M) g_L) \right] K_M a_M} \]  \hfill (4-29)

\[ q_M = \frac{q_{Mm} K_M a_M [1 - (1 - C_L M) g_L]}{1 + K_M a_M + g_L [(C_L - 1) + (C_L M - 1) K_M a_M]} \]  \hfill (4-30)
Note that in the absence of myoglobin, Eq. (30) reduces to the GAB isotherm and that in the absence of lysozyme, Eq. (31) reduces to the Langmuir isotherm, consistent with the proposed mechanism.

4.3.4. Data Regression.

The competitive adsorption data were fit to several forms of the model developed above. In total, six parameters can be estimated during the regression: \( q_{Lm}, q_{Mm}, C_L, K_M, C_{LM}, \) and \( f_L \). Five different formulations of the model were applied to evaluate the assumptions used during the model formulation.

Model 1: Competitive Langmuir. This is the simplest of the models, in which only mechanism steps M1 and M2 are assumed to proceed. This model thus disallows formation of lysozyme multilayers (\( f_L = 0 \)) and lysozyme adsorption onto myoglobin (\( C_{LM} = 0 \)), but allows different monolayer coverages. Under these assumptions, Eq. (4-29) and (4-30) reduce to the competitive Langmuir with \( -RT \ln K_i = \mu_{X1}^0 - \mu_i^0 \). This model has four parameters: \( q_{Lm}, q_{Mm}, K_L, \) and \( K_M \)

Model 2: Competitive GAB. In this model, lysozyme is assumed to adsorb to the silica following the GAB model (mechanism steps M1 and M3), myoglobin adsorbs following step M2, but there is no cooperative binding (step M4 is disallowed). Cooperative binding is disallowed by setting \( C_{LM} \) to zero. This model has five parameters: \( q_{Lm}, q_{Mm}, C_L, K_M, \) and \( f_L \)

Model 3: Cooperative Adsorption, No Lysosyme Multilayers. In this model, lysozyme and myoglobin compete for the adsorbent surface (steps M1 and M2); it is assumed that lysozyme multilayers do not form (no step M3), but that lysozyme can adsorb on adsorbed myoglobin (step M4 for \( i = 1 \)). For this model, Eq. (4-29) and (4-30) simplify to the forms:

\[
q_L = \frac{K_L a_L q_{Lm} + K_M K_{LM} a_M q_{Mm}}{1 + K_L a_L + K_M a_M + K_M K_{LM} a_L a_M} \quad (4-31)
\]
\[
q_M = \frac{K_M a_M q_{M_m} + K_M K_{LM} a_M q_{M_m}}{1 + K_L a_L + K_M a_M + K_M K_{LM} a_M a_M}
\] (4-32)

Here \(-RT \ln K_{LM} = \mu_L^* - \mu_L^0 = -RT \ln (C_{LM} f_L)\). This model has five parameters: \(q_{L_m}, q_{M_m}, K_L, K_M,\) and \(K_{LM}\).

**Model 4: Cooperative GAB with uniform \(q_m\).** This model is identical to the full formulation of Eq. (4-29) and (4-30) except that the same monolayer coverage is used for both proteins. This model has five parameters: \(q_m (= q_{L_m} = q_{M_m}), C_L, K_M, C_{LM},\) and \(f_L\).

**Model 5: Full Model.** This is the full formulation of the model as presented in Eq. (4-29) and (4-30). This model has six parameters: \(q_{L_m}, q_{M_m}, C_L, K_M, C_{LM},\) and \(f_L\).

Model parameters were determined by fitting Eqs. (30) and (31) to the experimental data using nonlinear regression to minimize simultaneously the residual variances for both lysozyme and myoglobin adsorption. Since the amount of lysozyme adsorbed was typically greater than the amount of myoglobin adsorbed, the residual variance, \(RV\), was scaled based on the average concentration of each protein (\(\bar{q}_L\) or \(\bar{q}_M\)) using the relationship

\[
RV = \left[ \sum_{i=1}^{N_L} \left( \frac{q_L^{(i)} - q_{L_C}}{\bar{q}_L} \right)^2 + \sum_{i=1}^{N_M} \left( \frac{q_M^{(i)} - q_{M_C}}{\bar{q}_M} \right)^2 \right] / DF
\] (4-33)

\(N\) is the number of data points, the superscript \((i)\) indicates individual data points, and the subscript \(C\) represents a calculated values. \(DF\) is the number of degrees of freedom, calculated as the total number of data points less the number of model parameters. Trivial points, where the protein liquid-phase activity is zero, were not included.

A two-stage regression algorithm was used. First, differential evolution was used to perform an unconstrained search of the parameter space (for example, \{\ln q_L, \ln q_M, \ln C_L, \ln K_M, \ln C_{LM}, \ln f_L\}) using a modified version of the MATLAB DE code published by Price and Storn.[186] The best five population members obtained from the differential evolution step were
averaged, and these average values were used as the initial estimate for a gradient descent search of the parameter space (for example, \{q_L, q_M, \ln C_L, \ln K_M, \ln C_{LM}, \ln f_L\}) using the MATLAB function \textit{fminunc} with the “quasi-newton” algorithm.

For the differential evolution step, a population size of 200 times the number of parameters was used, with a weighting parameter of 0.5 and a crossover coefficient of 0.85. Convergence was specified as 30 consecutive generations without at least a 0.5% decrease in the relative residual variance, defined as the residual variance at the best population parameter value divided by the residual variance at default parameter values. The initial population was determined by randomly sampling a broad parameter space (1 \leq (\ln q_L \text{ and } \ln q_M) \leq 6 \text{ and } 2 \leq (\ln C_L, \ln K_M, \ln C_{LM}, \text{ and } \ln f_L) \leq 20.

The topography of the search space requires a regression methodology more complex than simple gradient descent. It was confirmed through numerical experimentation that \textit{fminunc} alone was far less effective in minimizing the objective function than was differential evolution followed by \textit{fminunc}.

The standard errors of the final parameter estimates were obtained from the diagonals of the variance-covariance matix, \textit{cov}. The variance-covariance matrix is calculated from the Jacobian matrix, \textit{J}, and the mean squared error of the regression, \textit{MSE}:

\[
cov = [J']^{-1}MSE = \begin{bmatrix}
\sigma_{\beta_1}^2 & \cdots & \sigma_{\beta_1}\sigma_{\beta_6} \\
\vdots & \ddots & \vdots \\
\sigma_{\beta_6}\sigma_{\beta_1} & \cdots & \sigma_{\beta_6}^2
\end{bmatrix}
\]

\[
J = \begin{bmatrix}
\frac{\partial(e_1^2)}{\partial \beta_1} & \cdots & \frac{\partial(e_1^2)}{\partial \beta_6} \\
\vdots & \ddots & \vdots \\
\frac{\partial(e_n^2)}{\partial \beta_1} & \cdots & \frac{\partial(e_n^2)}{\partial \beta_6}
\end{bmatrix}
\]
\[
MSE = \left[ \sum_{i=1}^{N_L} \left( \frac{q_L^{(i)} - \bar{q}_L}{\bar{q}_L} \right)^2 + \sum_{i=1}^{N_M} \left( \frac{q_M^{(i)} - \bar{q}_M}{\bar{q}_M} \right)^2 \right] \left( N_L + N_M \right) = RV \frac{DF}{N_L + N_M} \quad (4-36)
\]

Here \(e_i\) is the residual error of measurement \(i\), \(\beta\) is the vector of parameters, and \(\sigma_j\) is the standard error of parameter \(j\).

### 4.3.5. Regression Results.

Regression results (parameter values, parameter standard deviations, and residual variances) obtained using each model are presented in Table 4-2.

Table 4-2. Statistical results for regression of one- and two-protein adsorption onto MCF silica using five regression models. All experiments were conducted in pH 7 0.02 M potassium phosphate buffer at 25°C.

<table>
<thead>
<tr>
<th>Model</th>
<th>Model 1 Competitive Langmuir</th>
<th>Model 2 Competitive GAB</th>
<th>Model 3 Cooperative Langmuir</th>
<th>Model 4 Extended GAB ( q_{Lm} = q_{Mm} )</th>
<th>Model 5 Extended GAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>(q_{Lm}, \mu \text{mol g}^{-1})</td>
<td>30.5 ± 5.9</td>
<td>30.5 ± 6.0</td>
<td>25.3 ± 3.4</td>
<td>19.3 ± 2.7</td>
<td>20.4 ± 4.8</td>
</tr>
<tr>
<td>(q_{Mm}, \mu \text{mol g}^{-1})</td>
<td>18.0 ± 3.2</td>
<td>18.0 ± 3.2</td>
<td>19.4 ± 4.5</td>
<td>18.6 ± 4.4</td>
<td>18.6 ± 4.4</td>
</tr>
<tr>
<td>(\mu_{L}^{*} - \mu_{L}^{0}) =</td>
<td>- (RT \ln f_{L}), kJ mol(^{-1})</td>
<td>8 ± 130</td>
<td>-11.0 ± 2.3</td>
<td>-11.1 ± 2.5</td>
<td>-11.1 ± 2.5</td>
</tr>
<tr>
<td>(\mu_{XL}^{0} - \mu_{LL}^{0}) =</td>
<td>- (RT \ln C_{L}), kJ mol(^{-1})</td>
<td>-45.6 ± 128.1</td>
<td>-18.2 ± 2.2</td>
<td>-18.3 ± 2.4</td>
<td>-18.3 ± 2.4</td>
</tr>
<tr>
<td>(\mu_{XM}^{0} - \mu_{LM}^{0}) =</td>
<td>- (RT \ln K_{M}), kJ mol(^{-1})</td>
<td>-35.5 ± 1.8</td>
<td>-35.5 ± 1.8</td>
<td>35.8 ± 1.4</td>
<td>35.8 ± 1.4</td>
</tr>
<tr>
<td>(\mu_{LM}^{0} - \mu_{LL}^{0}) =</td>
<td>- (RT \ln C_{LM}), kJ mol(^{-1})</td>
<td>-25.8 ± 1.3</td>
<td>-25.8 ± 1.3</td>
<td>-25.6 ± 1.8</td>
<td>-25.6 ± 1.8</td>
</tr>
<tr>
<td>(\mu_{L}^{*} - \mu_{L}^{0}) =</td>
<td>- (RT \ln K_{L}), kJ mol(^{-1})</td>
<td>-18.6 ± 1.7</td>
<td>-16.8 ± 1.9</td>
<td>[-29.2 ± 4.5]†</td>
<td>[-29.4 ± 4.9]†</td>
</tr>
<tr>
<td>(\mu_{LM}^{0} - \mu_{LM}^{0}) =</td>
<td>- (RT \ln K_{LM}), kJ mol(^{-1})</td>
<td>-19.7 ± 2.2</td>
<td>[-36.8 ± 3.6]†</td>
<td>[-36.7 ± 4.3]†</td>
<td>[-36.7 ± 4.3]†</td>
</tr>
<tr>
<td>(RV)</td>
<td>0.0990</td>
<td>0.1013</td>
<td>0.0240</td>
<td>0.0137</td>
<td>0.0133</td>
</tr>
<tr>
<td>(DF)</td>
<td>43</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>(F_{12} = 1.02)</td>
<td>(F_{13} = 4.22)</td>
<td>(F_{34} = 1.75)</td>
<td>(F_{45} = 1.03)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Calculated from other regression parameter values.
The regression results using the full model (Model 5), which had the lowest residual variance, were used to generate the curves for lysozyme and myoglobin shown in Figure 4-1 and Figure 4-2. Model performance is more clearly visualized using parity plots showing calculated adsorption as a function of measured adsorption; these plots are shown, for all five models, in Figure 4-5.

(A)
Figure 4-5. Observed vs predicted values for (A) Competitive Langmuir, (B) Competitive GAB, (C) Cooperative sans LL, (D) Same binding footprint, (E) Full model
To compare model performance objectively, $F$-tests were performed on the residual variance at a 95% level of confidence. For 41-43 degrees of freedom, the critical value of $F$ is 1.66-1.68; $F$ values above the critical value are statistically significant. The performances of Models 1, competitive Langmuir, and 2, competitive GAB, are effectively indistinguishable ($F = 1.02$). Model 3, which allows for cooperative adsorption of lysozyme on myoglobin but does not allow for multilayer formation, outperforms both Model 1 and Model 2; (for comparison of Models 1 and 3, ($F = 4.22$). This result is not surprising given the inadequacy of simple site competition models to explain the trends in the data; this point was discussed in Section 3.1 above. The regression results confirm that allowing cooperative adsorption of lysozyme adsorption onto myoglobin is critical to explaining the protein behavior in this system.

Models 4 and 5, which allow for both cooperative adsorption and the formation of multilayers, show comparable statistical performance ($F = 1.03$). The regression results thus suggest that lysozyme and myoglobin have similar footprints on the adsorbent surface. However, both of these models outperform Model 3 (for comparison on Models 3 and 4, $F = 1.75$), suggesting that lysozyme multilayers exert significant influence on the observed adsorption behavior.

Model 4 assumes equal saturation capacities, $q_{im}$, for the two components, while Model 5 allows for the possibility that $q_{iL} \neq q_{iM}$. Since Model 5 has more adjustable parameters than Model 4, it was expected that Model 5 would provide a better fit to the experimental data. However, the additional parameter did not improve the fit. This is a surprising result since it suggests that an adsorbed myoglobin molecule covers the same surface area as a somewhat smaller lysozyme molecule.
4.3.6. Adsorption Energetics.

The overall Gibbs free energy change of adsorption for lysozyme adsorption obtained using the extended GAB \( \mu_{XL}^0 - \mu_L^0 = -RT \ln K_L, -29.2 \pm 4.5 \text{ kJ mol}^{-1} \) using Model 4) is consistent with previously reported Gibbs free energy of lysozyme adsorption on silica from a single-protein solution in 10 mM Hepes buffer at pH 7 \( 25.5 \pm 0.6 \text{ kJ mol}^{-1} \).[187] For the conditions in this study, the strongest adsorption interactions are myoglobin with the silica surface \( \mu_{XM}^0 - \mu_M^0 = -RT \ln K_M = -36.2 \pm 1.3 \text{ kJ mol}^{-1} \) and lysozyme onto myoglobin \( \mu_{LM}^* - \mu_L^0 = -RT \ln K_{LM} = -36.8 \pm 3.6 \text{ kJ mol}^{-1} \). Thus, lysozyme binds more strongly to previously adsorbed myoglobin than it does to previously adsorbed lysozyme \( \mu_{LL}^* - \mu_L^0 = -RT \ln f_L = -11.0 \pm 2.3 \text{ kJ mol}^{-1} \). Consequently, the thermogram for lysozyme adsorption was unaffected by previously adsorbed myoglobin, as shown in trace 3 of Figure 4-4.

The additional multilayer lysozyme adsorption onto adsorbed lysozyme and myoglobin causes a greater release of enthalpy that would otherwise not be taken into account using multicomponent Langmuir. If Model 1 was true for this system, then trace 3 in Figure 4-3 would simply be average of traces 1 and 2, which is not the case. The higher enthalpy of combined adsorption is due to the adsorption energies from all four mechanism steps.

4.4. Conclusions

A model of multicomponent multilayer adsorption has been successfully developed to describe the simultaneous adsorption of lysozyme and myoglobin on MCF silica; the model is based on competition for surface area, the formation of lysozyme multilayers, and the adsorption of lysozyme on previously adsorbed myoglobin. Regression analysis demonstrates that all of these effects must be accounted for to explain the observed adsorption behavior. The model is consistent with adsorption data and measured enthalpies of adsorption. Interestingly, only a
single parameter was needed to account for lysozyme-myoglobin interactions in this system. The same derivations model can further be used for other protein systems.
Chapter 5 Effects of Surface Curvature on Lysozyme Adsorption on Silica Surfaces

A.M. Darwish, R.J. Desch, S.W. Thiel, “Thermodynamic effects of surface curvature on lysozyme adsorption on silica surfaces” Microporous and Mesoporous Materials, in review (2014)
5.1. Introduction

To understand adsorption mechanisms, the factors that can affect adsorption must be considered. Many of these factors have been thoroughly studied, such silica surface charge,[157] surface chemistry[106] and pH and molarity of the solution.[148] For the different types of chromatography, the main separation principle for selectivity is an obvious main focus of the study. For size-exclusion chromatography, that main separation principle is pore size.[87-89] The effect of pore size, and in effect pore curvature, is often ignored except in size-exclusion chromatography. When pore size is considered in a chromatographic context, it is generally considered in terms of accessible surface area but not in terms of its impact on protein adsorption equilibrium.[69-76] Due to the complexity of proteins and their specificity of protein interaction with other surfaces, changes in surface curvature may impact adsorption. Surface curvature was in fact found to impact the biological activity of cells[90] and proteins[91] on normal[92] and reverse phase[93] adsorption media. Thus, the impact of the physical characteristics of chromatographic materials on protein adsorption should be considered.

In addition, materials with bimodal pore size distributions, including perfusion chromatography adsorbents or monoliths, are a current trend in stationary phase development.[91, 188] A bimodal pore size distribution will include large pores, for fast mass transport, that are lined with small pores, for high surface area. In this way, the mass transfer resistance through the film into the pores, and through the pores is reduced.[189] Such advancements in protein adsorption are promising, but the possible impact of pore curvature is yet to be understood. Pore curvature can impact protein adsorption energetics; and geometric considerations can impact the development of multiple attachments and impact protein conformational changes upon adsorption to the surface.[190, 191] Studying the surface curvature
effects would provide insight into adsorption mechanisms and the potential for optimizing proteins separations.

In the research reported in this chapter, the effect of adsorbent pore size on adsorption characteristics was studied and the thermodynamics of adsorption to each surface were analyzed. The adsorption of lysozyme onto acid-washed silica, amine-functionalized silica, and glycine-functionalized silica with two different pore sizes, 500 Å and 1000 Å was studied in 0.02 M phosphate buffer at pH 7 and 25°C. The 500 Å pores are at the highest limit of the mesoporous range and the 1000 Å pores are in the macroporous range. The thermodynamics of the adsorption process were studied using batch adsorption to measure changes in chemical potential and flow microcalorimetry (FMC) to measure changes in the enthalpy of adsorption.

5.2. Experimental

5.2.1. Materials

The silica adsorbents used were Matrex normal phase liquid chromatography media Si-500A-10µm (#87076508, Amicon, Billerica, MA, USA) and Matrex normal phase liquid chromatography media Si-1000A-10µm (#87098747, Amicon, Billerica, MA, USA). Lysozyme (95%) from MP Biomedicals Inc. was purchased through Fisher Scientific (Pittsburgh, PA) and used without further purification. Potassium phosphate monobasic, potassium phosphate dibasic, trifluoroacetic acid (TFA), protected glycine (Fmoc-Gly-OH, ≥98% by TLC and HPLC), N,N'-diisopropylcarbodiimide, 99% (DIC) and nitric acid 50-70% were purchased from Fisher Scientific (Fair Lawn, NJ). Sulfuric acid, extra pure, 96% solution in water was purchased from Acros Organics (New Jersey, USA). N-N-Dimethylformamide (DMF) was purchased from the Tedia Company. Acid-washed samples of these materials are referred to below as Si-500-AW
and Si-1000-AW, respectively. Amine- and glycine-functionalized 500 Å and 1000 Å silica adsorbents are referred to as Si-500-NH2, Si-1000-NH2, Si-500-G, and Si-1000-G respectively.

### 5.2.2. Methods

Silica samples were acid washed before use; acid washing cleans the surface, increases the number of silanol groups on the silica surface and increases reproducibility of protein adsorption to the surface. The method of acid washing, amine, and glycine functionalizations are also discussed in Section 2.2.1. Nitrogen adsorption and desorption measurements were performed at 77 K using a Micromeritics ASAP 2010 volumetric adsorption analyzer (Norcross, GA, USA). The Brunauer–Emmett–Teller (BET) method was used to determine the surface area. The details have been previously presented in Section 2.2.3.1.

Batch adsorption measurements were completed using lysozyme from chicken egg white dissolved completely in 0.02 M potassium phosphate buffer at pH 7 at 25°C. The heat of adsorption was measured directly using a flow microcalorimeter (FMC, Gilson Instruments, Westerville, OH, USA).[94, 106, 156] The adsorbate solution consisted of lysozyme at a concentration of 2 mg mL\(^{-1}\) (0.14 µmol mL\(^{-1}\)), 8 mg mL\(^{-1}\) (0.56 µmol mL\(^{-1}\)), or 20 mg mL\(^{-1}\) (1.4 µmol mL\(^{-1}\)) in 0.02 M potassium phosphate buffer pH 7. Details for batch adsorption and FMC experiments are presented in Section 2.2.4.1. The bed was packed with 38.9 – 48.5 mg Si-500-AW or 35.8 – 43.2 mg Si-1000-AW. Experiments were conducted in duplicate and thermograms were averaged. The flow rate was 1.85 mL h\(^{-1}\), the adsorbate sample volume was 1.36 mL, the mobile phase was 0.02 M potassium phosphate buffer pH 7, and the temperature was 25°C.
5.3. Results and Discussion

5.3.1. Characterization

Textural properties of acid-washed silica, aminopropyl-functionalized, and glycine-functionalized silica adsorbents were determined. The surface areas and pore volumes of the acid-washed silica adsorbents were determined using nitrogen adsorption and desorption data; results are shown in Table 5-1. Pore sizes were reported by the manufacturer. The nitrogen adsorption and desorption curves for Si-500-AW and Si-1000-AW showed negligible hysteresis, indicating negligible microporosity. These figures are shown in appendix.

Table 5-1. Surface area (m² g⁻¹) and pore volume (cm³ g⁻¹) for Si-500-AW and Si-1000-AW from nitrogen adsorption and desorption analysis

<table>
<thead>
<tr>
<th></th>
<th>Si-500-AW</th>
<th>Si-1000-AW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface area (m² g⁻¹)</td>
<td>94</td>
<td>50</td>
</tr>
<tr>
<td>Pore volume (cm³ g⁻¹)</td>
<td>0.632</td>
<td>0.201</td>
</tr>
</tbody>
</table>

FTIR, DSC and TGA data for Si-500-NH2 and Si-500-G were previously presented in Table 3-1. The TGA results for Si-500-NH2, Si-500-G, Si-1000-NH2 and Si-1000-G are presented in Table 5-2. Si-1000-NH2 and Si-1000-G showed higher ligand densities than Si-500-NH2 and Si-1000-G. Si-500-NH2, Si-500-G, and Si-1000-G all have approximately the same charge per area. Si-1000-NH2 has twice the charge per area of any of the other functionalized surfaces.
Table 5-2. Surface coverage, $N_s$, and ligand density, $D$, for surface amine-, carboxyl-, and amino acid-functionalized silica. Surface coverage calculated from TGA weight loss between 250°C and 800°C; ligand densities based on the BET surface area of 94.4 m$^2$ g$^{-1}$; ligand spacings estimated assuming uniform spacing on surface; charge estimated based on ligand density and degree of dissociation of terminal and side groups at pH 7.

<table>
<thead>
<tr>
<th></th>
<th>TGA %</th>
<th>$M_{ligand}$</th>
<th>$N_s$, µmol g$^{-1}$</th>
<th>$D$, ligand nm$^{-2}$</th>
<th>pKa</th>
<th>Charge C m$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-500-NH2</td>
<td>1.81</td>
<td>58</td>
<td>155</td>
<td>0.99</td>
<td>10.53 [163]</td>
<td>+0.159</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.34 (-COOH)</td>
<td>[165]</td>
</tr>
<tr>
<td>Si-500-G</td>
<td>2.52</td>
<td>117</td>
<td>139</td>
<td>0.89</td>
<td>9.6 (-NH2)</td>
<td>+0.145</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.34 (-COOH)</td>
<td>[165]</td>
</tr>
<tr>
<td>Si-1000-NH2</td>
<td>1.96</td>
<td>58</td>
<td>179</td>
<td>2.08</td>
<td>10.53 [163]</td>
<td>+0.333</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.6 (-NH2)</td>
<td>[165]</td>
</tr>
<tr>
<td>Si-1000-G</td>
<td>2.59</td>
<td>117</td>
<td>138</td>
<td>1.60</td>
<td>2.34 (-COOH)</td>
<td>+0.160</td>
</tr>
</tbody>
</table>

5.3.2. Equilibrium Loading

The dimensions of lysozyme are 19 x 25 x 43 Å and the hydrodynamic radius of lysozyme is 19.7 Å.[193] When $\lambda$, the ratio of the adsorbate diameter to the pore diameter, is less than 0.2 the adsorption geometry can often be modeled as a sphere–plane system.[194, 195] The surfaces of 500 Å ($\lambda = 0.08$) and 1000 Å ($\lambda = 0.04$) are often considered to be planar for the purpose of modeling lysozyme adsorption, thus ignoring the effects of surface curvature. In this study, different lysozyme adsorption behaviors onto the 500 Å and 1000 Å surfaces were observed. The equilibrium batch adsorption isotherms for acid-washed, amine-functionalized and glycine-functionalized adsorbents are shown in Figure 5-1, Figure 5-2, and Figure 5-3 respectively. The adsorption data for Si-500-AW, Si-500-NH2 and Si-500-G were fit to the
Langmuir model as described in Chapter 3, where \( q_m \) is fixed for adsorption onto all three functionalizations as shown in Eq. (5-1).

\[
q_j = q_m \frac{\exp(-\frac{\Delta G_j^0}{RT}) x}{1 + \exp(-\frac{\Delta G_j^0}{RT}) x} 
\]  

(5-1)

In this isotherm, \( x \) is the equilibrium mole fraction of the protein in the liquid phase, \( q_j \) is the protein surface coverage (\( \mu \text{mol g}^{-1} \)) on surface \( j \), \( q_m \) is the monolayer surface coverage (\( \mu \text{mol g}^{-1} \)), \( \Delta G_j^0 \) is the standard Gibbs free energy change for adsorption onto surface \( j \) (J mol\(^{-1} \)), \( R \) is the ideal gas constant (8.314 J mol\(^{-1} \) K\(^{-1} \)), and \( T \) is the temperature (298 K). As noted by Darwish and Thiel,[196] when the surface ligand spacing is less than or comparable to the dimensions of the adsorbed protein, the monolayer coverage is determined by accessible surface area rather than the surface ligand density. Consequently, in this work a single value of the lysozyme monolayer coverage, \( q_m \), was used for all adsorbents with 500 Å pores regardless of functionalization. This assumption was tested statistically using an \( F \)-test on the residual variances obtained using a single value of \( q_m \) versus those obtained using a separate value of \( q_m \) for each 500 Å surface; the \( F \) value was 1.02, indicating that using a functionalization-dependent values of \( q_m \) did not improve the descriptive power of the model. The Langmuir model failed to converge for the 1000 Å surfaces, consequently, the results for the 1000 Å were fit to the Guggenheim-Anderson-deBoer (GAB) isotherm [95] shown in Eq. (5-2), where \( q_m \) is fixed for all surfaces.

\[
q_j = q_m \frac{\exp(-\frac{\Delta G_j^0}{RT})\exp(-\frac{\Delta \tilde{G}_j^0}{RT}) x}{(1 - \exp(-\frac{\Delta G_j^0}{RT}) x)(1 - (1 - \exp(-\frac{\Delta \tilde{G}_j^0}{RT}) \exp(-\frac{\Delta G_j^0}{RT}) x))} 
\]  

(5-2)
The GAB model accounts for differences between the energy of adsorption to the surface and the energy of adsorption to already attached adsorbate molecules. In this equation, $\Delta \bar{g}^{0}_{fj}$ is the standard Gibbs free energy change for adsorption from the bulk onto an adsorbed layer on the surface $j$ (J mol$^{-1}$), $\Delta \bar{g}^{0}_{cj}$ is the standard Gibbs free energy change between the adsorbent on the surface $j$ and the subsequent layer (J mol$^{-1}$).

Figure 5-1. Equilibrium lysozyme adsorption onto Si-500-AW and Si-1000-AW in 0.02 M phosphate buffer at pH 7 and 25°C as a function of liquid-phase lysozyme concentration. Points are experimental results; curves are fit to Langmuir and GAB isotherms respectively.
Figure 5-2. Equilibrium lysozyme adsorption onto Si-500-NH2 and Si-1000-NH2 in 0.02 M phosphate buffer at pH 7 and 25°C as a function of liquid-phase lysozyme concentration. Points are experimental results; curves are fit to Langmuir and GAB isotherms respectively.

Figure 5-3. Equilibrium lysozyme adsorption onto Si-500-G and Si-1000-G in 0.02 M phosphate buffer at pH 7 and 25°C as a function of liquid-phase lysozyme concentration. Points are experimental results; curves are fit to Langmuir and GAB isotherms respectively.
As was done with the results for adsorbents with 500 Å pores, a single value of the lysozyme monolayer coverage, $q_m$, was used for all adsorbents with 1000 Å pores regardless of functionalization. This assumption was also tested statistically using an $F$-test on the residual variances obtained using a single value of $q_m$ versus those obtained using a separate value of $q_m$ for each 1000 Å surface; the $F$ value was 1.02, indicating that using a functionalization-dependent values of $q_m$ did not improve the descriptive power of the model. The regression results for all six surfaces are shown in Table 5-3.

Table 5-3. GAB isotherm fits for lysozyme adsorption on Si-1000-AW, Si-1000-NH2 and Si-1000-G. Langmuir isotherm fits for lysozyme adsorption on Si-500-AW, Si-500-NH2 and Si-500-G. Solution conditions: 0.02 M potassium phosphate buffer at pH 7 and 25°C; initial lysozyme concentrations 0 - 2.8 µmol mL$^{-1}$.

<table>
<thead>
<tr>
<th></th>
<th>$q_m$ (µmol m$^{-2}$)</th>
<th>$\Delta G^0_{CJ}$ (kJ mol$^{-1}$)</th>
<th>$\Delta G^0_{kJ}$, (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-1000-AW</td>
<td>0.370 ± 0.039</td>
<td>-6.67 ± 0.57</td>
<td>-23.3 ± 0.22</td>
</tr>
<tr>
<td>Si-1000-NH2</td>
<td>0.370 ± 0.039</td>
<td>-3.66 ± 0.55</td>
<td>-22.6 ± 0.29</td>
</tr>
<tr>
<td>Si-1000-G</td>
<td>0.370 ± 0.039</td>
<td>-2.81 ± 1.33</td>
<td>-22.4 ± 0.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>$q_m$ (µmol m$^{-2}$)</th>
<th>$\Delta G^0_{k}$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-500-AW</td>
<td>0.210 ± 0.018</td>
<td>-29.5 ± 0.65</td>
</tr>
<tr>
<td>Si-500-NH2</td>
<td>0.210 ± 0.018</td>
<td>-21.6 ± 0.73</td>
</tr>
<tr>
<td>Si-500-G</td>
<td>0.210 ± 0.018</td>
<td>-26.4 ± 0.56</td>
</tr>
</tbody>
</table>

Similar monolayer loadings per area were shown by Daly et al., who reported a lysozyme monolayer coverage of 2.5 mg m$^{-2}$ on a flat silica surface at from 5 mM triethanolamine hydrochloride buffer at pH 7.4 and 25°C.[197]
The average area taken by a lysozyme molecule on the surface assuming uniform monolayer adsorption, $A_L$ (Å$^2$), can be calculated from the specific surface area of the adsorbent, $A_S$ (m$^2$ g$^{-1}$), and $q_m$ (μmol g$^{-1}$):

$$A_L = \frac{A_S \times 10^{20}}{q_m \times N_a}$$  \hspace{1cm} (5-3)

where $N_a$ is Avogadro’s number (6.022 x 10$^{17}$ molecules μmol$^{-1}$). $A_L$ was found to equal 790 Å$^2$ for the 500 Å surfaces and 450 Å$^2$ for the 1000 Å surfaces. In comparison, the dimensions of the lysozyme crystal structure indicate that lysozyme requires 475-1075 Å$^2$, depending on the orientation. Assuming the shape of the lysozyme stays intact, the surface area covered by each protein on the 1000 Å surfaces is only possible with a single orientation in which the lysozyme occupies the least amount of space on the surface. This has significant effects on the entropy of adsorption, which likely accounts for the difference in Gibbs free energy between the two surfaces. This is likely the reason the 1000 Å surfaces could not be fit to a Langmuir isotherm. If the lysozyme is already tightly packed, additional lysozymes would have to adsorb to the multilayer. The higher per area coverage onto the 500 Å surfaces would allow for additional lysozymes to adsorb into the monolayer.

At pH 7, lysozyme (pI=11.2) carries a net positive charge and Si-500-AW and Si-1000-AW carry a negative charge, suggesting that attractive electrostatic interactions between the protein and silica are likely at the low ionic strength (0.02 M) used for these experiments. The Debye length ($k_o^{-1}$), a measure of the distance a charge propagates through liquid medium, can be calculated. Lysozyme was first adsorbed in 0.02M potassium phosphate buffer at pH 7 and room temperature. These conditions were determined to keep the solution effects at a minimum. The Debye length, $k_o^{-1}$, (Å) was calculated using Eq. (5-4) and (5-5).[121]
\[
k_0^{-1} = \sqrt{\frac{k_B T \epsilon_0 \epsilon_r}{2 N_A e^2 I}}
\]  
(5-4)

\[
I = \frac{1}{2} \sum C_i Z_i^2
\]  
(5-5)

\[k_B = 1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}\] is the Boltzmann constant; \([\epsilon_0 = 8.85 \times 10^{-12} \text{ C}^2 \text{ m}^{-1} \text{ J}^{-1}]\) is the permittivity of free space;\([T = 298 \text{ K}]\); \(N_A\) is the Avogadro constant; \([e = 1.60 \times 10^{-19} \text{ C}]\) is the charge of an electron; and \([I = 20 \text{ mol m}^{-3}]\) is the ionic strength of the solution.

The relative dielectric constants, \(\epsilon_r = 45\) in different salt concentrations were estimated from the reported values.\([198]\) The Debye length was found to be 13.3 Å.

The positively-charged lysozyme had a stronger adsorption onto the negatively-charged surfaces of Si-500-AW \((\Delta G^0_{kSi-500-AW} = -29.5 \pm 0.65 \text{ kJ mol}^{-1})\) and Si-1000-AW \((\Delta G^0_{cSi-1000-AW} + \Delta G^0_{fSi-1000-AW} = -30.0 \pm 0.79 \text{ kJ mol}^{-1})\) than all of the other remaining surfaces, which was an expected result. Si-500-NH2, Si-1000-NH2, Si-500-G, and Si-1000-G all had positive charges, as was shown in Table 5-2, which resulted in a lower lysozyme adsorption than on Si-500-AW and Si-1000-AW. Electrostatic interactions cannot be the only factor influencing lysozyme adsorption since the lysozyme adsorbs on Si-500-NH2, Si-1000-NH2, Si-500-G, and Si-1000-G despite the net positive charge on the surface. Strong van der Waals attractions have also been observed under similar conditions.\([148]\) Moreover, lysozyme had a stronger adsorption to Si-1000-NH2 \((\Delta G^0_{cSi-1000-NH2} + \Delta G^0_{fSi-1000-NH2} = -26.2 \pm 1.3 \text{ kJ mol}^{-1})\) than Si-1000-G \((\Delta G^0_{cSi-1000-G} + \Delta G^0_{fSi-1000-G} = -25.1 \pm 2.0 \text{ kJ mol}^{-1})\) despite having twice the net positive surface charge. This trend was different for the 500 Å surfaces, where lysozyme had a stronger adsorption to Si-500-G \((\Delta G^0_{kSi-500-G} = -26.4 \pm 0.56 \text{ kJ mol}^{-1})\) than Si-500-NH2.
\[ \Delta \bar{\Delta}^0_{k,\text{Si-500-NH}_2} = -21.6 \pm 0.73 \text{ kJ mol}^{-1} \]

for reasons that were previously discussed in Chapter 3. The change in pore size changed the method of adsorption onto the functionalized surfaces.

5.3.3. **Enthalpy of Adsorption**

To further elucidate trends observed in equilibrium studies, the enthalpy of lysozyme adsorption on the acid washed stationary phases was analyzed using flow microcalorimetry (FMC). FMC thermograms are direct measurements of adsorption enthalpy. In the equilibrium experiments, the isotherm plateau typically occurred in the protein concentration range of 0.56-1.40 µmol mL\(^{-1}\). The enthalpy of lysozyme adsorption was probed at three concentrations: below monolayer loading (0.14 µmol mL\(^{-1}\)), and at the lower (0.56 µmol mL\(^{-1}\)) and upper (1.4 µmol mL\(^{-1}\)) concentration limits of the protein loading plateau. If the protein molecules interact solely with the silica surface, the enthalpy of protein adsorption should be independent of protein concentration. By varying the protein concentration, it is possible to observe the protein-protein interaction effects and differentiate between single-layer and multilayer adsorption energetics. Further, by comparing these effects across silica samples with small and large pores, the impact of pore size on protein adsorption enthalpy can be determined. Thermograms for the adsorption of three concentrations of lysozyme (0.14 µmol mL\(^{-1}\), 0.56 µmol mL\(^{-1}\), and 1.4 µmol mL\(^{-1}\)) onto Si-500-AW and Si-1000-AW are shown in Figure 5-4 and Figure 5-5 respectively. The protein plug first encounters the packed bed at 15 minutes, causing an adsorption exotherm; the plug ends at 63 minutes, causing a desorption endotherm.
Figure 5-4. Thermograms for the adsorption of lysozyme at concentrations of 0.14 µmol mL$^{-1}$, 0.56 µmol mL$^{-1}$, and 1.4 µmol mL$^{-1}$ onto Si-500-AW in 0.02 M phosphate buffer pH 7 at 25°C. Sample loop volume: 1.36 mL, flow rate: 1.85 mL hr$^{-1}$, adsorbent mass: 38.9 – 48.5 mg.

Figure 5-5. Thermograms for the adsorption of lysozyme at concentrations of 0.14 µmol mL$^{-1}$, 0.56 µmol mL$^{-1}$, and 1.4 µmol mL$^{-1}$ onto Si-1000-AW silica in 0.02 M phosphate buffer pH 7 at 25°C. Sample loop volume: 1.36 mL, flow rate: 1.85 mL hr$^{-1}$ adsorbent mass: 35.8 – 43.2 mg.
Attractive electrostatic and van der Waals interactions are expected to result in a sharp exothermic adsorption peak in the thermogram. In addition to this initial adsorption peak, a broader and slower second exothermic peak was also observed, which is possibly associated with protein reorientation on the silica surface, protein conformation change or slow secondary attachments[148] as well as some mass transport-related peak distortion. Merged multipeak FMC thermograms have been previously observed for lysozyme adsorption onto silica.[120, 148] Finally, a desorption endotherm was observed at 70 – 90 min. These complex signals were deconvoluted into Gaussian peaks as shown in Figure 5-6.

Figure 5-6. Gaussian peak deconvolution of the thermogram for the adsorption of 1.4 µmol mL⁻¹ lysozyme onto Si-60-AW in 0.02 M phosphate buffer pH 7 at 25°C. The sum of the deconvoluted peaks (red) is compared to the original thermogram (dotted black). Adsorption exotherms I (blue) and II (green) and desorption endotherm III (fuchsia) are distinguished.
Increasing the lysozyme concentration from 0.14 to 0.56 µmol mL\(^{-1}\) enhanced the adsorption exotherm on Si-500-AW, but increasing the lysozyme concentration further had no effect. On Si-1000-AW, the adsorption exotherms were attenuated at both of the higher concentrations. Regardless of relative peak magnitude, the enthalpy of adsorption of dilute lysozyme was associated with a broad exotherm, while concentrated lysozyme adsorbed with a sharp narrow exotherm. A summary of liquid- and solid-phase protein concentrations, adsorption enthalpies, and the net enthalpy of adsorption are shown in Table 5-4.

Table 5-4. Initial lysozyme concentration (µmol mL\(^{-1}\)), loading (q_m, µmol g\(^{-1}\)), deconvoluted enthalpy of lysozyme adsorption (kJ mol\(^{-1}\)) and relative magnitude of the desorption peak (ΔH\(^{III}\) / ΔH_{ads}) on Si-500-AW and Si-1000-AW at 0.02 M potassium phosphate buffer at pH 7 and 25°C

<table>
<thead>
<tr>
<th>Silica Pore Size (Å)</th>
<th>Protein Conc. (µmol mL(^{-1}))</th>
<th>q_m (µmol g(^{-1}))</th>
<th>ΔH(^{I}) (kJ mol(^{-1}))</th>
<th>ΔH(^{II}) (kJ mol(^{-1}))</th>
<th>ΔH(^{III}) (kJ mol(^{-1}))</th>
<th>ΔH(^{III}) / ΔH_{ads}</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0.14</td>
<td>7.29</td>
<td>-4.36</td>
<td>-14.93</td>
<td>0.00</td>
<td>-19.3</td>
</tr>
<tr>
<td>500</td>
<td>0.56</td>
<td>11.03</td>
<td>-13.06</td>
<td>-14.22</td>
<td>6.61</td>
<td>-27.3</td>
</tr>
<tr>
<td>500</td>
<td>1.40</td>
<td>16.95</td>
<td>-12.44</td>
<td>-13.43</td>
<td>8.44</td>
<td>-25.9</td>
</tr>
<tr>
<td>1000</td>
<td>0.14</td>
<td>5.69</td>
<td>-8.52</td>
<td>-30.91</td>
<td>5.61</td>
<td>-39.4</td>
</tr>
<tr>
<td>1000</td>
<td>0.56</td>
<td>14.49</td>
<td>-7.55</td>
<td>-12.81</td>
<td>3.10</td>
<td>-20.4</td>
</tr>
<tr>
<td>1000</td>
<td>1.40</td>
<td>24.00</td>
<td>-5.59</td>
<td>-11.61</td>
<td>3.61</td>
<td>-17.2</td>
</tr>
</tbody>
</table>
The total heat of adsorption per mole of lysozyme ($\Delta H_{ads}$) was calculated from measured adsorption enthalpies ($\Delta H^I$ and $\Delta H^{II}$, Eq. 7) at three protein concentrations representing low loading (0.14 µmol mL$^{-1}$), the low concentration side of the loading plateau (0.56 µmol mL$^{-1}$), and the high concentration side of the loading plateau (1.4 µmol mL$^{-1}$).

$$\Delta H_{ads} = \Delta H^I + \Delta H^{II} \quad (5-6)$$

Below monolayer coverage at 0.14 mg mL$^{-1}$, Si-500-AW showed a lower enthalpy of adsorption ($\Delta H_{ads} = -19.3$ kJ mol$^{-1}$) than Si-1000-AW ($\Delta H_{ads} = -39.4$ kJ mol$^{-1}$). The Gibbs free energy of adsorption for Si-500-AW ($\Delta G_{Si-500-AW}^0 = -29.5 \pm 0.65$ kJ mol$^{-1}$) and Si-1000-AW ($\Delta G_{Si-1000-AW}^0 + \Delta G_{f Si-1000-AW}^0 = -30.0 \pm 0.79$ kJ mol$^{-1}$) are comparable, so the difference in the enthalpy of adsorption also indicates a difference in the entropy of adsorption. This is consistent with the previous finding that lysozyme packs tightly onto the surface of Si-1000-AW.

The entropy of adsorption can be calculated using Eq. (5-7).

$$\Delta G_j = \Delta H_j - T\Delta S_j \quad (5-7)$$

The entropy of adsorption for Si-500-AW ($T\Delta S_{Si-500-AW}^0 = 10.2$ kJ mol$^{-1}$) and Si-1000-AW ($T\Delta S_{Si-1000-AW}^0 = -9.4$ mol$^{-1}$) depends on the pore size. This is an interesting result; adsorption onto Si-500-AW is driven by both entropic and enthalpic effects, but adsorption to the Si-1000-AW is driven by enthalpic effects. This shows that lysozyme adsorption is in fact different for Si-500-AW and Si-1000-AW.

The enthalpy of adsorption per mole levels off on Si-500-AW for the concentration in the monolayer 0.56 µmol mL$^{-1}$ ($\Delta H_{ads} = -27.3$ kJ mol$^{-1}$) and 1.4 µmol mL$^{-1}$ ($\Delta H_{ads} = -25.9$ kJ mol$^{-1}$), which is consistent with the Langmuir isotherm. The enthalpy of adsorption per mole continues
to drop on Si-1000-AW for the concentrations at the monolayer 0.56 µmol mL\(^{-1}\) \((\Delta H_{ads} = -20.4\) kJ mol\(^{-1}\)\) and beyond the monolayer coverage 1.4 µmol mL\(^{-1}\) \((\Delta H_{ads} = -17.2\) kJ mol\(^{-1}\)\), which is consistent with the decreasing availability of the surface and the constant enthalpy of adsorption in the multilayers that would be expected in the GAB model.

### 5.3.4. Functionalized Surface Interactions

Pore size impacts the number of ligands that come in contact with the lysozyme. The positive charge per surface area doubled the amine functionalized silica from Si-500-NH2 (+0.159 C m\(^{-2}\)) to Si-1000-NH2 (+0.333 C m\(^{-2}\)). However, Si-500-NH2 a lower monolayer adsorption capacity (0.21 µmol m\(^{-2}\)) and lower energy of adsorption \((\Delta G_{k500-NH2}^0 = -21.6\) kJ mol\(^{-1}\)) than Si-1000-NH2 \((0.370 \pm 0.041\) µmol m\(^{-2}\)\), \((\Delta G_{c1000-NH2}^0 + \Delta G_{fSi1000-NH2}^0 = -26.3\) kJ mol\(^{-1}\)). This is an interesting result considering the overall positive charge on lysozyme, which would be expect to be more strongly repelled from Si-1000-NH2. However, lysozyme does have a small negative region that can be attracted to a positive surface. The larger pore size gives the lysozyme molecules more freedom of motion to attach to at a specific orientation thus increasing the probability of an attachment. This is similar to the specific interactions between the lysozyme and the glycine functionalization. Si-500-G \((\Delta G_{kSi-500-G}^0 = -26.4 \pm 0.56\) kJ mol\(^{-1}\)) was shown to adsorb lysozyme at certain residues (Chapter 3). Si-1000-G \((\Delta G_{c1000-G}^0 + \Delta G_{fSi1000-G}^0 = -25.1 \pm 2.1\) kJ mol\(^{-1}\)) showed a similar interaction energy as expected. Any difference between Si-500-G and Si-1000-G would likely be based on the ligand spacing. However, this interaction was shown to be surface area driven and not site specific in Chapter 3. This further validates the similar adsorption isotherms for Si-500-G and Si-1000-G.
5.4. Conclusions

Lysozyme adsorption onto silica surfaces with different functionalizations was found to be based on the available surface area and not specific to the number of ligands. A pore size effect is observed for lysozyme adsorption onto different functionalizations. The difference in pore size changed the shape of the adsorption isotherm. Entropic considerations such as the range of motion of the protein attachment to the surface were found to the impact the energy change of adsorption. The consideration of different pore sizes of chromatographic materials should not be limited to mass transport and surface area effects, but should also include the pore size effect on protein adsorption.
Chapter 6 Venom Separation

6.1. Introduction

By the end of 2011, there were six FDA-approved drugs derived from venomous sources. Most venoms are mixtures of proteins and peptides; the proteins present in venom target biological structures with high specificity and are resistant to the body’s immune responses. These unique properties make venom proteins and peptides valuable as potential pharmacological therapeutic agents. For example, integrins are membrane proteins that facilitate the attachment of cells and proteins. One third of known integrins, bind to the RGD (arginine-glycine-aspartic acid) sequence present in collagen and other structural proteins that are found in the extra cellular matrix. Disintegrins, which are present in nearly all snakes venoms that affect hemostasis, can disrupt integrin function in the body. Most disintegrins contain the RGD motif. They bind with high affinity to integrins, thus blocking normal integrin function. Due to their ability to penetrate tissues, disintegrins have many potential pharmaceutical applications. Most notably, disintegrins can inhibit tumor growth and are therefore the subject of research for cancer therapies. Moreover, complexes involving the RGD sequence bind cells together in tissues, increasing their viscosity. Thus, disintegrins are promising for blood thinning medications and for targeted drug delivery through tissue.

Due to the high cost of venom, highly selective separation methods are needed to separate disintegrins from the remainder of the venom. However, highly selective separation methods, such as affinity chromatography, require a higher material costs than normal or reverse phase chromatography. Therefore, material design for the venom separation must consider cost and selectivity. Amino acid-functionalized surfaces have been considered as a chromatographic stationary phase. Although the synthesis and use of amino acid-functionalized surfaces is not widespread, a few studies have shown that amino acids grafted to silica surfaces had a significant
effect on the adsorption of biomolecules. In a study conducted by Xu et al. Leucine functionalized SBA-15 was used a drug carrier.[101] Poly-lysine and poly-glutamic acid were used as a surface functionalization to prevent protein adsorption onto a silica surface, which is important for biomaterials that may be used in vivo.[103] In a previous study, amino acids were grafted onto the surface through a peptide bond to study the influence of the amino acid functionalization on the adsorption of lysozyme.[196] In the study reported here, the same functionalization method was used to prepare a surface for the selective separation of metalloproteinase from venom, a complex protein mixture.

The venom used in this study was obtained from the red diamond rattlesnake, Crotalus ruber ruber, a species of viper commonly found in Southern California and in Mexico,[54] produces venom that consists of 76% protein on a dry basis.[129] A more thorough analysis of C. r. ruber venom showed the presence of a metalloproteinase, Rubelysin or HT-2,[201] with disintegrin properties.[130] The sequence of HT-2 is available in the scientific literature, and is characterized by a chelating zinc ion.[202] Metalloproteinases are characterized by the presence of a metal ion in the center of the protein molecule.[135] Metalloproteinases attack cell adhesion complexes,[131] decreasing the viscosity of the tissue and thereby enhancing the penetration of the venom through tissue.

The goal of the study reported here was to test amino acid-functionalized adsorbents for the selective adsorption of a metalloproteinase from C. r. ruber venom under conditions that mimic normal biological conditions in mammals. The amino acids involved in integrin/disintegrin interactions were used for the surface functionalizations. Hence, individual arginine, glycine and aspartic acid ligands as well as a random mixture of all three amino acid ligands were grafted onto a commercially-available mesoporous silica surface to adsorb selectively the
metalloproteinase. Total protein adsorption and metalloproteinase adsorptions were measured for all adsorbents to determine the selectivity of each surface functionalization. Silica was used as a base material due to its mechanical stability and controllable surface chemistry and morphology.[60, 64-68]

6.2. Experimental

6.2.1. Materials

(3-Aminopropyl) triethoxysilane (APTES, 99%), ethyl (hydroxyimino)cyanoacetate (oxyma), piperidine (ReagentPlus®, 99%), dichloromethane (DCM) thioanisole purum, ≥99.0%, 1,2-ethanedithiol ≥98.0%, casein from bovine milk, 2 N Folin & Ciocalteu’s phenol reagent, trichloroacetic acid, sodium carbonate anhydrous, and L-tyrosine ≥98%, were obtained from Sigma–Aldrich (St. Louis, MO). Potassium phosphate monobasic and potassium phosphate powder, trifluoroacetic Acid (TFA), protected glycine (Fmoc-Gly-OH, ≥98% by TLC and HPLC), protected arginine (Fmoc-Arg(Pbf)-OH, ≥98% by TLC and HPLC) protected aspartic acid (Fmoc-Asp(OtBu)-OH, Anaspec), Anisole (99%, Acros Organics) and N,N'-diisopropylcarbodiimide,99% (DIC) were purchased from Fisher Scientific (Pittsburg, PA). N-N-Dimethylformamide (DMF) was purchased from the Tedia Company. Normal phase granular silica (Si-1000Å-10µm) was purchased from Matrex Liquid Chromatography Media. Nalgene Polyethersulfone Sterile Syringe Filter Membrane, 25mm, 0.2 Micron were purchased from Amazon (Seattle, WA). *C. ruber ruber* was generously provided by Dr. Sharath Krishna from Central State University (Wilberforce, Ohio).
6.2.2. Methods

The methods used for surface functionalization of Acid-washed silica (Si-1000-AW), aminopropyl-functionalized silica (Si-1000-NH2), glycine-functionalized silica (Si-1000-G), aspartic acid-functionalized silica (Si-1000-D), arginine-functionalized silica (Si-1000-R), and arginine-glycine-aspartic acid-functionalized silica (Si-1000-RGD) are presented in Section 2.2.1.

Nitrogen adsorption and desorption measurements were performed on all three adsorbents. The Brunauer–Emmett–Teller (BET) method was used to determine the surface area as discussed. The functionalizations were verified using Fourier Transform Infrared Spectroscopy (FTIR). Thermogravimetric Analysis (TGA) was used determine the surface coverage of the functional groups, and Differential Scanning Calorimetry (DSC) was used to determine the decomposition rate of the functional groups. Details of these methods and the instruments used have been previously presented in Chapter 2.

An isotherm was generated to determine the amount of venom required to cover a single layer of the adsorbent surface. Solid lyophilized venom was dissolved completely in 50 mM potassium phosphate buffer pH 7.5 and 37 °C. Details of generating the isotherm have been previously presented in Chapter 2. A schematic of the surface selectivity experimental procedure is shown in Figure 6-1.
The metalloproteinase activity was determined before and after adsorption using a casein assay as previously described in Chapter 2. The activity of the metalloproteinase was distinguished from other possible proteinases through the addition of EDTA to the venom. This was also previously described in Chapter 2.

6.3. Results and Discussion

6.3.1. Surface Functionalization

DSC was used to determine that the amino acids attached to the aminopropyl group rather than to the surface. The DSC data shown in Figure 6-2 show the same trends for Si-1000-NH2, Si-1000-G, Si-1000-D, Si-1000-R, and Si-1000-RGD. Inflection points occur in the DSC curves for all four materials at approximately 100°C and 550°C. This similarity indicates that the amino
acid ligands decompose at the same temperature as the aminopropyl ligands.[162] This result indicates that the amino acids attached to the amine through a peptide bond without further interaction between the amino acids and uncovered surface silanols as was discussed in Section 3.4.1.

![DSC graph](image)

Figure 6-2: DSC for (1) Si-1000-AW, (2) Si-1000-NH2, (3) Si-1000-R, (4) Si-1000-D, (5) Si-1000-RGD, and (6) Si-1000-G under argon gas with 20°C min⁻¹ heating

TGA was used to determine the surface coverage, ligand density and fraction of surface amines bonded with amino acids. The weight loss curves obtained in the TGA characterizations are shown in Figure 6-3; summary results are shown in Table 6-1.
Table 6-1: Surface coverage, $N_s$, and ligand density, $D$, for Si-1000-NH2, Si-1000-R, Si-1000-G, Si-1000-D, and Si-1000-RGD. Surface coverage calculated from TGA weight loss between 250°C and 800°C; ligand densities based on the BET surface area of 51.9 m² g⁻¹; % amine groups covered by amino acids and specific selectivity for each adsorbent

<table>
<thead>
<tr>
<th>% Weight loss</th>
<th>$M_{\text{ligand}}$</th>
<th>$N_s$ (µmol g⁻¹)</th>
<th>$D$ (molecule nm⁻²)</th>
<th>% APTS coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-1000-NH2</td>
<td>1.96</td>
<td>58</td>
<td>179</td>
<td>2.08</td>
</tr>
<tr>
<td>Si-1000-R</td>
<td>3.78</td>
<td>216</td>
<td>151</td>
<td>1.75</td>
</tr>
<tr>
<td>Si-1000-G</td>
<td>2.59</td>
<td>117</td>
<td>138</td>
<td>1.60</td>
</tr>
<tr>
<td>Si-1000-D</td>
<td>3.40</td>
<td>175</td>
<td>157</td>
<td>1.83</td>
</tr>
<tr>
<td>Si-1000-RGD</td>
<td>3.75</td>
<td>169.3¹</td>
<td>182</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Surface coverages for Si-1000-NH2, Si-1000-G, Si-1000-D, Si-1000-R and Si-1000-RGD ranged from 138 to 182 µmol g⁻¹. The corresponding ligand densities ranged from 1.6 to 2.1 ligands nm⁻². The amino acid coverage, defined as the percentage of surface amines bound to an amino acid, was 84% for Si-1000-R, 77% for Si-1000-G, 88% for Si-1000-D, and 101% for Si-1000-RGD. The extra 1% for Si-1000-RGD is likely due to noise in the data. Ligand densities were based on the BET surface area determined from the nitrogen adsorption/desorption measurements, 51.9 m² g⁻¹.
Figure 6-3: TGA for (1) Si-1000-AW, (2) Si-1000-NH₂, (3) Si-1000-G, (4) Si-1000-D, (5) Si-1000-R, and (6) Si-1000-RGD under argon gas with 20°C min⁻¹ heating

6.3.2. Batch adsorption

An isotherm was generated for venom on Si-1000-NH₂ as shown in Figure 6-4. The difference in venom adsorption on Si-500-NH₂ between 1 mg ml⁻¹ and 2 mg ml⁻¹ was within error, so it was assumed that monolayer was reached. Therefore, 2 mg ml⁻¹ was as a sufficient liquid concentration as a single point adsorption within the monolayer region of the isotherm. 2 mg ml⁻¹ was used as an initial concentration for a single point adsorption on all surfaces.
Figure 6-4: Venom adsorption onto Si-1000-NH2 in 50mM potassium phosphate buffer pH 7.5 and 37 °C

6.3.3. Venom Activity

The casein assay is conducted on the lyophilized venom and on a venom EDTA mixture. The EDTA would chelate the zinc ion from metalloproteinase causing it to lose its function, thus the venom-EDTA mixture activity would determine if other proteinases besides the metalloproteinase are present in the venom. The results of the casein assay on the venom and venom-EDTA mixture are shown in Figure 6-5. The activity of the venom was completely eliminated with the addition of the EDTA. Therefore, it is concluded that all proteinase activity is due to the metalloproteinase.
Figure 6-5: Venom activity in 50 mM potassium phosphate buffer pH 7.5 and 37 °C with and without EDTA

6.3.4. Surface Selectivity

The venom adsorption results were used to determine the overall protein adsorption to surface. The venom activity was determined before and after adsorption to the different surfaces. As previously discussed, all the casein digestion in the venom is attributed to the metalloproteinase. Therefore the difference in activity based on the casein digestion can be safely taken as the difference in metalloproteinases adsorption to the surface. The results of the overall venom adsorption and the metalloproteinase adsorption for each surface are shown in Table 6-2.
Table 6-2. Overall protein adsorption metalloproteinase adsorption from an initial 1 mL of 2 mg/mL venom solution for adsorption onto 2 mg of Si-1000-NH2, Si-1000-R, Si-1000-G, Si-1000-D, and Si-1000-RGD based on the venom positive control activity in 50 mM potassium phosphate buffer pH 7.5 and 37 °C.

<table>
<thead>
<tr>
<th></th>
<th>$C_{Liquid}$ (mg mL$^{-1}$)</th>
<th>$C_{Solid}$ (mg g$^{-1}$)</th>
<th>$a_V$</th>
<th>Casein activity</th>
<th>$a_M$</th>
<th>$K_M$</th>
<th>$K_V$</th>
<th>$S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-1000-AW$^1$</td>
<td>1.95</td>
<td>28.4</td>
<td>2.89</td>
<td>0.95</td>
<td>17.1</td>
<td>0.21</td>
<td>0.030</td>
<td>7.0</td>
</tr>
<tr>
<td>Si-1000-NH2$^1$</td>
<td>1.94</td>
<td>33.3</td>
<td>3.29</td>
<td>1.01</td>
<td>11.4</td>
<td>0.13</td>
<td>0.034</td>
<td>3.8</td>
</tr>
<tr>
<td>Si-1000-R$^2$</td>
<td>1.97</td>
<td>65.2</td>
<td>5.32</td>
<td>1.09</td>
<td>12.3</td>
<td>0.14</td>
<td>0.056</td>
<td>2.5</td>
</tr>
<tr>
<td>Si-1000-G$^2$</td>
<td>2.00</td>
<td>44.7</td>
<td>3.59</td>
<td>1.10</td>
<td>11.5</td>
<td>0.13</td>
<td>0.037</td>
<td>3.5</td>
</tr>
<tr>
<td>Si-1000-D$^2$</td>
<td>2.01</td>
<td>41.4</td>
<td>3.30</td>
<td>0.96</td>
<td>23.2</td>
<td>0.30</td>
<td>0.034</td>
<td>8.9</td>
</tr>
<tr>
<td>Si-1000-RGD$^3$</td>
<td>1.98</td>
<td>14.5</td>
<td>2.09</td>
<td>1.00</td>
<td>9.41</td>
<td>0.10</td>
<td>0.021</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Venom positive control activity: $^1$ 1.14 activity units $^2$ 1.24 activity units $^3$ 1.10 activity units

The adsorption coefficient, $K_j$, for venom and the metalloproteinase is defined by Eq.(6-1) The selectivity, $S$, is determined from the metalloproteinase adsorption coefficient, $K_M$, and the venom adsorption coefficient, $K_V$ using Eq. (6-2) These results are also shown in Table 6-2.

$$K_j = \frac{a_j}{1 - a_j}$$  \hspace{1cm} (6-1)

$$S = \frac{K_M}{K_V}$$  \hspace{1cm} (6-2)
Si-1000-NH2, Si-1000-R and Si-1000-G had similar metalloproteinase adsorption coefficients ($K_M=0.13$). Si-1000-D had the highest metalloproteinase adsorption coefficient ($K_M=0.30$), than twice that of the other surfaces. When compared to the adsorption coefficient of the venom, Si-1000-NH2, Si-1000-G and Si-1000-D had similar values ($K_V=0.034$). Si-1000-R had the highest venom adsorption coefficient ($K_V=0.056$). The combination of both these values resulted in the highest selectivity for Si-1000-D and the lowest selectivity for Si-1000-R. Si-1000-R may be selectively adsorbing another component of the venom, which would explain the higher value of $K_V$. Determining which protein in the venom preferred the Si-1000-R would require deeper study of that particular protein.

The selectivity coefficient shows that the metalloproteinase greatly prefers Si-1000-D over any other surface investigated in this study. It can be argued that this affinity was the result of the zinc ion being removed from the metalloproteinase. However, if the zinc ion was removed, the metalloproteinase would activity would drop without dropping the overall venom adsorption. This would result in a similar result as the venom-EDTA assay, which was not the case. This could also be attributed to electrostatic effects. However, Si-1000-AW is a more negative surface than Si-1000-D due to the terminal amine present on the Si-1000-D surface. Despite this charge difference, Si-1000-AW had a lower metalloproteinase adsorption coefficient than Si-1000-D. Therefore, it cannot be concluded that metalloproteinase adsorption to Si-1000-D is only due to electrostatic interactions between the positive zinc ion and the negative carboxyl group on aspartic acid. This result is best explained by a biological affinity between the metalloproteinase and aspartic acid. Previous literature reports that the aspartic acid residue has the highest binding impact between disintegrins and the surface.[140] The metalloproteinase had an adsorption coefficient onto Si-1000-D that was almost an order of magnitude greater than the venom.
adsorption coefficient on the same surface. This is an encouraging result considering the relatively simplicity of adding a single amino acid to the surface compared to the synthesis of more complex chromatographic materials such as grafting antibodies to the surface for affinity chromatography.

The original hypothesis was that Si-1000-RGD would have a higher selectivity for the metalloproteinase than would the single amino acid surface since, based on ligand spacing, it is possible that the distances between the amino acids on Si-1000-RGD could be comparable to side group spacings in the RGD peptide. However, Si-1000-RGD had a lower selectivity than both Si-1000-D and Si-1000-G. Moreover, Si-1000-RGD had the lowest adsorption coefficients for both venom and metalloproteinase. This result shows that amino acid functionalized surfaces that do not have specific biological interactions can exhibit lower protein adsorption than traditional surfaces such as Si-1000-AW and Si-1000-NH2. This is further supported by poly-lysine and poly-glutamic acid being used to prevent protein adsorption onto a silica surface.[103]

These results make a case for using single amino acid functionalizations to obtain selective protein adsorption. Metalloproteinase adsorption onto Si-1000-D had a selectivity of 8.9 compared to normal and reverse phase adsorbents, Si-1000-AW and Si-1000-NH2, which had selectivities of 7.0 and 3.8. Moreover, amino acid functionalization provides a more specific adsorption at a low cost. For chromatographic materials, increased complexity of the surface functionalization can produce higher specificity,[22] but also results in higher costs.[86] Affinity chromatography, which uses a biologically related agent, or affinity ligand, as a stationary phase to selectively retain analytes or to study biological interactions,[22] is extremely specific. However, synthesis of stationary phases for affinity chromatography requires a complex procedure to generating and purifying antibodies. Stationary phases using simpler surface
functionalities such as amine and carboxyl functionalization are far less expensive to produce than stationary phases for affinity chromatography, but can provide only simple separations based on charge, hydrophobicity, or size. The amino acid functionalized surfaces used in this study provide low-cost functionalization with biomimetic qualities.

6.4. Conclusion

Disintegrins are pharmaceutically valuable proteins. Amino acid functionalized surfaces were designed for the selective adsorption of a metalloproteinase with disintegrin properties from *C. r. ruber* venom. Arginine, glycine and aspartic acid were chosen based on biological disintegrin interactions. A peptide synthesis method was used to attach the amino acids to the amine functionalized surface through a peptide bond. The aspartic acid functionalized surface had an adsorption coefficient for metalloproteinase that was an order of magnitude higher than that of the overall venom. These results are promising for amino acid functionalized surfaces providing selective adsorbents at a fraction of the cost of affinity chromatography. This functionalization method resulted in a low cost selective adsorbent for a complex biological solution. These results are promising for the selective adsorption of key components from other complex biological mixtures.
Chapter 7 Summary and Conclusions
Effective protein separation methods are invaluable to the pharmaceutical industry. The goal of this research was to study the separation materials and the surrounding competing proteins in order to optimize the protein purification process. It included studies of the effect of physical and chemical variations of the silica on lysozyme adsorption. Chapter 3 addressed amino acid functionalized surfaces for lysozyme adsorption. The effect of competing proteins was also studied. Chapter 4 reported the results of lysozyme adsorption on silica in the presence of different concentrations of myoglobin. Chapters 5 addressed the effects of surface curvature on lysozyme adsorption. Finally, Chapter 6 reported the separation of metalloproteinase from snake venom using specifically designed silica surfaces.

Silica was successfully functionalized with amino acids using a peptide synthesis method and the effect of amino acid functionalization on lysozyme adsorption to silica surfaces was determined. Due to the high ligand density at the adsorbent surface, monolayer coverage is determined by the available surface area and not by the number of ligands. Lysozyme adsorption could be modeled using a single monolayer coverage for all the functionalizations synthesized in this study. The net charge difference between the surface and the adsorbed protein was the main factor influencing the adsorption of lysozyme onto carboxyl- and amine-functionalized silica. Surprisingly, net charge difference did not affect lysozyme adsorption onto amino acid-functionalized silica. Rather, specific interactions between lysozyme and the amino acid functionalities determined adsorption behavior.

A model of multicomponent multilayer adsorption has been developed to describe the simultaneous adsorption of lysozyme and myoglobin on MCF silica; the model is based on competition for surface area, the formation of lysozyme multilayers, and the adsorption of lysozyme on previously adsorbed myoglobin. Regression analysis demonstrates that all of these
effects must be accounted for to explain the observed adsorption behavior. The model is consistent with adsorption data and measured enthalpies of adsorption. Interestingly, only a single parameter was needed to account for lysozyme-myoglobin interactions in this system. The same derivations model can further be used for other protein systems.

Lysozyme adsorption onto silica surfaces with different functionalizations was found to be based on the available surface area and not specific to the number of ligands. A pore size effect is observed for lysozyme adsorption onto different functionalizations. The difference in pore size changed the shape of the adsorption isotherm. Entropic considerations such as the range of motion of the protein attachment to the surface were found to the impact the energy change of adsorption. The consideration of different pore sizes of chromatographic materials should not be limited to mass transport and surface area effects, but should also include the pore size effect on protein adsorption.

Finally, amino acid functionalized surfaces were designed for the selective adsorption of a metalloproteinase with disintegrin properties from *C. r. ruber* venom. Arginine, glycine and aspartic acid were chosen based on biological disintegrin interactions. A peptide synthesis method was used to attach the amino acids to the amine functionalized surface through a peptide bond. The aspartic acid functionalized surface had an adsorption coefficient for metalloproteinase that was an order of magnitude higher than that of the overall venom. These results are promising for amino acid functionalized surfaces providing selective adsorbents at a fraction of the cost of affinity chromatography. This functionalization method resulted in a low cost selective adsorbent for a complex biological solution. These results are promising for the selective adsorption of key components from other complex biological mixtures.
Chapter 8 Recommendations for Future Work
Because proteins are essential to cell function, proteomics has become a large and growing field of study. [1-3] Therefore, the purification of proteins is essential to the study of proteins and to product development in the pharmaceutical industry.[2, 4] The application of proteomics in the pharmaceutical industry is dependent on efficient, cost effective separations.[3] Because liquid chromatography has already been established as the industry standard separation method,[11] the most cost effective modification is modifications of liquid and stationary phases for more selective and efficient adsorption.[12]

The goal of this research was to study the thermodynamic equilibria of protein adsorption to silica and modified silica surfaces. The study of such interactions can provide a general basis for understanding protein separations as well as insight into the mechanisms of the separations. Efficient separations can improve the understanding of different ailments and improve the ability to find effective, personalized cures.[2] To achieve such developments, the current understanding of separations must be supplemented with an understanding of equilibrium thermodynamics of the separation components.[13] The future directions of silica surface modifications for more selective protein separations are now discussed.

8.1. Lysozyme Adsorption on Amino Acid-Functionalized Silica

Amino acids have proven to be an effective surface functionalization for selective protein adsorption. The next, more exciting step is to test these adsorption materials in an actual separation. Another test mixture could use the separation of lysozyme from competing proteins in the hen egg white mixture. More complex systems that would benefit from selective separations include venom, stroke[57] and Alzheimer’s[58] biomarkers from blood plasma and intracellular recombinant cutinase from E. coli cell homogenate. Amino acids were specifically chosen as biological functionalizations that are cheaper than affinity chromatography, but still
more selective than traditional stationary phases. A cost-benefit analysis of producing amino acid functionalized surfaces must be conducted before they can be considered for applications in the industry. If they prove effective, cost efficient stationary phases they can help solve the high cost of production for new and developing drugs.

8.2. Competitive Adsorption of Lysozyme and Myoglobin on Mesostructured Cellular Foam Silica

An extension of the GAB models has proved to be an effective predictor of multicomponent, multilayer protein adsorption. It also produced values for thermodynamic variables such including the Gibbs free energy of adsorption. A more thorough calorimetric study with a greater number of initial protein concentrations can produce values of enthalpy of adsorption for the competing proteins. Then entropies can be calculated for each adsorbed species, thus providing a complete picture of the thermodynamics of competitive adsorption. By understanding the thermodynamics of competitive protein adsorption, chromatographic separations can be better designed to reach the optimum purification results.

8.3. Effects of Surface Curvature on Lysozyme Adsorption on Silica Surfaces

Surface curvature was shown to impact the thermodynamics of adsorption. Smaller pore sizes were found help the reversibility of adsorption. Desorption studies for each surface curvature would be useful in determining the activity of the adsorbed protein before and after it is adsorbed to the surface. This way the effect of the reorientation of the protein on the larger pores can be determined. Circular dichroism (CD) can used to study the change in secondary and tertiary protein structure and nuclear magnetic resonance (NMR) can be used to study the changes in amino acid residues before and after adsorption the surface.[191] Moreover, a statistical analysis of different adsorption methods should be conducted for each surface to
determine which model best describes the protein adsorption. Larger pores have been shown to affect the protein structure. It could influence protein reorientation on the silica surface, protein conformation change or slow secondary attachments[148] as well as some mass transport-related peak distortion. The extent of that effect should be determined. Molecular dynamics would be useful to determine the effect of ligand distance and curvature on the adsorbed protein.

8.4. **Selective Metalloproteinase Adsorption from Viper Venom**

The addition of the amino acid functionalization through a peptide synthesis method was effective in achieving high surface coverage. Selective metalloproteinase adsorption is possible through amino acid functionalized silica surfaces. The next step in such surface modifications should be the addition of short peptides to increase surface selectivity to mimic already existing biological attachments. The process of building peptides on silica surfaces must first be studied to ensure the formation of the correct peptide on the surface. The effective separation of disintegrins such as metalloproteinases will be beneficial in understanding the biological pathways of such proteins and how they can be stopped. Other invasive biological agents such as cancer cells have been difficult to predict and control. A more thorough understanding of their migration through the body is key to finding a cure.
References


Appendix: Nitrogen Adsorption and Desorption

**Si-500-AW**

![Graph showing nitrogen adsorption and desorption for Si-500-AW](image)

**Si-1000-AW**

![Graph showing nitrogen adsorption and desorption for Si-1000-AW](image)