To my family
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Zirconium phosphate and zirconium phosphonate particles can specifically anchor many phosphorylated molecules, including oligonucleotides or phospholipids for bioanalytical studies. Recent research in our group has shown that DNA binds directly to zirconium phosphonate modified surfaces through a naturally occurring 5' terminal phosphate and we have demonstrated that this interaction can be used to immobilize oligonucleotide probes for DNA array applications. However, we can use this DNA binding system for other biological applications. The first half of the dissertation investigates the interactions of DNA molecules with zirconium phosphate and zirconium phosphonate particles for affinity chromatography applications. The particles are prepared via a simple and inexpensive synthesis that gives high product yields. We report the optimal conditions to bind probe ssDNA to these types of particles. Furthermore, we determine if probe DNA could hybridize with its complementary strand after immobilization on the surface of the particles. After simple rinsing procedures, non-specific interactions of DNA on zirconium phosphate and zirconium phosphonate particles are much less compared to the amount of DNA specifically bound to the surface via the 5'-terminal
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In the second half of this dissertation, we report methods to bind histagged proteins to modified zirconium phosphonate surfaces. Phytic acid can form monolayers on metal surfaces and can it form complexes with common immobilized affinity chromatography metals including Cu(II), Co(II) and Ni(II) ions. We used this knowledge to investigate immobilization of histagged proteins on metal-phytic acid modified zirconium phosphonate surfaces. These experiments produced moderate results, however, the use of NTA-functionalized bisphosphonate linkers on zirconium phosphonate surfaces for histagged proteins binding compared favorably against many commercially available Ni-NTA slides.
CHAPTER 1
SPECIFIC IMMOBILIZATION OF PROTEINS AT INTERFACES

Introduction

Proteomics is the study of protein function, expression and localization on a cellular scale. For proteome studies, high-throughput methods for protein characterizations are of great importance. Protein arrays have become a major tool used in proteomics research, having been successfully used to identify the differential expression of biomarker proteins in response to stimuli.\(^1\)-\(^5\) They have also been used for drug screening and medical diagnostic applications. Proteomics, however, is still an emerging technology and there is still a need for more selective surface immobilization methods for array-based techniques.\(^6\),\(^7\)

The immobilization of proteins on surfaces presents a significant challenge in the development of protein microarrays. Random orientations of immobilized proteins have been known to affect the protein's activity and function, which decreases detection sensitivity.\(^8\),\(^9\) Furthermore, non-specific adsorption of proteins on surfaces is often associated with problems such as protein denaturation, high background signal, and significant loss of protein probes after stringent surface washes.\(^10\) In order to fully retain biological activity, proteins should be attached to surfaces with high affinity and specificity.

Recently in the Talham group, zirconium phosphonate surfaces have been used to specifically bind phosphate-terminated oligonucleotides. This concept was used to develop DNA bioarrays on which DNA binding proteins could be immobilized without loss of protein function. In this dissertation, recent developments are expanded to bind proteins using two new approaches: (1) protein binding to DNA-modified zirconium
phosphate and zirconium phosphonate nanoparticles, (2) phosphopeptide binding to zirconium phosphate and zirconium phosphonate particles and (3) binding histidine tagged proteins to modified zirconium phosphonate surfaces. This chapter introduces common ways to specifically immobilize proteins as well as a general overview of the new approaches that will be discussed in this dissertation.

**Approach 1: DNA-Modified Zirconium Phosphate and Phosphonate Surfaces for Protein Capture Experiments**

DNA arrays have emerged as a convenient tool in molecular biological research for rapid and accurate gene mapping, DNA sequencing, mRNA transcription analysis to diagnose genetic diseases and monitor gene regulation. Common surface modifications for DNA immobilization include (1) adsorption to porous materials (usually polyacrylamide or agarose gels) bound to glass or polymer coated surfaces or (2) layers of surface bound functional groups for covalent bonding with oligonucleotides. In this work, we focus on the latter immobilization strategy.

Oligonucleotides are usually modified at the 5'-end for specific immobilization to functionalized surfaces. Common organic functional groups on surfaces include carboxylic acid, aldehydes, epoxides, and thiols (Figure 1-1). Amine-terminated oligonucleotides can be specifically bound to surfaces through carbon-nitrogen bonds on carboxylic acid, aldehydes, or epoxides. Thiol modified DNA can bind to thiol modified surfaces through a disulfide bridge. Biotin modified oligonucleotides can be specifically immobilized on streptavidin terminated surfaces. With these specific linkages, the oligonucleotide probes are attached in a homogeneous manner with good surface coverage. The probes, however, require specific chemical modifications of the 5'-terminal oligonucleotides which can be expensive and time-consuming.
Some organic surfaces are used to circumvent the need for any DNA modification. Unmodified oligonucleotides have been electrostatically attached to amine terminated surfaces via the phosphate backbone of the DNA.\textsuperscript{26-27} The potential drawback for this type of DNA/surface interaction is that electrostatic binding causes the probes to lie on the surface, reducing the ability of hybridization with complementary DNA.

In contrast to the organic covalent linkages mentioned above, the Talham group has recently reported a metal/ligand interaction to immobilize DNA. These types of "organic-inorganic" interactions remain relatively unexplored with the exception of thiol modified DNA binding to gold surfaces.\textsuperscript{28-31}

In the Talham group’s approach, substrates are modified with a zirconium phosphonate monolayer surface layer that is known to strongly immobilize phosphate or phosphonate functionalized molecules through a coordinate covalent interaction between the zirconium ions and the terminal ROPO\textsubscript{3}\textsuperscript{2-} and RPO\textsubscript{3}\textsuperscript{2-} functional groups.\textsuperscript{32-37} Taking advantage of this chemistry, phosphate terminated oligonucleotides were shown to selectively bind to zirconium phosphonate monolayer surfaces for the fabrication of efficient DNA microarrays (Figure 1-2 A).\textsuperscript{38} High specificity of oligonucleotide probes anchoring onto the support was demonstrated as well as good sensitivity of the probes for detecting their complementary oligonucleotide target strands. More recently, the metal/ligand approach to immobilizing oligonucleotide probes was extended to the binding of dsDNA probes for the detection of protein targets with good sensitivity and specificity (Figure 1-2 B).\textsuperscript{39}
In addition to arrays, DNA can be immobilized to solid supports for other applications. For example, the surface of nanoparticles can be customized using the same functionalization methods previously discussed for chromatography applications. DNA affinity chromatography has been extensively used for purification for separation of target DNA from proteins or target DNA specifically complexed to protein partners. Non-specific DNA columns are commonly used in protein purification, but the DNA-protein interactions are mainly electrostatic and tend to offer little selectivity for purification purposes.\textsuperscript{40} Single-stranded DNA affinity columns have been fabricated to bind single-stranded DNA binding protein or DNA and RNA polymerase enzymes using fragmented DNA\textsuperscript{41} or chemically synthesized oligonucleotides.\textsuperscript{42} Proteins such as transcription factors and restriction enzymes show high specificity for specific dsDNA sequences and can be purified using dsDNA affinity columns (sequence specific DNA affinity).\textsuperscript{43-47}

A straightforward, cost effective chromatography system based on the design of zirconium phosphate and bisphosphonate particles for affinity chromatography is discussed in Chapter 2 of this dissertation. Like DNA modified zirconium phosphonate arrays, the main advantage of using zirconium phosphate and phosphonate particles to target DNA-protein interactions is that the surface is specific for the 5'-phosphate of DNA, thus, there is no need for the particles, DNA or proteins to undergo further chemical modifications. Immobilized DNA on these particles can hybridize complementary DNA demonstrating that the DNA-modified zirconium phosphate and phosphonate particles have biological activity. Biomolecules can easily be removed from zirconium phosphate and zirconium phosphonate particles, which is also an
important attribute of these particles for affinity chromatography applications. Also, the size, chemical composition, and polydispersity of the nanoparticles can be tailored and the performance of the chromatography system can be controlled\textsuperscript{48-50} offering more control of the biomolecule immobilization to the particles.

**Affinity Interactions of Proteins Directly to Zirconium Phosphonate Surfaces**

In Approach 1, the study of protein binding to surfaces through DNA-protein interactions was discussed. However, protein binding directly to surfaces can also be achieved though direct binding of amino acid residue side chains to surfaces. This approach is advantageous because it can be accomplished without the need of introducing a foreign functional group to the proteins, as is the case when using DNA-modified surfaces. In Approach 2 and 3, proteins are bound directly to zirconium phosphonate particles and planar surfaces.

Four main approaches for immobilizing proteins on supports are 1) adsorption, 2) physical entrapment, 3) covalent linking, and 4) affinity attachment.\textsuperscript{15} (Figure 1-3) Protein binding by surface adsorption is simple and convenient because it is easy to generate these surfaces and does not require any chemical modifications of capture agents, but the attached proteins lay on the surface in random orientation, may be denatured, and can be removed under stringent washing conditions. In addition, the background noise level is usually higher because of the non-specific adsorption. Proteins can be physically or chemically entrapped into platforms such as agarose\textsuperscript{51} or polyacrylamide\textsuperscript{52, 53} gel pads, sol-gel-encapsulated biomolecules patterned within multi-well PDMS films,\textsuperscript{54} and hydrogels.\textsuperscript{55, 56} The 3-D structure of these substrates results in higher protein binding capacity and lower protein denaturation due to the homogenous environment of the gel as compared to flat surfaces. However, gels can act as a
structural barrier to diffusion and molecular recognition events may require a long incubation time. Protein binding via absorption or entrapment methods often occur in a random or non-specific orientation which may reduce the activity or biochemical properties of immobilized proteins relative to covalent linking and affinity-based strategies have been used to immobilize proteins in an oriented and specific manner.

In this work, protein-binding strategies are rooted in covalent linkage and affinity based methods drawing from work with immobilized metal affinity chromatography (IMAC). IMAC was introduced in 1975 by Jerker Porath. This method consists of derivatizing a resin with iminodiacetic acid (IDA) and chelating metal ions to the resin. Proteins bind to the metal ions through unoccupied coordination sites and are immobilized on the column. In subsequent work, researchers have used ligands other than IDA, such as nitrilotriacetic acid (NTA), to chelate metal ions. IMAC is an attractive chromatography method because it is relatively inexpensive and operates under mild conditions, preventing protein denaturation. The key to the IMAC technique is selecting appropriate ligand-metal ion combinations to immobilize proteins.

The preferential affinity of a particular metal ion for an electron donor ligand is explained by the Principle of Hard and Soft Bases (HSAB), which was first developed by R.G. Pearson in 1965. Classification using this system is based on an atom or molecules' polarizability, the ability of the molecule for charge separation when placed in an energy field. Hard acids and bases are usually small and highly charged and not easily polarized. Conversely, soft acids and base are large and have a low charge density; these atoms and molecules are highly polarizable. Acids and bases that exhibit both hard and soft characteristics are classified as "borderline".
According to the HSAB principle, hard acids prefer to coordinate with hard bases and soft acids prefer to coordinate with soft bases. Hard acids bind to hard bases by ionic forces since the distance between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) is large; no electron transfer occurs and the interaction between acid and base is primarily electrostatic. Bonds between soft acid and soft bases are primarily covalent since the distance the HOMO and LUMO is small allowing electron donation to the metal orbitals from the unfilled orbitals of the ligand. Borderline species can exhibit both ionic and covalent interactions with ligands.

Many different metal ions have been tested for use in IMAC chemistry. Metals can be divided into 3 categories based on their preferential reactivity towards nucleophiles: hard, soft and borderline metals. The most common metals used, however, are "borderline metals", such as Cu(II), Co(II), Ni(II), and Zn(II). These metal ions preferential bind with aromatic nitrogen, oxygen and sulfur. These metals are used in the binding of histagged proteins on surfaces with IMAC. More recently, IMAC has been used with "hard metals", such as Fe(III), Ga(III), Ca(II), Mg(II), Sn(IV), Ti(IV), and Zr(IV). These metals have an affinity for oxygen, aliphatic nitrogen, and phosphates. Interactions that will be explored in this dissertation include Zr(IV) with phosphate (hard acid and hard base) and Ni(II), Co(II) and Cu(II) with the imidazole rings on histagged proteins (borderline acids and borderline bases).

**Approach 2: Affinity Chromatography With Hard Metal Ions: Interactions of Zr(IV) with Phosphate**

In Chapter 3 of this dissertation, phosphopeptide binding to zirconium phosphate and phosphonate nanoparticles will be discussed. Several affinity based peptide enrichment methods have been developed. Phosphorylation is an important post-
translational modification in eukaryotic cells in which a serine, threonine or tyrosine amino acid residue is phosphorylated by a protein kinase. This modification is involved in many regulatory mechanisms such as gene expression, signal transduction, enzyme activation and cell division. The development of sensitive and selective methods to quantify and characterize protein phosphorylation sites is needed because phosphopeptides are typically present in lower abundance than other peptides.

Methods to study protein phosphorylation can generally be divided into two categories: chemical modification and chromatography-based approaches. Although highly efficient, chemical modification is not the preferred approach because it requires a number of protein transformations, which can cause noise in the mass spectrometry analysis.61 Besides IMAC, other chromatography-based methods include strong cation (anion) exchange chromatography, metal oxide affinity chromatography (MOAC), and hydrophilic interaction chromatography. Of these, IMAC has been the most widely used method, but, more recently, enrichment strategies have employed the MOAC approach.

Fe(III)-IMAC is the most commonly used IMAC technique for phosphopeptide selection.62-65 Porath et al. showed that Fe(III)-IMAC can be used to selectivity absorb phosphoproteins and phosphopeptides.66, 67 Despite its widespread use, Fe(III)-IMAC are known to non-specifically absorb peptides mainly through carboxylate, thiol, amine and imidazole functional groups on amino acid side chains; these interactions compete with phosphate groups for Fe(III) sites.61, 66-68 More recently, however, Ga(III)-IMAC has been noted to be more advantageous in terms of higher phosphoprotein selectivity and ease of protein elution but there are still problems with non-specific peptide binding.69
Methyl esterification of carboxylates greatly reduces competition, but the chemistry is not always complete and can complicate analysis methods.\textsuperscript{61,64,68,70,71}

Metal oxide affinity chromatography (MOAC) employs the use of high valence metals oxides for protein separation such as zirconia (ZrO\textsubscript{2}),\textsuperscript{68,72,73} titania (TiO\textsubscript{2}),\textsuperscript{61,74,75} aluminum dioxide (Al\textsubscript{2}O\textsubscript{3}),\textsuperscript{75} tin dioxide (SnO\textsubscript{2}),\textsuperscript{76} and niobium(V) oxide (Nb\textsubscript{2}O\textsubscript{5}).\textsuperscript{77} The most popular MOAC medium, TiO\textsubscript{2}, was introduced as an enrichment medium as a pre-column for electrospray ionization liquid chromatography tandem mass spectrometry.\textsuperscript{78} The oxide surface charge changes with pH; at acidic pH, metal oxides are positively charged which allows greater selectively of phosphorylated molecules.\textsuperscript{61,68} ZrO\textsubscript{2} surfaces are more selective for monophosphorylated peptides whereas TiO\textsubscript{2} is more selective for multiphosphorylated peptides.\textsuperscript{61,79} ZrO\textsubscript{2} and TiO\textsubscript{2} were found to be more selective for phosphopeptides over acidic peptides as compared to Fe(III)-IMAC with the use of hydroxyl acids in the loading and washing buffers.\textsuperscript{74} This use of hydroxyl acids in washing buffers will be discussed further in Chapter 3.

Recently, zirconium phosphate and phosphonate surfaces have been used for phosphopeptide enrichment. A Zr(IV)-IMAC technique was developed by Zou and coworkers where Zr\textsuperscript{4+} ions were bound to phosphonate-modified polymer beads. The high specificity of this absorbent was demonstrated by effectively enriching phosphopeptides from a digested mixture of phosphoprotein (α or β-casein) and bovine serum albumin (BSA) at a molar ratio of 1:100.\textsuperscript{68,80} Alternatively, instead of using zirconium phosphonate modified beads, Zare and coworkers successfully enriched phosphopeptides with crystalline α-zirconium phosphate with higher sensitivity and lower detection limits compared to Fe(III)-IMAC, TiO\textsubscript{2}, or Zr(IV)-IMAC.\textsuperscript{81}
Alpha-zirconium phosphate crystals are layered structures with each zirconium atom sharing six oxygen atoms, all from different phosphates groups. In turn, each phosphate shares its three oxygen atoms with three different zirconium atoms (Figure 1-4A). The covalently bound metal-phosphate layers are connected by weaker hydrogen bonding. These structures are also formed by zirconium phosphonates, which replace HOPO$_3^{2-}$ with RPO$_3^{2-}$ ligands (where R is an organic group). The advantage in moving towards phosphonates is that changing to a phosphonate ligand as opposed to a phosphate changes the way zirconium ions are presented on the surface of the nanoparticles, thus affecting the affinity of phosphopeptide binding at these interfaces.

Figure 1-4B shows the ideal crystal structure of zirconium methylene diphosphonic acid, one of the materials synthesized for preliminary experiments of phosphopeptide enrichment. These materials could lead to increased phosphate binding capacity or enhanced phosphate selectivity because distinctive coordination of zirconium with phosphate can prevent non-specific binding of acidic peptides. Instead of an electrostatic attraction between a positively charged surface and an anionic molecule, zirconium phosphate and phosphonate surfaces form strong coordinate covalent bonds to dibasic phosphates (Figure 1-5). The divalent phosphate in a phosphopeptide is capable of displacing the terminal oxides and hydroxides on a hydrated surface to form covalent linkages to the Zr$^{4+}$ ions, similar to the IMAC mechanism. Acidic peptides have carboxylate side chains that are not basic enough to displace oxide and hydroxide from the zirconium surface. They will only form nonspecific electrostatic bonds to the oxide-like surface, which is similar to the MOAC mechanism. Subsequently, acidic peptides can be removed by buffer rinses.
Zirconium phosphonate particles can be prepared in a similar manner to phosphate particles. We would like to extend the study of phosphopeptide enrichment on the surface of zirconium phosphonate particles. In Chapter 3, preliminary results show the use of zirconium phosphate and phosphonate particles for phosphopeptide enrichment with the use of matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and HPLC/(-)ESI tandem mass spectrometry for analysis.

**Approach 3: Affinity Chromatography with Borderline Metals: Ni(II), Co(II) and Cu(II) Binding to Imidazole Groups on Histagged Proteins**

Histidine is the amino acid with the strongest affinity to metal ions\(^\text{85,86}\) although tryptophan and cysteine contribute to the binding of metal ions. Binding increases with an increasing number of histidyl side chains.\(^\text{85}\) Throughout the years, many different lengths of histidine tags have been employed, however, the most popular choice for histidine tags (histags) consists of six consecutive histidine residues.\(^\text{62,87}\) Histags are usually genetically engineered on the N-terminus of proteins of interest although C-termini histags have also been used.

Specific binding of histagged proteins can be accomplished using metal ions chelated to multidentate ligand supports (Figure 1-6). First, the ligand supports (columns, beads or surfaces) are charged with metal ions such as Ni(II), Co(II) or Cu(II). Available metal coordination sites occupied by water molecules can then be exchanged by electron donor groups (i.e. imidazole) for specific immobilization of proteins.\(^\text{62}\)

There are many types of ligands used for these experiments. Pentadentate ligands have been examined for protein immobilization thought histag moieties, but the most commonly used chelators are tridentate or tetradentate. Pentadentate ligands such as triscarboxymethyl ethylenediamine (TED) are the strongest absorbers of metal
ions followed by tetradeutate ligands (such as NTA) and then tridentate ligands (IDA). Ligands that coordinate with metal ions more strongly leave a smaller number of sites for protein binding to occur. For example, on a metal-IDA complex, there are at least two, usually three, free sites for protein binding, while there is only a single free site available for protein binding on a metal-TED complex. NTA, IDA and TED ligands have been used in commercially available resins for histagged protein purification.

In many studies, including one method that is discussed in this thesis, surfaces modified with Ni-NTA are used for histagged protein capture. Despite the moderate affinity ($K_d \sim 1-10 \mu M$) and stability of individual Ni-NTA: oligohistidine complexes, this strategy has been successfully used in microarray format for the immobilization of 6000 histag labeled yeast proteins, of which more than 80% were shown to retain their biological activity. However, most NTA surfaces used for the design of protein arrays are obtained by spreading a functionalized polymer matrix on a glass surface, leading to inhomogeneous surface coatings without rigorous control of the NTA density.

In this thesis, we study histagged protein binding to zirconium phosphonate modified surfaces. The surfaces are modified with phytic acid, a phosphoester ligand possessing six phosphate groups that are able to form soluble and insoluble complexes with a variety of borderline and hard metal ions (Figure 4-1). Profiling results by Martin and Evans suggest that phytic acid form soluble complexes with Ni(II) in basic conditions and Co(II) and Cu(II) form soluble complexes with phytic acid in acidic conditions. Bebot-Brigaud et al. found that phytic acid formed monocoordinated
soluble complexes with Co(II) and Zn(II) at low pH and bicoordination species at pH greater than 9. Similar results were seen by Torres et al. with Mg(II)-phytic acid complexes; furthermore, they found that the complexing ability of phytic acid with Mg(II) and Ca(II) is comparable to that of ethylenediaminetetraacetic acid (EDTA). Recently, phytic acid has also gained attention as a possible anti-corrosion and cleansing agent to remove rust from metal surfaces. Monolayers of phytic acid on iron, silver, copper, and cupronickel roughened electrode surfaces have been investigated using surface enhanced Raman spectroscopy (SERS).

Because phytic acid has been shown to bind metal ions and form monolayers on metal surfaces, we investigated whether phytic acid could be used to modify zirconium phosphonate surface for histagged protein binding. Chapters 4 and 5 of this dissertation discuss the use of immobilized Ni(II)-phytic acid complexes as a means to provide specific anchoring of histagged proteins on zirconium phosphonate surfaces.

**Scope of the Research**

The goal of this research is to explore ways to specifically immobilize DNA and proteins to surfaces. The first part of the dissertation investigates affinity interactions of zirconium phosphate and phosphonate particles with biomolecules. We report the optimal conditions to bind ssDNA to these types of particles and perform hybridization experiments to determine if the DNA-modified particles are biologically functional. We also use these particles to enrich phosphopeptides from a tryptically-digested solution of β-casein. Phosphopeptides can be eluted from the particles and analyzed with mass spectrometry. In the second part, we report methods to bind histagged proteins to phytic acid modified-zirconium phosphonate surfaces. Immobilization of histagged proteins on these surfaces produced moderate results. However, the use of NTA-
functionalized bisphosphonate linkers on zirconium phosphonate surfaces for binding of histagged proteins compared favorably with many commercially available Ni-NTA slides. Finally, we quantify the probe density of DNA on zirconium phosphonate surfaces using quartz crystal microbalance (QCM) analysis. These DNA-modified surfaces have been used successfully to study DNA-protein interactions.39

Figure 1-1. Amine-terminated oligonucleotides can be specifically bound to the surfaces through carbon-nitrogen bonds on carboxylic acids, aldehydes or epoxides. Thiol modified DNA can bind to thiol-modified surfaces through a disulfide bridge. Unmodified oligonucleotides can be immobilized on polymeric or self-assembled amine-terminated via the phosphate backbone of DNA.
Figure 1-2. Schematic for DNA binding to zirconium phosphonate surfaces (left) and the extension of this work for protein recognition with immobilized dsDNA (right).
Figure 1-3. Four main types of protein binding to various types of surfaces are (A) adsorption, (B) physical entrapment, (C) covalent linking, and (D) affinity attachment. Protein binding by absorption or entrapment often occur in a random or non-specific orientation which may reduce the activity or biochemical properties of immobilized proteins whereas covalent linking and affinity-based strategies have been used to immobilize proteins in an oriented and specific manner. This figure was adapted from a scheme found in Ref 15.
Figure 1-4. Idealized structures of (A) zirconium phosphate, Zr(HPO₄)₂·H₂O and (B) zirconium dimethylene phosphonic acid, Zr[O₃P(CH₂)PO₃]. Both zirconium phosphates and zirconium bisphosphonates are composed of layered galleries with each Zr sharing six oxygen atoms all from different phosphate groups. Each phosphate or phosphonate group shares its three oxygen atoms with three different metal atoms. This bonding is extremely strong and may lead to increase phosphopeptide selectivity on these surfaces. The figure was drawn based on previously published work on metal phosphate and metal phosphonate crystallographic data.⁸²,¹²⁰
Figure 1-5. Scheme illustrating the difference between the covalent linkage to the zirconium phosphate surface formed by dibasic phosphate (top) and the non-specific bonding experienced by weaker bases, such as carboxylates (bottom).

Figure 1-6. IMAC consists of derivatizing a support matrix with a ligand and chelating metal ions to the resin. Common ligands include (A) IDA, (B) NTA, or (C) TED. The metal chelating ligands can be tridentate (IDA), tetradentate (NTA) or pentadentate (TED). Proteins bind to the metal ions through unoccupied coordination sites.
CHAPTER 2
DNA-MODIFIED ZIRCONIUM PHOSPHATE AND ZIRCONIUM PHOSPHONATE NANOPARTICLES

Introduction

Affinity chromatography is a technique commonly utilized for separating complex mixtures of proteins. A commonly used affinity method for protein separation is avidin-coated polymer beads, which specifically bind proteins functionalized with biotin. Beads can also be functionalized with nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA), which, when chelated with a metal ion (usually Ni\(^{2+}\) or Co\(^{2+}\)), can bind proteins modified with six histidine amino acids (histag). However, protein modification with biotin or histags can be tedious and expensive and could also lead to protein denaturation. If protein(s) of interest cannot be modified, then other protein separation methods are needed. Recently, there has been interest in using DNA modified particles for DNA biosensors\(^1\)\(^2\)\(^,\)\(^3\) and affinity chromatography of DNA-binding proteins.\(^4\)\(^5\)\(^\text{122}-\text{125}\) However, the DNA and the particles used in all of these studies still have to be functionalized to ensure that DNA was immobilized in a specific orientation.

Previous research in our group has shown that DNA binds directly to zirconium phosphonate modified surfaces through a naturally occurring 5’ terminal phosphate and we have demonstrated that this interaction can be used to immobilize oligonucleotide probes for DNA arrays. We would like to extend this chemistry to zirconium phosphate nanoparticles (ZrPN). Using confocal laser scanning microscopy, we demonstrate that single-stranded, phosphorylated oligonucleotides specifically bind ZrPN that are terminated in Zr\(^{4+}\) ions. Furthermore, DNA-modified ZrPN can hybridize with its complementary stand. We also synthesize zirconium phosphonate particles for biomolecule immobilization and detection. Zirconium bis- and triphosphonate particles
can be prepared in a similar manner as zirconium phosphate nanoparticles. Particles of zirconium nitrilotri(methylene)triphosphonate (Zr-NTMP) do not show specificity for binding phosphorylated oligonucleotides, however, DNA immobilization experiments show specific binding of 5'-phosphorylated DNA with Zr\(^{4+}\) treated-zirconium methylenediphosphonate (Zr-MDP) particles. Like ZrPN, DNA-modified Zr-MDP can also hybridize with its complementary sequence. Non-specific DNA interactions on ZrPN and Zr-MDP are also examined using control experiments. Our experiments show that particles must be both terminated in Zr\(^{4+}\) ions and incubated with phosphorylated DNA for significant amounts of DNA binding to occur.

**Experimental Section**

**Materials**

All DNA was custom synthesized and purchased from Sigma-Aldrich (St. Louis, MO) and used as received. For ssDNA binding studies, desalted fluorescien (Flc)-labeled 5'-phosphorylated and non-phosphorylated ssDNA (5'-phos-TTTTTTTTTTTTTTTTTTTTT-Flc-3' and 5-TTTTTTTTTTTTTTTTTTTT-Flc-3') was obtained from Sigma. For hybridization studies, a 5'-phosphorylated and non-phosphorylated probe and its complementary target strand were used. The strands are as follows: phosphorylated probe (5'-[phos]-ATCTAACTGCTGCGCCCGCC-3'), non-phosphorylated probe (5'-ATCTAACTGCTGCGCCCGCCG-3'), and target (5'-[Flc]-CGGCGGCAGCGATAGTTAGAT-3'). The oligonucleotide strands were HPLC-purified by Sigma before use. The appropriate amount of 1X sodium saline citrate (SSC) buffer (pH 8) was added to oligonucleotides to make 100 μM solutions and then the oligonucleotides were aliquoted into one-time-use volumes (10 μl). The aliquots were
stored at -20°C. All other reagents were of analytical grade and used as received from commercial sources unless otherwise noted.

**Synthesis of Zirconium Phosphate and Zirconium Phosphonate Nanoparticles**

Zirconium phosphate nanoparticles (ZrPN) were synthesized using a procedure previously reported with slight modifications.\(^{81,126}\) Briefly, 10 g zirconyl chloride was refluxed with 100 ml of 3 M H\(_3\)PO\(_4\) at 100°C for 24 h. After the reaction, 25 ml of products were placed in 50 ml falcon tubes (4 tubes total), washed with water, and collected by centrifugation at 2900 g for 20 minutes. Centrifugation was repeated twice. The ZrPN particles were dried at 65°C overnight and then ground with a mortar and pestle into a fine powder. ZrPN particles (10 mg) were rinsed with 1 ml of water and centrifuged at 8800 g for 5 minutes twice. The supernatant was removed and the ZrPN particles were resuspended in acetonitrile (ACN)/water (1:1, v/v). After rinsing for 5 minutes, the particles were collected by centrifugation (8800 g for 5 minutes) and the supernatant was again removed. The particles were then resuspended in 1 ml of water to form a uniform ZrPN suspension. The ZrPN solution was then treated with 20 mg/mL ZrOCl\(_2\) at room temperature overnight with shaking to terminate the particles in Zr\(^{4+}\) ions for optimal binding with phosphorylated DNA. The ZrPN particles were collected by centrifugation, washed with water twice to remove excess zirconyl chloride, and then resuspended in water. Particles were buffered to pH 2 by adding 10 µL of 5% HCl to 5 mg/ml particle solution (total volume = 1 ml) because the particles are more readily suspended in acidic solutions.

Zr-MDP was synthesized by adding 100 ml of 3 M methylenediphosphonic acid to 10 g of zirconyl chloride at 80°C for 2 hours. The Zr-MDP was dried at 65°C overnight.
and then ground with a mortar and pestle into a fine powder. 10 mg/ml of Zr-MDP particles were washed and treated with Zr\(^{4+}\) using the same procedure described for the zirconium phosphate nanoparticles.

Zr-NMTP was synthesized by refluxing 7.7 g of zirconyl chloride with 100 ml of nitrilotris(methylene)triphosphonic acid solution at 95°C overnight. The Zr-NMTP was dried at 65°C overnight and then ground with a mortar and pestle into a fine powder. Zr-NMTP particles (10 mg/ml) were washed and treated with Zr\(^{4+}\) using the same procedure described for the zirconium phosphate nanoparticles.

**Single-stranded DNA Binding to ZrPN Nanoparticles**

A 250 nM ssDNA stock solution was prepared by diluting 10 μl of aliquoted phosphorylated or non-phosphorylated DNA with 4 ml of nanopure water. One hundred microliters of zirconium phosphate or zirconium phosphonate particles (5 mg/ml) were incubated in 400 μl of stock phosphorylated or non-phosphorylated DNA solution in deionized water for 2-4 hours at room temperature (total volume = 1ml). Non-specifically bound DNA was removed from the particles by either: (1) consecutive rinsing with 20 μl of 1X phosphate-buffered saline (PBS) pH 7, water and 0.1% phosphoric acid (one time each) or (2) consecutive rinsing with 2X SSC/0.1% SDS (pH 7.8) for 2 minutes and 0.2X SSC/0.1% SDS (pH 7.8) for 2 minutes. After each rinse step, particles were collected by centrifugation at 8800g for 5 minutes. Particles were then resuspended in either 20 μl water for confocal laser scanning microscopy (CLSM) analysis or hybridization solution for further experiments (see next section).

**Hybridization of DNA-Modified Nanoparticles**

A 125 nM target DNA stock solution was prepared by diluting 10 μl of aliquoted target DNA with 8 ml of water. DNA modified zirconium phosphate and zirconium
phosphonate particles were suspended for 2-4 hours in a hybridization solution (8 ml of 0.5 nM complementary target DNA in 3.5X SSC (pH 7.4), 0.03% SDS, 5X Dernhardt's solution, and 10% Tris-EDTA). Non-specifically bound DNA was removed by rinsing the particles with 2X SSC, 0.1% SDS (pH 7.8) for 2 minutes and 0.2X SSC, 0.1% SDS (pH 7.8) for 2 minutes. Each rinse step included sonication. After each rinse step, particles were collected by centrifugation at 8800 \( g \) for 5 minutes. Non-hybridized targets were removed by washing with 2X SSC/0.1% SDS for 2 minutes, 0.2X SSC/0.1% SDS for 2 minutes and then particles were reconstituted in water for CLSM analysis.

**Instrumentation**

ZrPN, Zr-MDP, and Zr-NMTP were imaged using a Hitachi S-400 FE scanning electron microscope (SEM). Ten microliters of an aqueous solution of particles (5 mg/ml) after treatment with zirconyl chloride were spotted on silicon slides. Images were taken after slides had dried in air overnight.

The surface charge of the zirconium phosphate and zirconium phosphonate particles was measured with a ZetaPlus (Brookhaven) using the PALS Zeta Potential Analyzer software. An electric field (10-11 V) was applied across a disperse particle solution (500 \( \mu g/ml \)). The charged particles move towards the electrode of opposite polarity. When a laser beam (532 nm) is passed through the particle solution, the frequency of the scattered light from the moving particles can be measured. Electrophoretic mobility was calculated in the Zeta Potential Analyzer software given the values of the frequency of the scattered light, the wavelength of the laser and the scattering angle. The software calculated zeta potential measurements from the electrophoretic mobility using the Smoluchowski equation.
Florescent DNA modified phosphate or phosphonate particles were imaged using an IX81 confocal microscope (Olympus, Japan) with Fluoroview software. The particles were imaged with 10x magnification with a PMT voltage set to 660x at 30% laser intensity unless otherwise noted. Fluorescien has an excitation wavelength of 494 nm and an emission of 521 nm. The fluorescein-labeled DNA was excited with 488 nm Argon laser and emission was detected at all wavelengths greater than 505 nm using a 505 nm band pass interference filter (505IF). All samples were compared to a blank solution to ensure that fluorescence intensities are only attributed to the DNA immobilized on the particles. The blank solution consisted of particles that went through the DNA modification procedure sans fluorescent DNA. The fluorescence intensities are color-coded from blue (low) to green, yellow, red and then white (saturation). Fluorescence images shown in the text are of 20 μl solutions of DNA-modified particles in microwell plates. Each image was cropped to show the fluorescence intensity of the background of the well versus the fluorescence intensity of a solution of DNA-modified particles. Quantification of fluorescence intensities was done using the ImageJ software (freely available from the NIH). The average and standard error of at least three DNA binding experiments for each system was determined after background subtraction.

Results

Nanoparticle Synthesis

Zirconium phosphate nanoparticles were prepared by refluxing ZrOCl₂ in H₃PO₄ overnight. SEM images show that the particles are nanoplatelet-like structures of uniform size (Figure 2-1), which is in good agreement with published results. After synthesis, the surfaces of the particles can be terminated in either Zr⁴⁺ or PO₄³⁻ ions. To maximize the covalent binding of phosphorylated DNA, the platelets were treated with
excess ZrOCl₂ overnight to ensure that the particles were completely terminated in Zr⁴⁺ ions. Zeta potential measurements were used to determine the surface charge of the particles before and after treatment with zirconyl chloride (Table 2-1). Before treatment, the particles have a zeta potential of -32 mV. After ZrOCl₂ treatment, the zeta potential increases to 13 mV showing that after treatment with zirconyl chloride, the charge density of the particles increased due to the binding of Zr⁴⁺ ions.

**Single-Stranded DNA Binding and Hybridization of Target DNA to the ZrPN**

For DNA binding studies with ZrPN (pH 2), four oligonucleotide-modified particle systems were analyzed: (1) particles that were not pretreated with ZrOCl₂ and incubated in non-phosphorylated DNA, (2) particles that were pretreated with ZrOCl₂ and incubated in non-phosphorylated DNA, (3) particles that were not pretreated with ZrOCl₂ and incubated in phosphorylated DNA, (4) particles that were pretreated with ZrOCl₂ and incubated in phosphorylated DNA (Figure 2-2). Systems 1-3 are the control systems used to analyze non-specific binding of DNA to the zirconium phosphate particles. Fluorescence data shows that there is the greatest binding affinity of phosphorylated DNA with Zr⁴⁺-treated ZrPN as compared to any of the other DNA-particle systems studied. Fluorescence intensities indicate that non-specifically bound DNA was removed from the particles following washes with PBS and dilute phosphoric acid (Figure 2-3). Particles not pretreated with ZrOCl₂ showed some areas of saturated fluorescence (size > 10 μm), which is assumed to be aggregates of particles with non-specifically bound DNA. This result is not entirely unexpected because, although the surface consists mainly of PO₄³⁻ binding sites, thus the surface of the ZrPN particles will still have some Zr⁴⁺ sites that will readily interact with phosphorylated oligonucleotides.
The next test performed was hybridization studies with DNA-modified ZrPN nanoparticles to determine if probe DNA could hybridize with its complementary strand after immobilization on zirconium phosphate particles. Zirconium treated and non-treated particles were subjected to the ssDNA binding experiment as described above. Then particles were incubated with a solution of fluorescently labeled target DNA and then imaged with CLSM (Figure 2-4). In this experiment, the PMT voltage of the fluorescence microscope was increased to 895x because the fluorescence intensity after hybridization was much less than after probe DNA binding directly to particle surface. Despite this, it was clear that the greatest amount of hybridization occurred on DNA-modified ZrPN particles modified with phosphorylated probe DNA.

We wanted to extend this project to include DNA-binding to zirconium phosphonate particles. Changing the zirconium binding ligand from a phosphate to a phosphonate could have dramatic effects in probe binding. Changes in the ligands allow variation of electronic properties of the zirconium ions and can also affect the crystalline lattice constants and metal ion spacing in the structures. Each of these effects could alter DNA interactions at the surface.

**Characterization of Zirconium Phosphonate Particles**

Zirconium bisphosphonate and zirconium triphosphonate particles were synthesized using phosphonate precursors methylenediphosphonic acid (MDP) and nitrilotris(methylene)triphosphonic acid (NMTP) (Figure 2-5). NMTP is commonly used as a biological and environmental chelator of metal ions, thus it was expected to form particles when refluxed with ZrOCl₂. The synthesis of Zr-NMTP particles was similar to the ZrPN nanoparticle synthesis. SEM images of Zr-NMTP show particles of
a similar size and shape. Zr-MDP particles vary in size and shape (Figure 2-6). Particles up to 2 μm in diameter were seen in SEM images of Zr-MDP particles.

Zeta potential measurements indicate that the Zr-MDP and Zr-NMTP particles can be functionalized with zirconium ions (Table 2-1). Like with ZrPN, the particles were treated with excess ZrOCl₂ to give a more positive surface charge. Before ZrOCl₂ treatment, Zr-MDP particles had a surface charge of -43 mV and after treatment the surface charge increased to 2 mV. A dramatic change in surface charge was also seen with the Zr-NMTP; before ZrOCl₂ treatment the particles exhibited a surface charge -49 mV and after treatment, the zeta potential increased to 11 mV.

**DNA Binding and Hybridization on Zirconium Phosphonate Particles**

Single-stranded DNA was immobilized to phosphonate particles using the same method as the ZrPN particles; fluorescence images of DNA modified phosphonates paraticles are shown in Figure 2-7. The fluorescence on the Zr-MDP surface showed significant amounts of non-phosphorylated DNA binding to zirconium treated particles, however, there is more fluorescence intensity from the phosphorylated DNA binding to the zirconium treated particles. DNA binding to Zr-MDP not treated with ZrOCl₂ showed some aggregates of particles with non-specifically bound phosphorylated DNA as seen with ZrPN particles (included in Figure 2-7 for comparison). DNA binding to the Zr-NMTP particles showed significant binding of both phosphorylated and non-phosphorylated DNA to zirconium-functionalized particles. There is also binding of the phosphorylated DNA to the non-zirconated particles. Despite this, the fluorescence intensities indicate that there is still more phosphorylated DNA binding with Zr⁴⁺ treated surfaces.
Phosphate-buffered saline and dilute phosphoric acid was used to remove non-specifically bound DNA. The belief here is that in very dilute amounts, phosphate anions would displace any non-specifically bound DNA because it has a greater affinity for the zirconium ions on the surface. This rinse procedure, however, was not as efficient at removing non-specifically bound DNA on the phosphonates particles as it was for ZrPN particles. This could be due to the electronic properties of zirconium binding sites in phosphate vs. phosphonate structures. Rinsing with sodium saline citrate (SSC) was used as an alternative to help improve specificity of phosphorylated DNA on zirconium phosphonate particles. Figure 2-8 shows the fluorescence intensities of DNA bound to ZrPN, Zr-MDP and Zr-NMTP after rinsing with SSC buffer. Using the new rinsing procedure, much of the non-specific DNA binding on the zirconium treated Zr-MDP was eliminated, while maintaining a high specificity for phosphorylated DNA. There is about 10 times more binding of the phosphorylated DNA on Zr\(^{4+}\) treated Zr-MDP than with the other Zr-MDP systems (Figure 2-9B). On Zr-MDP particles with no zirconium pretreatment, the fluorescence was minimal, indicating the need for Zr\(^{4+}\) terminated surfaces for DNA binding. As a comparison, DNA modified ZrPN particles were analyzed after SSC buffer rinsing. Similar results were seen with these particles; there is more phosphorylated DNA binding to the Zr\(^{4+}\) treated ZrPN particles. There is 3 times more fluorescence intensity of Zr\(^{4+}\)-treated ZrPN with phosphorylated DNA than with any of the other ZrPN control experiments (Figure 2-9A). For the Zr-NMTP, there seems to be no specificity of the DNA binding to the surface. After either phosphate or SSC buffer rinsing, there are still large amounts of non-specific interactions of DNA binding on the non-treated surfaces. Although DNA binding to Zr-NMTP exhibits a
similar trend to DNA binding to ZrPN and Zr-MDP particles, the amount of non-specific binding on the surface is significant. The error bars for this system are also very large, indicating that DNA binding is not uniform or controlled using this probe immobilization procedure (Figure 2-9C). For this reason, hybridization studies were not conducted on Zr-NMTP surfaces.

Figure 2-10 shows the CLSM images of the hybridization studies that were conducted on DNA modified ZrPN and Zr-MDP particles after rinses with SSC buffer to remove non-specifically bound DNA. Both DNA-modified ZrPN and Zr-MDP show specificity for target DNA. There is some non-specific binding of the target strand on Zr$^{4+}$ treated surfaces, but the hybridization of phosphorylated DNA on Zr$^{4+}$ treated ZrPN is about three times higher than that of phosphorylated probe DNA on non-treated ZrPN (Figure 2-11A). Likewise, the fluorescence intensity is ten times larger on DNA-modified Zr-MDP particles that were pretreated with Zr$^{4+}$ ions (Figure 2-11B). The control experiments show that the fluorescence intensities of the target DNA on non-treated phosphate and phosphonate particles were very small emphasizing the need for ZrOCl$_2$ pretreatment of the surfaces for DNA-immobilization to occur.

**Discussion**

Previous research in the Talham group suggests that zirconium phosphonate modified surfaces will only form covalent linkages with terminal phosphates (ROPO$_3^{2-}$) on DNA. The same principle applies for DNA binding to ZrPN and Zr-MDP particles. The phosphodiester backbone [(RO)$_2$PO$_2^{-}$] of a DNA molecule is not basic enough to displace oxides and hydroxides that terminate a hydrated zirconium phosphate or phosphonate surface. The resulting zirconium phosphate/phosphonate after DNA immobilization is structurally similar to the layers of the solid state zirconium phosphate.
The phosphate terminated DNA bound to the particles stabilizes the lattice energy associated with the resulting network structure. ZrPN and Zr-MDP particles treated with ZrOCl$_2$ shows specificity for 5'-phosphorylated DNA molecules. Pretreated Zr-NMTP does not show as much specificity for phosphorylated DNA. Phosphorylated DNA binding was significant on Zr-NMTP not treated with ZrOCl$_2$, which is indicative of uncontrolled binding and random orientation of DNA on the surface.

The hybridization experiments determine if probe oligonucleotides maintain activity after immobilization on the particles. Confocal microscopy images give evidence that phosphorylated probe oligonucleotides bound to the Zr$^{4+}$ treated surfaces are functional because more DNA hybridization occurs on these particles. Zr-MDP shows promising results; after DNA modification, this surface is ten times more specific for phosphorylated DNA than non-phosphorylated DNA. The DNA-modified Zr-MDP particles also show ten times more fluorescence intensity after hybridization with the target DNA strand.

**Conclusions**

We have demonstrated that zirconium phosphate and phosphonate particles can specifically bind phosphate-terminated oligonucleotides. The particles are easy and inexpensive to prepare and the synthesis gives high product yields. Oligonucleotide binding of these particles occurs through a coordinate covalent linkage with the naturally occurring terminal 5'-phosphate found on all DNA molecules. Therefore, binding to these particles does not require any modification of the DNA molecule to obtain specific immobilization. After simple rinsing procedures, non-specific interactions of DNA on ZrPN and Zr-MDP are much less compared the amount of specifically bound DNA.
Furthermore, the DNA-modified particles are able to hybridize with its complementary sequence demonstrating that these particles maintain biological activity. All of these factors are advantageous when using DNA-modified materials as tools for affinity chromatography of biomolecules. Because of the specific interactions of DNA with zirconium phosphate and phosphonate particles, these surfaces could potentially be used as resins for sequence-specific affinity chromatography of DNA-binding proteins (Figure 2-12).

![SEM images of α-ZrPN nanoparticles. The particles are about 100 nm in size, which is in agreement with published data.](image)

Table 2-1. Zeta potential measurements of zirconium phosphate and phosphonate particles before and after treatment with ZrOCl₂.

<table>
<thead>
<tr>
<th></th>
<th>ZrPN</th>
<th>Zr-MDP</th>
<th>Zr-NTMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Potential Before ZrOCl₂ Treatment</td>
<td>-32 mV</td>
<td>-43 mV</td>
<td>-49 mV</td>
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<tr>
<td>Zeta Potential After ZrOCl₂ Treatment</td>
<td>13 mV</td>
<td>2 mV</td>
<td>11 mV</td>
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Figure 2-2. Visual representation of the DNA-particles systems studied. These systems help to understand the specificity of DNA binding to the zirconium phosphate or phosphonate surface.

Figure 2-3. CLSM images of ssDNA binding to zirconium phosphate nanoparticles. DNA particles were stored in a dilute HCl solution before DNA immobilization, which kept zirconium functionalized particles from aggregating in solution. After changing the rinsing procedure to remove non-specifically bound DNA, more binding of phosphorylated DNA to zirconium functionalized particles is observed as compared to the non-functionalized particles.

Figure 2-4. CLSM images of hybridization on DNA-modified zirconium phosphate nanoparticles. Particles were imaged with different fluorescence microscopy settings. The single-stranded DNA particles cannot be compared, however,
there is more fluorescence on the zirconium-functionalized particles with phosphorylated DNA indicating more hybridization on these particles.

Figure 2-5. Structures of (A) nitrilotris(methylene)triphosphonic acid and (B) methylenediphosphonic acid. Bisphosphonate and triphosphonate compounds were used to ensure that synthesized materials could be terminated in Zr⁴⁺ ions.

Figure 2-6. SEM images of Zr-MDP (A) and Zr-NMTP (B). Both Zr-MDP and ZrNMTP particles are about 100 nm, however, some bigger particles were observed in other SEM images.
Figure 2-7. Single-stranded DNA immobilized to zirconium phosphate and phosphonate particles. There is more non-phosphorylated DNA binding on zirconium functionalized phosphonate particles indicating non-specific binding of DNA through phosphodiester backbone. On all surfaces, the greatest amount of binding occurred when on zirconium-functionalized particles was treated with phosphorylated DNA.

Figure 2-8. Single-stranded DNA on the particles after using SSC buffer. Instead of PBS and H$_3$PO$_4$, SSC buffer was used to rinse non-specifically bound DNA. With this treatment there is less non-specifically bound DNA on the Zr-MDP particles. There is still more DNA binding to Zr-NMTP particles indicating that the phosphorylated DNA is not as specific for Zr-NMTP as with the ZrPN and Zr-MDP.
Figure 2-9. Quantification of ssDNA binding to (A) ZrPN, (B) Zr-MDP, and (C) Zr-NMTP. For both the ZrPN and Zr-MDP, there is ten times more binding of phosphate-terminated DNA to zirconium treated particles. For Zr-NMTP, there is a significant amount of non-specific binding of phosphate modified DNA on particles that have not been treated with Zr$^{4+}$ ions. The error bars in this system are large indicating that DNA binding on Zr-NMTP is not uniform or controlled. All calculations are background corrected.
Figure 2-10. Hybridized DNA on particles on ZrPN and Zr-MDP particles. There is some non-specific binding on the zirconium functionalized particles, but the more binding was seen on the particles treated with phosphorylated DNA.
Figure 2-11. Quantification of DNA hybridization on (A) ZrPN and (B) Zr-MDP. For ZrPN, there is 2.5 times more hybridization at Zr$^{4+}$ treated surfaces modified with phosphorylated DNA as compared to non-phosphorylated DNA. This implies there is non-specific binding of the target DNA to the Zr$^{4+}$ treated surfaces. For the Zr-MDP surface, there is 10 times more hybridization to the Zr$^{4+}$ treated surfaces modified with phosphorylated DNA as compared to non-phosphorylated DNA. Furthermore, there is little to no hybridization on surfaces not treated with Zr$^{4+}$ ions. All calculations are background corrected.
Figure 2-12. Proposed scheme for protein immobilization to DNA-modified zirconium phosphate or phosphonate nanoparticles. Particles are first functionalized with dsDNA and then added to a complex protein mixture. After non-specifically bound proteins are washed away, the DNA binding protein remains bound to the DNA modified particles through the dsDNA until they are eluted.
CHAPTER 3
PHOSPHOPEPTIDE BINDING TO ZIRCONIUM PHOSPHATE AND ZIRCONIUM PHOSPHONATE NANOPARTICLES

Introduction

Phosphorylation is an important post-translational modification in eukaryotic cells in which a serine, threonine or tyrosine residue is phosphorylated by a protein kinase. This modification is involved in many regulatory mechanisms such as gene expression, signal transduction, enzyme activity, and cell division. The development of sensitive and selective methods to quantify and characterize protein phosphorylation sites is needed because phosphopeptides are typically present in lower abundance than other peptides.

Fe(III)-immobilized metal affinity chromatography (IMAC) is a commonly used technique for phosphopeptide selection. With this technique, Fe$^{3+}$ ions are immobilized to a support matrix and phosphoproteins will displace the water on Fe(III) binding sites (Figure 3-1). One of the downfalls of Fe(III)-IMAC is that peptides can non-specifically absorb to the Fe$^{3+}$ binding sites mainly through carboxylate, thiol, amine and imidazole functional groups on amino acid side chains. The use of metals with a higher affinity for phosphate groups has greatly improved metal-IMAC techniques for phosphopeptide enrichment. Metal (IV) ions such as Ti (IV) and Zr (IV) have a strong affinity for phosphorylated molecules. ZrO$_2$ and TiO$_2$ were found to be more selective for phosphopeptides over acidic peptides as compared to Fe(III)-IMAC with the use of hydroxyl acids in the loading and washing buffers. A Zr(IV)-IMAC technique was developed by Zou and coworkers where Zr$^{4+}$ ions were bound to phosphonate-modified polymer beads. The high specificity of this absorbent was demonstrated by effectively enriching phosphopeptides from a digested mixture of phosphoprotein (α or
β-casein) and bovine serum albumin (BSA) at a molar ratio of 1:100.\textsuperscript{68,80} Alternatively, instead of using zirconium phosphonate modified beads, Zare and coworkers successfully enriched phosphopeptides with crystalline α-zirconium phosphate with higher sensitivity and lower detection limits compared to Fe(III)-IMAC, TiO\textsubscript{2}, or Zr(IV)-IMAC.\textsuperscript{81}

Recently in the Talham group, the Zr\textsuperscript{4+}-phosphonate interaction has been used to specifically anchor oligonucleotides, phospholipids, and other phosphorylated molecules to solid supports for bioanalytical studies.\textsuperscript{32-39,133} The current investigation extends these studies to zirconium phosphonate particles as a tool for phosphorylated peptide enrichment. These materials could lead to increased phosphopeptide binding capacity or enhanced phosphopeptide selectivity. The distinctive coordination of zirconium with phosphate may greatly improve selectivity of phosphopeptides by preventing acidic peptide binding over other common peptide enrichment media, such as Fe-IMAC and zirconium or titanium oxides.\textsuperscript{68}

Mass spectrometry (MS) is used to examine phosphopeptide enrichment due to sensitivity and speed of the analysis, which allows high-throughput investigations. In a typical analysis, protein samples are enzymatically digested into peptides for MS analysis. After digestion, phosphorylated peptides are present in very low concentrations because of the overwhelming amounts of non-phosphorylated peptides in the solution. After the digested peptide solution is treated with enrichment media, the enriched phosphorylated peptides are eluted and analyzed.

Figure 3-2 shows the general scheme of phosphopeptide enrichment on zirconium phosphonate particles. The analysis methods used in these experiments was matrix
assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or high performance liquid chromatography with negative-mode electrospray ionization mass spectrometry (HPLC/(-)ESI-MS). Zirconium phosphate particles (ZrPN) have been successfully used for phosphopeptide enrichment without the need for dihydroxybenzoic acid, which has been shown to help prevent binding of non-specific amino acids on enrichment media. Significant amounts of phosphopeptides were enriched with zirconium methylenediphosphonate particles (Zr-MDP) and zirconium nitrilotris(methylene)triphosphonate particles (Zr-NMTP). The ability to selectively bind and elute biomolecules is an important aspect of affinity chromatography media.

**Experimental Section**

**Materials**

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Deionized water used for all experiments was purified with Barnstead E-Pure purification system (Thermo Fisher Scientific, Waltham, MA).

**Tryptic Digestion of Bovine β-Casein**

One hundred micrograms of Promega modified trypsin was reconstituted in 100 μl of 50 mM acetic acid. A 3.2 mg/ml mother solution bovine β-casein was prepared by adding 1.6 mg of β-casein to 500 μl of 100 mM ammonium bicarbonate. The digest was prepared by adding 2.5 μl of the reconstituted trypsin to 31.5 μl of the bovine β-casein mother solution. The digest was diluted to 100 μl with ammonium bicarbonate and sat for 18 hours at room temperature. The final concentration of β-casein in the tryptic digest was 1 mg/ml.
Particle Synthesis

Zirconium phosphate nanoparticles (ZrPN), zirconium methylenediphosphonate (Zr-MDP) and zirconium nitrilotris(methylene)triphosphonate (Zr-NMTP) particles were prepared as described in Chapter 2.

Initial Experiments for Capture of Phosphopeptides Using ZrPN

The experimental procedure was taken from Xu et al. with slight modifications.\textsuperscript{81} The peptides analyzed in this experiment come from bovine β-casein, a well-characterized phosphoprotein that can be digested with trypsin to form phosphorylated peptides. Beta casein is routinely used to test new phosphopeptide enrichment procedures because the digests are well-studied and easy to prepare; the lack of cysteine residues make the digest protocol extremely simple for this protein because there is no need for reduction and alkylation steps before treatment with trypsin. The amino acid sequence of β-casein is shown in Figure 3-3. Trypsin cleaves proteins after arginine (R) and lysine (K) residues. With the enzyme to substrate ratio used in this experiment (1:40), the most abundant phosphopeptide of β-casein after tryptic digestion has molecular weight of 2062 g/mol. Higher amounts of trypsin in the digest give a faster and more complete digestion. The peptide is seen in all the mass spectrometry experiments reported in this chapter. However, there are other phosphopeptides of β-casein that have been reported but were not seen in the experiments discussed in this dissertation (Table 3-1).

A 10 μl volume of the tryptic digest of β-casein (0.1 mg/ml) was diluted with a 90-μl solution of 80% acetonitrile (ACN) containing 5% trifluoroacetic acid (TFA) as a loading solution. The loading solution (8 μl) was mixed with 10 μl of the ZrPN suspension (5 mg/ml in 80% ACN containing 0.1% TFA), and then 200 μl of 80% acetonitrile
containing 5% TFA was added. This allows the phosphopeptides of the β-casein to bind to the nanoparticles. After incubation for 30 min with shaking, the mixture was centrifuged at 13200g for five minutes and the supernatant removed to remove non-specifically bound peptides. The pelleted ZrPN were further washed with 100 μl of 80% ACN/5% TFA once, followed by 80% ACN containing 0.1% TFA twice. Finally, the trapped phosphopeptides on the ZrPN were released by eluting with 10 μl of a 1:1 ACN:water solution containing 0.1 M ammonia hydroxide in a bath sonicator for 2h. One microliter of the eluted phosphopeptide solution was added to 9 μl of the matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 80% ACN with 0.1% TFA). One microliter of this mixture was spotted on a gold MALDI plate and dried at room temperature before MALDI-TOF MS/MS analysis.

Capture of Phosphopeptides after Enrichment with ZrPN, Zr-MDP and Zr-NMTP Particles

A 50 μl volume of the tryptic digest of β-casein (0.5 mg/ml) was diluted with a 50 μl solution of 80% acetonitrile (ACN) containing 5% TFA as a loading solution. The loading solution (8 μl) was mixed with 10 μl or 50 μl of the ZrPN, Zr-MDP, or Zr-NMTP particle suspension (5 mg/ml in 80% ACN containing 0.1% TFA), and then 200 μl of 80% ACN containing 5% TFA was added. After incubation for 30 min with shaking, the mixture was centrifuged at 13200g for 5 min, and the supernatant removed to remove non-specifically bound peptides. The pelleted particles were further washed with 100 μl of 80% ACN solution containing 5% TFA once. The particles were centrifuged at 13200g for 5 minutes and the supernatent was removed. The particles were further washed with a solution of 80% ACN containing 0.1% TFA twice. After each wash, the particles were centrifuged at 13200g for 5 minutes. Finally, the trapped phosphopeptides on the
particles were released by eluting with 1:1 ACN:water solution containing 1.5% H₃PO₄ (10 μl) in a bath sonicator for 2h. The solution was centrifuged at 13200g and the supernatent was collected. This elution step had been optimized for the phosphopeptide release from Fe³⁺-IMAC; the combination of phosphoric acid and acetonitrile generated an excellent IMAC eluent for subsequent MALDI- or ESI-MS analysis. The samples prepared in this experiment were analyzed by HPLC/(-) ESI-MS analysis. The results were compared to a standard consisting of 8 μl of loading solution with 2 μl 1:1 ACN:water solution containing 1.5% H₃PO₄.

**Mass Spectrometry Analysis**

Basri Gulkaban, graduate student at the University of Florida, did MALDI-TOF experiments on an ABI 4700 Proteomics analyzer (Applied Biosystems, Framingham, MA, USA) equipped with an Nd:YAG laser (355 nm, 3- to 7-ns pulses) was operated in the reflector, positive-ion mode. The acceleration voltage was 20 kV. The laser was operated at a fixed fluency approximately 5% above the threshold for ionization; its rate was 200 Hz and the laser-firing pattern was set to “uniform.” Both sample plate and laser were aligned before spectral acquisition. For each spot, spectra were obtained from 1000 laser shots (40 subspectra in different positions, 25 shots per subspectrum) and averaged to form a single spectrum. Spectra were smoothed by “noise filter” and baseline corrected with Data Explorer 4.0.

Dr. Jodie V. Johnson analyzed samples on HPLC/(-) ESI-MS. Samples were analyzed when the effluent from the HPLC column (Phenomenex, Torrence, CA) was introduced to a ThermoFinnigan (San Jose, CA) quadrapole ion trap mass spectrometer (LCQ) via reverse phase gradient C18 HPLC (Agilent, Palo Alto, CA) with negative mode electrospray ionization. An HPLC 1100 series binary pump was used with water
and methanol mobile phases and flow rate of 0.2 ml/min. ESI parameters: sheath gas (N2) = 65; aux gas (N2) = 3; spray voltage = 3.2 kV; cap temp = 250 C; cap volt = -16 V; tube lens offset = 0 V.

Results

To characterize enrichment, MALDI-TOF MS analysis was done on a protein solution before and after treatment with zirconium phosphate nanoparticles. Bovine β-casein (MW = 23.6 kDa), a standard protein consisting of well-characterized phosphopeptides, was enriched with zirconium phosphate as per the procedure adapted by Xu et al. Figure 3-4 shows the spectra of tryptically-digested β-casein before and after enrichment with zirconium phosphate nanoparticles. The MS analysis shows enrichment of phosphopeptides from the digested solution. In the mass range examined, only the phosphopeptide with m/z = 2062 is expected to be seen. There are other peaks at m/z = 1660 and m/z = 1951 peaks due to presence of phosphopeptide impurities from the alpha form of casein. The amino acid sequences of the phosphorylated peptides seen in the MALDI MS spectrum are shown in Table 3-1.

HPLC/(-) ESI-MS was also used to analyze phosphopeptide enrichment on zirconium phosphonate surfaces. 760 nM of β-casein was enriched with 10 μl and 50 μl of ZrPN, Zr-MDP, and Zr-NMTP (5 mg/ml). With the MALDI experiments, 15 nM β-casein was treated with 10 μl ZrPN; when the same procedure was used for LC-MS analysis with phosphate and phosphonate particles, peaks at m/z = 2062 were too weak to be accurately detected. The chromatogram peak corresponding to m/z = 2062 was seen when the concentration was increased. The mass detected for this peptide was at m/z ~1030, which corresponds to the double-negatively charged phosphopeptide, [M-2H]²⁻ with M = 2062.
Figure 3-5 shows the singly phosphorylated peptide of β-casein before enrichment (A) and after enrichment with 50 μl or 10 μl ZrPN (B and C), Zr-MDP (D and E) and Zr-NMTP (F and G). The percentage of recovered phosphopeptide is taken from the peak area of each enriched spectra and compared to the peak of unenriched β-casein. For each volume of particles used, the highest percentage of recovered peptide was seen with the ZrPN. After enrichment with 50 μl of Zr-NMTP, 87% of phosphopeptides were recovered. There was about a two-fold decrease in the recovery after enrichment with 10 μl of Zr-NMTP. The amount of recovered phosphopeptides after enrichment with 10 μl of Zr-MDP was greater than after enrichment of 50 μl of the particles. The retention time of the peak (BP = 1029.8) was shifted from ~29 minutes to ~23 minutes because a different gradient was used, however, the peak area is corrected for the m/z = 1030.

Discussion

Fe(III)-IMAC media are known to non-specifically adsorb peptides mainly through carboxylate, thiol, amine and imidazole functional groups from amino acid side chains; these interactions compete with phosphate groups on phosphopeptides for Fe(III) binding sites.\(^{61, 66-68}\) Dihydroxybenzoic acid, DHB (Figure 3-6A), is known to reduce the binding of nonphosphorylated peptides by competing with these peptides for metal-binding sites on enrichment media. DHB will displace non-specifically bound peptides, however, it cannot displace immobilized phosphopeptides because phosphate groups have a higher affinity for Fe\(^{3+}\) ions.\(^{74}\) DHB is usually added to the solution used to elute phosphopeptide from the support matrix. Also, DHB is a commonly used matrix for MALDI mass spectrometry, which makes it an ideal reagent for analysis of phosphopeptide enrichment with MALDI-TOF-MS. Original experiments of phosphopeptide enrichment with zirconium phosphate particles performed by Xu et al.
used DHB in elution buffer. However, attempts to repeat this experiment in this lab proved unsuccessful because DHB completely saturated the MALDI MS signal, attenuating the phosphopeptide signal. Instead of using DHB, phosphopeptides were washed with common solvents (ACN:TFA:water) and eluted with dilute phosphoric acid. The MALDI matrix, α-cyano-4-hydroxycinnamic acid (Figure 3-6B) was added to the already-enriched protein solution for MS analysis. Using this method, phosphopeptides from α- and β-casein were observed. This suggests that there may not be a need for a competitive binder such as DHB that competes with acidic peptides for binding sites on the particles. Phosphate groups on the peptides are more specific for zirconium phosphate particles because these groups are able to displace surface oxide and hydroxide to form covalent linkages to the Zr⁴⁺ ions (Figure 1-5). Carboxylate functional groups on acidic peptides cannot displace oxide and hydroxide; thus, they can only participate non-specific electrostatic interactions on zirconium phosphate surfaces. Any non-specifically bound proteins are removed from particles with common solvent rinses. This behavior is similar to the DNA-modified particles discussed in Chapter 2. On the same particles, non-specifically adsorbed DNA was easily removed with dilute phosphoric acid or sodium saline citrate buffer, which again emphasizes the specificity of phosphate molecules with zirconium binding sites on phosphate and phosphonate surfaces.

A singly phosphorylated peptide of β-casein (m/z = 2062) can be enriched with zirconium phosphate and phosphonate and detected with HPLC/(-)ESI-MS. The tetraphosphorylated peptide (RELEELNVPGEIϕeVESLPϕSPϕSϕSPϕSEESITR, m/z = 3121) of β-casein was also monitored with HPLC/(-) ESI-MS, however, no signal corresponding
to this peptide could be detected. This could be because LC-ESI-MSMS shows known bias towards monophosphorylated peptides. The recovery of phosphopeptides from the zirconium phosphate and phosphonate lays emphasis on the fact that these particles could be used for affinity chromatography techniques. Phosphopeptides are eluted with dilute phosphoric acid. After peptide elution, the particles could be regenerated with Zr$^{4+}$ for another round of enrichment experiments, similar to other IMAC techniques.

**Future Work**

The purpose of this project is to explore the limits of zirconium phosphate and zirconium phosphonate to significantly enhance phosphopeptide enrichment strategies. Using the knowledge gained from previous research in the Talham group, we would like to use this class of materials to analyze, understand, and control peptide affinity at the zirconium phosphonate surface. The future goals for this project include determining the limits of detection of phosphopeptide enrichment with zirconium phosphonate particles. Zare and coworkers were able to detect phosphopeptides in a 1:2000 dilution using zirconium phosphate. Using TiO$_2$, casein phosphopeptides were only detected in dilutions of approximately 1:50 and 1:100. After determining the sensitivity of the phosphopeptide enrichment with zirconium phosphonate particles, the next step will be to quantify the efficiency of peptide recovery.

Other work with this project should be directed towards understanding the thermodynamics of phosphorylated protein binding to the zirconium phosphate and zirconium phosphonate particle surfaces. Duff and Kumar recently published a report on the thermodynamics of positively and negatively charged protein binding to negatively charged $\alpha$-zirconium phosphate surfaces. In their studies, zirconium
phosphate was synthesized using a method similar to the one described in this dissertation (Chapter 2), however zirconium phosphate surfaces were not terminated in Zr⁴⁺ ions. As discussed in Chapter 1, α-zirconium phosphate crystals are layered structures with each zirconium atom sharing six oxygen atoms, all from different phosphates groups.⁸²,⁸³ In turn, each phosphate shares three oxygen atoms with three different zirconium atoms. The fourth oxygen carries a proton which can be dissociated at neutral pH.¹³⁴ Using solution based studies with isothermal titration calorimetry (ITC), Duff and Kumar concluded that protein binding is coupled to proton release or absorption on zirconium phosphate surfaces because the binding of charged proteins to charged surfaces alters the pKₐ of ionizable groups at the protein surface.¹³⁴ Upon binding of positively charged proteins to negatively charged zirconium phosphate surfaces, the solution pH is altered and subsequently, the pH of the solution needs to be re-adjusted for further studies.¹³⁴ In turn, the binding of negatively charged proteins to the zirconium phosphate surfaces requires charge neutralization at the protein-solid interface by either the adsorption of counter ions to the surface (acquired in the buffer solutions), by protonation of polar groups from side chains of the amino acids adsorbed at the interface or by some combination of the two events.¹³⁴ Unfortunately, only electrostatic interactions of protein binding is discussed in this study; it did not include phosphorylated protein binding to zirconium phosphate surfaces. Future experiments of phosphopeptide enrichment studies on zirconium phosphate and phosphonate surfaces in our work will go towards understanding the thermodynamics of coordinate covalent bonding of dibasic phosphates to zirconium phosphate and zirconium phosphonate surfaces. In conjunction with data acquired in the work of Duff and Kumar, the
thermodynamics of specific vs. non-specific interactions of phosphopeptide binding to zirconium phosphate and zirconium phosphonate surfaces can be better understood.

Figure 3-1. Fe(III)-IMAC support medium with iminodiacetic acid as the metal chelating ligand. Peptides displace water molecules on the Fe$^{3+}$ binding sites. Peptides are adsorbed to the support mainly through carboxylate, thiol, amine and imidizole amino acid side chains.

Figure 3-2. General scheme of phosphopeptide enrichment with zirconium phosphate or phosphonate particles. In this study, Zirconium phosphate nanoparticles, zirconium methylenediphosphonate and zirconium nitrilotris(methylene)triphosphonate particles are used to enrich phosphopeptides from a β-casein digest.
Figure 3-3. Protein sequence of bovine β-casein using 1-letter abbreviation codes for the amino acids. The protein consists of 224 amino acids. The O-phosphorylation sites are underlined.

Figure 3-4. MALDI mass spectra of tryptic digested β-casein with before enrichment with zirconium phosphate nanoparticles (top) and after treatment with zirconium phosphate nanoparticles (bottom). The asterisks mark
phosphopeptides at m/z 1660 and 1951, which are known phosphopeptides from α-casein.

Table 3-1. Amino acid sequence of the phosphopeptides observed in the MALDI MS after enrichment of tryptically-digested β-casein with zirconium phosphate nanoparticles. The peptides marked with (*) are phosphopeptides of β-casein not observed in our analysis. The peptides marked with (**) are observed in our analysis and are thought to be due to impurities of α-casein.

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</tbody>
</table>
Figure 3-5. HPLC/(-)ESI-MS chromatograms of (A) singly phosphorylated peptide of β-casein peptide (MW = 2062) with no enrichment and after enrichment with (B) 50 μl and (C) 10 μl of ZrPN, (D) 50 μl and (E) 10 μl of Zr-MDP, and (F) 50 μl and (G) 10 μl of Zr-NMTP. The BP reported is the doubly negative ion (MW = 1030). The label on each chromatogram gives the percentage of the amount of recovered phosphopeptide after enrichment with each zirconated material.
DHB is known to reduce the binding of nonphosphorylated peptides by competing with acidic peptides for binding sites on the particles. DHB is also a common MALDI matrix. Original experiments of phosphopeptide enrichment with zirconium phosphate particles done by Xu et al. used DHB in washing buffer. However, attempts to repeat this experiment with DHB done in this lab proved unsuccessful. After enrichment and elution of phosphopeptide with no hydroxyl acid wash, α-cyano-4-hydroxycinnamic acid was used as a MALDI matrix. Phosphopeptide enrichment using this method was successful.
CHAPTER 4
PART I: METALLATED PHYTIC ACID AS A NOVEL WAY TO IMMOBILIZE PROTEINS
ON ZIRCONIUM PHOSPHONATE SURFACES

Introduction

One of the goals of the Talham group is to research and design novel ways to immobilize biomolecules to inorganic surfaces. Recent studies have shown that DNA binds directly to zirconium phosphonate modified surfaces through a naturally occurring 5’ terminal phosphate and it was demonstrated that this interaction can be used to immobilize oligonucleotide probes for DNA arrays. Furthermore, the zirconium phosphonate DNA arrays can be used to capture DNA-binding proteins. The next step in this research is to extend this surface chemistry for the capture of non-DNA binding proteins. A common way to specifically orient and capture proteins to surfaces is by using a technique called immobilized metal affinity chromatography (IMAC). This technique heavily relies on metal-ligand interactions between surface bound transition metals and ligating groups on the protein; IMAC is particularly successful when an appropriate affinity tag is used. A common affinity modification is a hexahistidine tag (histag), which consists of six consecutive histidine residues genetically engineered on proteins of interest. Previous attempts to bind histagged proteins directly to zirconium phosphonate surfaces proved unsuccessful due to the fact that the imidazole functional groups of histagged proteins are not basic enough to displace oxides and hydroxides that are coordinated to the zirconium on the surface. However, these proteins can chelate with many divalent and trivalent first-row transition metals immobilized to solid supports. A commonly used solid support for protein capture is a Ni-NTA modified surface (Figure 1-6B where Mn⁺ = Ni²⁺). The purpose of this project is to determine if protein immobilization chemistry can be accomplished
using a ligand that binds both the zirconium phosphonate surface and divalent metal ions to provide sites for histag coordination. The ligand used in these experiments was myo-inositol hexakisphosphate, also known as phytic acid or IP₆ (Figure 4-1).

Surface enhanced Raman spectroscopy studies showed that phytic acid forms monolayers on metal surfaces. At pH 5, the phytic acid molecules are assumed to be immobilized on metal surface via four coplanar phosphates to form self-assembled monolayers (SAMs). At pH 11.27, it is assumed that only one phosphate from each phytic acid molecule is adsorbed onto the iron surface. The same research group investigated IP₆ monolayers on silver surfaces and found conflicting results: at pH 13, phytic acid molecules are immobilized to the surface via four coplanar phosphates, whereas at pH 1.27, phytic acid absorbs to the surface through only one phosphate group. At pH 11.30, phytic acid molecules were chemically adsorbed onto the freshly exposed copper surface to form ordered monolayers through two co-planar phosphates. Furthermore, calorimetric and titrimetric studies have shown that phytic acid forms soluble complexes with such divalent and trivalent metal ions as Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺ and Fe³⁺. Histagged proteins can form soluble chelates with many of these metal ions but the ones most often used are Ni²⁺, Co²⁺, and Cu²⁺ ions. A schematic representing the potential binding scheme of phytic acid to zirconium-ODPA surface and it subsequent immobilization of histagged protein via a metal chelate is shown in Figure 4-2.

We proposed using phytic acid to modify zirconium phosphonate surfaces for histagged protein immobilization. The six phosphate groups on the phytic acid will allow the molecule to form layers on surface bound zirconium. After charging the phytic acid-
modified surfaces with metal ions, it was expected that the surface could then provide specific orientation of histagged proteins. The results show that although there is protein binding on the metal-phytic acid modified surface, the amount of bound protein is significantly less than the amount on commercially available slides. Metal-phytic acid complexes can bind to zirconium phosphonate surfaces and these surfaces are stable in water. However, after the surfaces are subjected to rinsing with common protein buffers such as tris-buffered saline (TBS), phytic acid molecules are removed from the zirconium phosphonate support.

In this chapter, the best deposition method of metal-phytic acid chelates on zirconium-ODPA surfaces is discussed. Next, using AFM and XPS, the most appropriate chelating metal for this experiment is determined. Finally, the amount of protein bound to metal-phytic acid is determined using fluorescence microscopy and the results are compared to protein binding on a commercially available Ni-NTA surface.

**Experimental Section**

**Materials**

Glass substrates were purchased from Gold Seal Products (Portsmouth, NH). Phytic acid dodecasodium salt, copper (II) nitrate, cobalt (II) nitrate, and nickel (II) nitrate were purchased from Sigma-Aldrich (St. Louis, MO). Iodoacetamide fluorescein was purchased from Molecular Probes (Carlsbad, CA). Microdialysis tubes used for removing excess iodoacetamine fluorescein label were purchased from Pierce (Rockford, IL). Gail E. Fanucci at the University of Florida generously donated histagged IA3 protein.
Zirconium Phosphonate Substrates

Glass slides were made hydrophobic using octadecyltrichlorosilane (OTS) following a method by Sagiv. Briefly, the glass slides were cleaned with piranha etch (3:1 H₂O₂:H₂SO₄) and followed by the RCA cleaning method (4:1:1 H₂O:NH₄OH:H₂O₂ then 5:1:1 H₂O:HCl:H₂O₂). For each step, the solutions boiled for 20 minutes. The slides were made hydrophobic by treating twice with a 5 mM solution of octadecyltrichlorosilane (OTS) in filtered bicyclohexyl for 2 minutes, rinsing with toluene for 30 seconds and then drying with N₂.

The zirconium phosphonate films were prepared by a Langmuir-Blodgett film transfer technique. The films were prepared by first spreading a 0.3 mg/ml solution of ODPA in chloroform on a 2.6 mM aqueous CaCl₂ subphase buffered to pH 7.8 with KOH. The ODPA was compressed at the rate of 10 mm/min to a pressure of 20 mN/m. Once the target pressure was reached, a hydrophobic glass slide was dipped into the subphase a rate of 8 mm/min. The slide was then lowered into a glass vial in the trough. The vial was removed from the trough and about 2 ml of 5 mM of zirconyl chloride (ZrOCl₂) solution was added. The slides sat for 7 days in the Zr⁴⁺ solution, rinsed thoroughly, and then stored in water. A schematic showing the deposition procedure for the preparation of zirconium octadecylphosphonate monolayer is shown in Figure 4-3.

Surface Preparation

Complex Deposition Method: Metal-phytic acid slides were prepared by submerging a fresh zirconium phosphonate slide in a solution consisting of 1 mM IP₆ (pH 5) and 0.3 mM Cu(NO₃) solution in DI water (pH ~ 5). The length of time for
deposition ranged from 1-9 hours depending on the experiment. Afterwards, slides were rinsed with water and dried with nitrogen.

Sequential Deposition Method: Zirconium phosphonate monolayer slides were deposited into a solution of phytic acid (pH 5) in water for 1 hour and then rinsed with nanopure water. Next, phytic acid-modified slides were deposited into a metal nitrate solution (Cu$^{2+}$, Ni$^{2+}$, or Co$^{2+}$) for 1 hour and then rinsed with water and dried with nitrogen.

Commercial Surface (for comparison to metal-phytic acid surfaces): Ni-chelate slides were used as received from Xenopore (Hawthorne, NJ). The slides were reused for further protein binding studies after a restoration treatment. The restoration procedure included washing the spotted slides in a 1 M EDTA solution for 4 hours to remove Ni$^{2+}$ ions and any bound protein molecules. Afterwards, the slides were washed with water and placed in a 1 mM NaOH solution for 5 minutes to deprotonate the NTA groups on the surface. The slides were immediately transferred to a 200 mM NiSO$_4$ solution for 1 hour. After thorough rinses with water and drying with N$_2$ gas, the slides were ready for protein immobilization experiments.

Only phytic acid modified slides were analyzed with XPS. Both phytic acid modified surfaces and commercially available slides were used for fluorescence microscopy studies.

**Protein Purification**

Expression and purification of the IA3 protein was done in the lab of Gail Fanucci with the help of Natasha Hurst, graduate student in the Fanucci lab at the University of Florida. Site-directed mutagenesis was done to mutate the 57$^{th}$ amino acid (tyrosine) of wildtype IA3 to a cysteine. The mutated construct is abbreviated as Y57C. The
QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) was used for this experiment. The primers used in the site-directed mutagenesis are:

Forward: 5'-CTATCAGGAGCAATGCAACAAGCTCAAAGG -3'
Reverse: 5'-CCTTTGAGCTTGTTGCATTGCTCCTGATAG -3'

The bacterial host strain for expression was BL21-CodonPlus (DE3) RIL competent cells (Stratagene, La Jolla, CA). Cells were grown at 37°C in M9 medium to an OD₆₀₀ of ~0.6 before induction with 1 mM IPTG. After expression had been carried out for 3 h, the cells were harvested by centrifugation and lysed by sonication. The soluble recombinant protein was purified using affinity chromatography and eluted in 50 mM Na₃PO₄ and 300 mM NaCl, pH 4.

**Fluorescent Labeling**

Protein was labeled on the mutated cysteine residue with 5-iodoacetamide fluorescien, 5-IAF, (Molecular Probes) in buffer (10 mM Tris, 50 mM NaCl and 1 mM DTT, pH 7.5). Excess fluorescent label and DTT were removed and the protein buffer was exchanged into a desalting buffer (50 mM tris, 250 mM NaCl, pH 7.5). The concentration of the labeled protein was analyzed using a Modified Lowry Assay Kit (Pierce). Protein concentrations were compared to a BSA standard curve at 550 nm and 750 nm. The amount of labeled protein was then determined by:

\[
\% \text{ labeled protein} = \frac{(MW_{\text{protein}} \times A)}{(\varepsilon_{\text{IAF}} \times C_{\text{protein}})}
\]

where \( A \) = absorbance of the fluorophore at its excitation wavelength (492 nm)

\( \varepsilon \) = extinction coefficient of the fluorophore 5-IAF (78,000 M⁻¹cm⁻¹)
C = concentration of the protein solution as found by the Lowry Assay.

The best confocal microscopy results were obtained when 23% or more protein was fluoroestently labeled with 5-IAF.

UV-Vis was used to show that the absorbance band of 5-IAF labeled IA₃ at 492 nm does not overlap with 550 nm, ensuring that the absorbance of the fluorophore was not contributing to values used in determining the concentration of the labeled protein during the Lowry Assay experiments.

**Protein Immobilization Studies**

Various concentrations of fluorescent-labeled IA3 protein in spotting buffer (50 mM tris base, 250 mM NaCl, pH 8) were spotted on metal-phytic acid modified zirconium phosphonate slides overnight at 4°C. Slides were rinsed three times in tris-buffered saline (TBS: 20 mM tris base, 50 mM NaCl, 50 mM KCl, pH 8) and water to remove non-specifically bound proteins. Slides were dried with nitrogen for analysis with confocal laser scanning microscopy (CLSM).

**Instrumentation**

A KSV 2000 LB double-barrier Teflon trough was used to form ODPA monolayers at the air-water interface. A filter paper Wilhelmy balance attached to a KSV microbalance was used to measure surface pressure.

XPS was performed using a UHV XPS/ESCA PHI 5100 system. Survey scan and multiplex scans (Zr 3d, P 2p, N 1s and Cu) were taken with a Mg or Al Kα x-ray source using a power setting of 300 W and a takeoff angle of 45° with respect to the surface. For samples with copper or nickel ions, the Mg x-ray source was used. For samples with cobalt ions, the Al x-ray source was used. Survey scans were taken for all samples with a pass energy of 89.4 eV and multiplex scans were taken with a pass energy of
22.36 eV. Using commercial XPS analysis software and Shirley background subtraction, the peak areas were determined. Elemental ratios reported are averages of at least three experiments.

Tapping-mode AFM was carried out on air-dried samples using a Multimode AFM with a Nanoscope IIa controller (Digital Instruments, Santa Barbara, CA) and commercially available silicon cantilever probes (Nanosensors, Phoenix, AZ). The thickness of the surface was estimated using AFM. The average surface height deviations measured from the mean plane (mean roughness) was also reported. All thickness and roughness data reported the average of at least three measurements from at least three different areas on the same surface.

Fluorescent spots were imaged with an Olympus 1X81 (Melville, NY) confocal laser scanning microscope (CLSM). Slides were scanned with 488 nm laser for FITC labeled proteins. The PMT was set to 785x at 100% laser intensity. 5-Iodoacetamidofluorescein (5-IAF) has an excitation wavelength of 494 nm and an emission of 515 nm, therefore the 5-IAF-labeled IA3 protein was excited with 488 nm Argon laser and emission was detected at all wavelengths greater than 505 nm with an 505 nm band pass interference filter (505IF). Quantification of the fluorescence intensities of bound protein was done using the ImageJ software (freely available from the NIH). The average and standard error of at least three DNA binding experiments for each system was determined after background subtraction.

**Results**

**Complex vs. Sequential Binding of IP6**

The first issue to address was determining the best way to bind phytic acid to zirconium phosphonate modified surfaces. Two approaches were considered: (1)
binding of a metal/phytic acid complex onto the slide or (2) a sequential deposition in, first, phytic acid and then in a metal ion solution.

Table 4-1 summarizes the XPS data for the key elements on the zirconated surface, using the Zr (3d), P (2p3), and Cu (2p3) signals. Each of these surfaces shows an increase in phosphorus on the surface suggesting that there is binding of phytic acid on the zirconium phosphonate surface. For a bare zirconium phosphonate monolayer surface, the P/Zr ratio is about 1. For the surface on which the copper phytic acid (CuIP₆) complex was deposited, there is over twice as much P as Zr in addition to a significant amount of copper. This indicates that not only did phytic acid bind, but there may be at least a monolayer of phytic acid on the surface. On the surface prepared by sequential deposition, there is also phytic acid on the surface because the P/Zr ratio has increased to 1.7. The immobilized phytic acid is binding the deposited Cu²⁺ anions. A full self-assembled monolayer of a molecule with one phosphorus atom would be expected to have about a P/Zr = 2. Phytic acid has six phosphorus atoms per molecule; if it self-assembles on the surface, the ratio would be greater than 2.

A stability test was done on the surface that was modified with the Cu/phytic acid complex. After the complex was deposited, the slide was left in water overnight and then analyzed with XPS. There is still much more phosphorus on the surface compared to zirconium (P/Zr = 1.8). This shows that the phytic acid modified surfaces are stable after constant exposure to water.

It has been suggested that in concentrations of 10 mM and greater phytic acid forms dinuclear complexes with itself.¹³⁶,¹³⁷ Thus, to eliminate this variable, only concentrations less than 10 mM were used for these experiments. Also, during the
complex deposition method, it is possible that metal-phytic acid complexes could form such that the metal is not in the position to bind histag proteins (Figure 4-4). Therefore, it was decided to use the sequential deposition for subsequent experiments.

There are many metals that are used to bind histag molecules. The protein affinity for the ions Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ increases with increasing number of $d$ electrons and decreases for Zn$^{2+}$. The most commonly used systems use Ni$^{2+}$ ions. Cobalt ions have the strongest affinity for histagged proteins and are also used in affinity chromatography of where high selectivity of histagged proteins is needed (i.e. complex protein solutions where histagged proteins are present in low concentrations).

Copper ions have the highest loading capacity of proteins in affinity chromatography of proteins, however, it is not as selective for histagged proteins as Co$^{2+}$ or Ni$^{2+}$ ions.

Analysis of each step of the metal-phytic acid modified zirconium phosphonate slides was analyzed using XPS and AFM. XPS was used again for elemental analysis and AFM was used to determine thickness and roughness of the modified surfaces. Figure 4-5 shows the scheme of the binding of phytic acid and metal ions on the surface.

As expected, XPS results for the Ni-phytic acid and Co-phytic acid surface show not only the presence of the respective metal ions on the surface, but there is also an increase in the amount of phosphorus on the slide (Table 4-2). There are a higher percentage of cobalt ions on the surface as compared to nickel ions. This value is expected because studies have shown that phytic acid has a slightly higher affinity towards Co$^{2+}$ than Ni$^{2+}$ ions in solution.
XPS from the CuIP₆ surface were not expected. After complexation of CuIP₆ on the surface, the P/Zr ratio decreases to 0.8. The most likely explanation for this ratio is that CuIP₆ complexes are being removed from the zirconated surface. AFM image of the CuIP₆ surface shows significant defects where it seems as though phytic acid was removed from the zirconated surface upon binding with copper ions (Figure 4-6A). The top layer of the surface has a mean roughness value of 0.27 (Table 4-3). The lower layer has a mean roughness of 0.12, which corresponds to the zirconium phosphonate surface. AFM images of Ni-phytic acid and Co-phytic acid surfaces do not show these defects in the layer (Figure 4-6 B and C). The increase in thickness supports the fact that there are layers of these metal-phytic acid complexes forming on zirconium phosphonate surfaces (Table 4-3).

Although, there are many factors involved, it has been widely noted, one of the most important factors for the retention of proteins on a surface is the metal ion affinity to the histag moiety.⁶² Cu-, Ni- and Co-phytic acid complexes could be acceptable choices for further study of this system. Because different metal ions have different affinities to proteins, the best test would be to use Ni(II)-phytic acid modified surfaces for comparison to the commercially available Ni-NTA surface. Using the same metal ion on both phytic acid and commercially available slides may eliminate experimental variables of due to the use of different metal ions.

**Protein Immobilization Studies**

Fluorescently-labeled (His)₆-IA3 proteins were immobilized on either commercially available Ni-NTA slides (Xenopore) or NiIP₆ and analyzed with confocal laser scanning microscopy (CLSM).
As expected, the Xenopore slides were very efficient at binding several different concentrations of IA3 protein. The same protein binding procedure was applied to the immobilized IA3 on the Ni-IP$_6$ surfaces. There was much less bound protein on the NiIP$_6$ surfaces as compared to the commercially available slides (Figure 4-7). An increase in the concentration of Ni$^{2+}$ solution from 1 mM to 200 mM did not facilitate an increase in protein binding on phytic acid modified surfaces. The results show a minimal amount of protein binding to the surface after the modification. In the case of spotting 1.368, 2.052, and 4.788 µg/ml protein concentrations, the fluorescence intensity of bound protein was less than that of the 5 mM IP$_6$: 1 mM Ni$^{2+}$ slides.

To investigate what was happening to the metal-phytic acid slide after protein binding, XPS was employed. Slides were prepared as described in the previous experiments except that the concentration of the phytic acid was 5 mM and the concentration of the Ni$^{2+}$ solution was 200 mM. The slides were treated with a "blank rinse" that consisted of binding washing the slides three times with TBS. This buffer is used to rinse non-specifically bound proteins from the metal-phytic acid surfaces. In the blank experiments, however, no protein is immobilized to the surface. This experiment was performed to determine what affect TBS buffer has on the phytic acid modified surfaces. XPS analysis showed that after rinsing the slide with TBS, the P/Zr ratio is about 1.1, which is considerably less than the data presented in Table 4-2. The percentage of Ni$^{2+}$ ions on the surface is much less than the amount before buffer rinses (Figure 4-8). TBS removes most of the Ni-phytic acid complex from the zirconoated surface causing low protein binding.
Discussion

Two different methods for deposition of phytic acid-metal ion complexes to zirconium phosphonate surface were tested. In the first method, zirconium phosphonate slides were modified Cu(II)-phytic acid complex solution. In the second method, zirconium phosphonate slides were first modified with phytic acid and then with a solution of Cu (II) ions. Two different deposition methods were used to determine if phytic acid binds to the zirconium phosphonate surface as a monolayer and subsequently available to bind metal ions (sequential deposition) or if soluble IP₆-metal complexes form in solution bind better to the zirconated surface (complex deposition).

The difference in the concentration of IP₆ and Cu(NO₃)₂ for each method was due to the ability of phytic acid to form insoluble complexes with metal ions. Phytic acid can bind up to six Cu²⁺ ions and depending on the pH and the concentration of the metal-IP₆ solution, insoluble precipitates can form. Therefore, to maintain soluble IP₆: Cu complexes in solution, lower concentrations of both IP₆ and Cu(NO₃)₂ were used.

Another aspect to consider is that insoluble metal phytate complexes are formed in the presence of excess metal ions. Thus, for both deposition methods, a molar excess of phytic acid used to prevent precipitation of metal phytate complexes.

The survey scan for the CuIP₆ complex deposition methods shows that the P/Zr ratio on the slide is 2.2. This is an encouraging result because a bare zirconium phosphonate slide has a P/Zr ratio of 1. This indicates that phytic acid binds to the zirconium phosphonate surface. Also, the presence of Cu peaks on the survey scan indicates the presences of Cu²⁺ ions on the phytic acid modified surface. Survey scan data of metal-phytic acid modified surfaces prepared using the sequential deposition
method show a P/Zr ratio of 1.5, indicating that zirconium phosphonate can be modified with phytic acid using this method also.

A stability test was conducted on a zirconium-ODPA slide that was modified with soluble CuIP₆ complexes. After the modification, the slide was placed in water overnight. XPS results indicated that P/Zr of 1.8 on this surface. This implies that phytic acid remains bound to the zirconated surface even after the slide was left in water for an extended period of time. Thus, it was concluded that soluble complexes of CuIP₆ are stable on zirconium phosphonate surfaces.

For all subsequent experiments the sequential deposition method was used to ensure that the bound metal ions are on the surface and are available for histag binding. During treatment of a zirconated slide in a solution of CuIP₆ complexes, there is a possibility that CuIP₆ forms complexes with itself before binding to the surface. While the phytic acid will still bind to the zirconium-ODPA slide in this form, the metal is not available on the surface to bind histagged proteins.

Phytic acid has been known to form dimers with itself in concentrations above 10 mM,¹³⁷ thus we used concentrations less than 10 mM for all further experiments of phytic acid modification of zirconium phosphonate surfaces. One goal of this work was to determine if phytic acid bound to zirconium phosphonate surfaces could concomitantly bind metal ions. Because Cu²⁺ is known to be toxic to some proteins, we expanded our research with other histag binding metals such as Co²⁺ and Ni²⁺ ions. Along with their ability to bind phytic acid, both cobalt and nickel are used in commercially available slides and chromatography columns for histag protein binding and purification.
XPS analysis shows that there is more phosphorus on the slide than zirconium on both CoIP₆ slides and NiIP₆ slides, however there is more metal ions on the Co-phytic acid surface. The CuIP₆ slides showed less phosphorus than zirconium on the surface because upon copper binding to phytic acid on the surface. This implies that the copper phytate complexes are removed from the surface because the affinity of copper binding to phytic acid is stronger than the affinity of phytic acid binding to the zirconium phosphonate surface. It is thought that presence of the copper ions in the XPS spectrum implies that copper ions are binding to the oxides and hydroxides that terminate the surface of zirconium phosphonate surfaces, thus attenuating the Zr signal.

Protein binding does not seem to occur on NiIP₆-modified zirconium phosphonate monolayer surfaces. XPS results show that metal-phytic acid complexes bind to zirconium phosphonate surfaces and are stable in water. However, XPS also shows that most of the phytic acid-metal complexes are removed from the surface after washing with TBS. There were also negligible amounts of Ni²⁺ on the slides. Previous work by Kang et al. employed the use of XPS for their Ni-NTA derivatized silica surfaces. They found that they could not detect Ni ions on the slide using XPS. However, given that phytic acid binding has been seen to increase phosphorus intensity on the zirconium phosphonate monolayer surface and fluorescence experiments correlate with the XPS findings, it was concluded that in the presence of TBS, most of the phytic acid-metal complex is removed from the zirconated slide.

**Conclusion**

It is essential that any protein binding microarray technology is able to withstand washes with buffer solutions. With histagged proteins especially, the buffer helps
control the binding of Ni-(His)$_6$ complex. Due to the pKa of the amino acid, histidine, the optimal binding pH is around 7-8. Even though the metal-phytic acid surfaces can be washed in nanopure water without deterioration, it must be able to withstand rinses with common buffers used in protein analysis. In the next chapter is a discussion of the pH-dependent interconversion of phytic acid. In the next chapter, we take advantage of the pH-dependent conformation of phytic acid to determine if the molecule could simultaneously immobilize to zirconium phosphonate surfaces and chelate metal ions for histagged protein coordination.

![Figure 4-1](image_url)  

Figure 4-1. Structure of phytic acid. Phytic acid has six phosphate groups on the cyclohexane ring, which make it a good candidate to bind zirconium phosphonate surfaces. It is also known to chelate with many metal ions namely Ni$^{2+}$, Co$^{2+}$ and Cu$^{2+}$, which are often used to immobilize histagged proteins.
Figure 4-2. Binding scheme of phytic acid to the zirconium phosphonate monolayer surface. The goal of this project is to employ the ability of phytic acid to chelate metal ions that bind histagged protein molecules. Depicted in this schematic is phytic acid chelated to Ni$^{2+}$, however, other metals ions tested in this experiment include Co$^{2+}$ and Cu$^{2+}$ ions.

Figure 4-3. Procedure for making the zirconium phosphonate monolayers. First, ODPA is spread at the air-water interface and then transferred onto a hydrophobic support. This is followed by the addition of zirconyl chloride, which forms a coordinate covalent network with the phosphonate head groups.
Table 4-1. Summary of the XPS analysis of Zr(3d), P(2p3), and Cu(2p3) peaks after binding of phytic acid/metal complexes to zirconium phosphonate monolayer surfaces using either the complex or sequential deposition method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Zr (3d)</th>
<th>P (2p3)</th>
<th>Metal Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuIP₆ complex deposition</td>
<td>26%</td>
<td>57%</td>
<td>17%</td>
</tr>
<tr>
<td>Sequential Deposition of IP₆ then Cu</td>
<td>32%</td>
<td>48%</td>
<td>20%</td>
</tr>
<tr>
<td>CuIP₆ complex deposition after overnight water rinse</td>
<td>29%</td>
<td>52%</td>
<td>19%</td>
</tr>
</tbody>
</table>

Figure 4-4. Possible formation of CuIP₆ complex in solution. As shown, if metal/phytic acid complexes are formed like this in solution before deposition on zirconium phosphonate monolayer surfaces, then the metal ions will not be available to bind histagged proteins.
Figure 4-5. Binding of phytic acid and various metal ions to the surface. Phytic acid surfaces can be modified with Ni$^{2+}$, Co$^{2+}$, or Cu$^{2+}$ proteins.

Table 4-2. Summary of XPS analysis of elemental percentages of the elements of interest on phytic acid modified zirconium phosphonate.

<table>
<thead>
<tr>
<th></th>
<th>Zr</th>
<th>P</th>
<th>Metal Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zirconium Phosphonate</td>
<td>50%</td>
<td>50%</td>
<td>--</td>
</tr>
<tr>
<td>5mM IP$_6$ pH 5</td>
<td>41%</td>
<td>59%</td>
<td>--</td>
</tr>
<tr>
<td>5mM IP$_6$ + 1mM Co$^{2+}$</td>
<td>29%</td>
<td>44%</td>
<td>27%</td>
</tr>
<tr>
<td>5mM IP$_6$ + 1mM Cu$^{2+}$</td>
<td>45%</td>
<td>38%</td>
<td>17%</td>
</tr>
<tr>
<td>5mM IP$_6$ + 1mM Ni$^{2+}$</td>
<td>30%</td>
<td>53%</td>
<td>17%</td>
</tr>
</tbody>
</table>
Figure 4-6. AFM of zirconium phosphonate modified surface after phytic acid and copper nitrate solution. The image shows that when CuIP$_6$ surfaces are formed major defects can form in the layer (A) whereas no major defects were seen on the NiIP$_6$ (B) or CoIP$_6$ (C) surfaces.
Table 4-3. Estimated thickness and roughness data of each layer of the phytic acid-metal deposition process as determined by AFM.

<table>
<thead>
<tr>
<th>Source</th>
<th>Estimated Thickness (Å)</th>
<th>Mean Roughness of Surface (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>--</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>OTS</td>
<td>--</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Zirconium Phosphonate</td>
<td>21</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>5mM IP₆ pH 5</td>
<td>23</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>5mM IP₆ + 1mM Co²⁺</td>
<td>24</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>5mM IP₆ + 1mM Ni²⁺</td>
<td>27</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>5mM IP₆ + 1mM Cu²⁺ (top layer)</td>
<td>27</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>5mM IP₆ + 1mM Cu²⁺ (lower layer)</td>
<td>--</td>
<td>0.12 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 4-7. Comparison of protein binding on 5mM IP₆:1mM Ni²⁺ surface (black), 5mM IP₆:200mM Ni²⁺ surface (red) and the commercially available Ni-NTA (blue) slides.
Figure 4-8. XPS of 5mM IP₆: 200mM Ni²⁺ slide rinsed with TBS pH 8. After rinsing with the protein buffer, most of the phytic acid is removed from the surface, leaving only a small amount of Ni ions on the surface.
Zirconium phosphonate modified surfaces are known to efficiently bind phosphate and phosphonate compounds. In the previous chapter, it was shown that phytic acid binds zirconium phosphonate surfaces in acidic conditions; however, upon rinsing with common protein buffers, the phytic acid was removed from the zirconium surface.

A property of phytic acid that had not been explored yet in this research is its ability to exist in two different stereochemical forms: the equatorial form or the axial form (Figure 5-1). The equatorial form of phytic acid is defined as phosphates 1, 3, 4, 5 and 6 are in the equatorial position and phosphate 2 is in the axial position (5eq/1ax). When the phosphates previously in the equatorial form are converted to the axial position and phosphate 2 is converted to the equatorial position, this is known as the axial form of phytic acid (5ax/1eq). It was suggested by Emsley and Niazi, that phytic acid only exists in the axial form between pH 5 -12; above and below this pH range, the equatorial form is favored. More recently, however, Murthy and coworkers used $^{31}$P NMR to suggest that phytic acid coverts from the equatorial form to the axial form at pH 9.0-9.5 in the presence on metal ions. At pH below 9.0, the equatorial form of phytic acid predominates, however, between pH 9.0 and 9.5, the stereochemistry of the phosphate groups is inverted. Murthy’s study was conducted with alkali metal ions, however, other groups have used this work as a basis to study phytic acid complex formation with other divalent and trivalent metal ions. This inversion to the 5ax/1eq form is believed to be due to complexation with metal ions, which reduces the electrostatic repulsion of the phosphate anions.
Yang and coworkers have studied the interconversion of phytic acid on silver and iron electrode surfaces using surface-enhanced Raman scattering spectroscopy (SERS).\textsuperscript{117,118} They demonstrated that self-assembled monolayers of phytic acid in either conformation demonstrate ordered and uniform features. It was suggested that phytic acid in the 5eq/1ax conformation form SAMs at metal surfaces via the P=O of one of the free phosphates. Phytic acid in the 5ax/1eq configuration form more stable monolayers.\textsuperscript{117,118} This is due to the strong reactions of the completely deprotonated IP\textsubscript{6} molecules with the metal surface via the P-O moieties of the four phosphates positioned in the axial configuration (Figure 5-1).

The hypothesis of this experiment is that the phosphate groups of the phytic acid in the 5ax/1eq form are in a better position to bind the zirconium phosphonate surface than the 5eq/1ax form because more of the phosphate groups are positioned vertically and will have more direct contact with the Zr\textsuperscript{4+} ions. In the axial form, the phytic acid will form monolayers on the zirconium phosphonate surfaces by either two or three phosphate groups simultaneously. The phytic acid will bind through the phosphates on carbons 1, 3, and 5 or through carbons 4 and 6. Thus, phytic acid should interact more strongly with zirconated film and, therefore, not wash off of the surface as easily after rinses with common protein buffers as seen in Chapter 4.

In this chapter, phytic acid is immobilized on the zirconium phosphonate surface and the amount and stability of metal-phytic acid complex binding was analyzed with XPS. The amount and specificity of protein binding to Ni- and Co-phytic acid modified surfaces was determined with fluorescence microscopy.
Although there was more protein adsorbed to surfaces modified with phytic acid in 5ax/1eq form, the amount of protein binding was much less than the amount on commercially available slides. Therefore, further protein binding studies were done on Co\(^{3+}\)-phytic acid surfaces. Hale published a report that showed that Co\(^{2+}\) can be oxidized to Co\(^{3+}\) to form “exchange inert” or irreversible complexes with bound proteins.\(^{143}\) In this work, proteins were bound to Co\(^{2+}\)-IDA in a reversible manner and after immobilization, the cobalt was oxidized with dilute hydrogen peroxide to form an irreversible complex with the bound proteins. We tried to replicate this work with Co\(^{2+}\)-phytic acid surfaces. Histagged proteins bound on Co\(^{2+}\)-phytic acid surfaces were treated with dilute hydrogen peroxide to form a Co\(^{3+}\) complex to determine if more histagged protein could be retained on the surface. Although, XPS results did show that Co\(^{2+}\) was oxidized to Co\(^{3+}\) after peroxide treatment, fluorescence experiments showed no increase in the amount of histagged protein binding on the surfaces.

**Experimental Section**

**Materials**

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

**Preparation of Metal-Phytic Acid Slides for XPS Studies**

Zirconium phosphonate films were prepared as previously described. Spots of 50mM, 10mM and 1mM phytic acid (30 µl) were printed on the zirconium phosphonate surface for nine hours at 4°C. The pH of the phytic acid solution used for spotting depended on which metal-phytic acid complex was used to modify the zirconium phosphonate surface. It has been suggested that Ni(II) forms soluble complexes with phytic acid when the molecule is in the axial form and Co(II) forms soluble complexes
with phytic acid when it is in the equatorial form.\textsuperscript{103} Therefore, the solution of phytic acid used to form NiIP\textsubscript{6} complexes on zirconated surfaces was of pH 10.82, ensuring phytic acid was in the axial configuration on the slide. To form CoIP\textsubscript{6} modified slides the zirconium phosphonate surfaces were, first, deposited the phytic acid solution of pH 9.01 for 1 hour. At this pH, phytic acid is either in the equatorial or axial form. Next, the slides were rinsed with water and then dipped in a 100 mM nickel (II) chloride or cobalt (II) chloride solution. The slides were then rinsed with water and tris-buffered saline, TBS, (pH 8) 3 times for 30 seconds. To remove residual salt, the slide was dipped into a vial of water one time. These surfaces were spin-dried and analyzed by XPS as described in Chapter 4.

**Preparation of Metal-Phytic Acid Surfaces for Analysis of Protein Binding Using Confocal Laser Scanning Microscopy (CLSM)**

A desired concentration of phytic acid (10 mM - 50mM, 1.5 µl) was spotted on the slide as described above. The slides were rinsed with water and then left in a 100 mM nickel or cobalt nitrate solution for 1 hour. Slides were rinsed again with water and dried with nitrogen. Next 3 µl of 1.4 µg/ml fluorescent IA3 protein (expressed and purified as described in Chapter 4) was spotted directly on top of the phytic acid spots already printed on the slide. Slides were incubated at 4°C overnight. Slides were rinsed with TBS (pH 8) three times for 30 seconds each time. The surface was rinsed for 10 minutes and then rinsed with water and dried with nitrogen. CLSM was used to analyze the amount of protein binding on the surface using the same instrumental parameters as described in the previous chapter.

To test for specific binding interactions of protein with the surface, metal-phytic acid modified slides were treated with florescent IA3 protein and incubated overnight at
4°C as described above. The slides were then rinsed with TBS (pH 8) three times for 30 seconds each time. Finally, the slides were rinsed with 200 mM imidazole in TBS (pH 8) for 2 minutes and then rinsed with water and dried with nitrogen. CLSM was used to analyze protein binding to the slides as described in Chapter 4.

**Oxidation from Co²⁺ to Co³⁺ on Surfaces**

Cobalt-phytic acid modified zirconium phosphonate surfaces were prepared as described above. To oxidize Co²⁺ to Co³⁺, the slides were placed in about 25ml of TBS pH 8 in a Petri dish. As the TBS in the Petri dish was stirred, 250 µl H₂O₂ was slowly dripped in the dish and the slide sat in the solution for 90 minutes. The slides were then rinsed with water and TBS (pH 8) 3 times for 30 seconds. Residual salt was removed from the slide by dipping it into a vial of water one time. These surfaces were spin-dried and analyzed by XPS as described in Chapter 4.

**Bisphosphonate Linker vs. Phytic Acid Linker for Histag Protein Binding**

Mathieu Cinier at the Université de Nantes in France synthesized a bisphosphonate linker with either one or two NTA moieties. Surface analysis of the linker binding to zirconium phosphonate monolayers was studied in our lab. Bisphosphonate slides were prepared using a Whatman Fast-Frame slide plate and a 2-pad incubation chamber. One pad of the zirconium phosphonate slide was immersed in 1 ml nanopure water as an experimental control. The immersed slides were kept at 4°C overnight. The slides were rinsed with water and then deposited in a solution of 100mM NiCl₂ for 1 hour. Finally, the slide was rinsed with nanopure water and spin-dried in air. Surfaces with the linker were analyzed using XPS with an Al Kα X-ray source with the procedure outlined in Chapter 4.
Results

The main concern when developing these slides was stability. Previous studies showed that the equatorial-configured phytic acid surfaces were stable in water, but upon rinsing with salt buffers, the phytic acid was removed from the surface. Surfaces with 50 mM, 10 mM, and 1 mM IP₆ after treatment with Ni²⁺ and rinsed with TBS buffer were analyzed using XPS. Table 5-1 summarizes the data.

Slides that were modified with 1 mM phytic acid solutions did not seem to retain any metal ions or any phytic acid after being rinsed with TBS buffer as evidenced by the P/Zr ratio of 1. However, for the 10mM surface, the P/Zr ratio increases to 1.2 and there are Ni ions on the surface after buffer rinsing. The P/Zr ratio is similar for the 50 mM surface. From this data, it does not seem as though there is any advantage of binding 50 mM phytic acid to the surface instead of 10 mM because there is similar amounts of nickel on the each surface. Figure 5-2 shows XPS spectra of the elements of interests.

Protein Binding Studies

Proteins were immobilized on Ni(II)-and Co(II)-phytic acid modified surfaces (Figure 5-3). It should be noted that the pH of the phytic acid solution used to form Ni(II)-phytic acid surfaces was 10.72, whereas to form the Co(II)-phytic acid slide, the pH of the phytic acid was 9.01. Previous attempts to bind soluble complexes of Co(II)-phytic acid formed when the phytic acid solution was pH 10.72 (axial form) proved unsuccessful. Fluorescence microscopy studies showed that there was no protein binding if Co²⁺ ions were deposited onto phytic acid surfaces at this pH. A possible reason for this is given by the pH profiling results of the formation of soluble complexes of Co(II)- and Ni(II)-phytate.¹⁰³ This work, conducted by Martin and Evans, showed that
Co$^{2+}$ prefers to bind to phytic acid in the equatorial position and Ni$^{2+}$ prefers to bind to phytic acid in its axial form. Therefore, it was concluded that there was probably little to no Co(II)-phytic acid complexes on these surface for histagged protein binding.

From the work of Murthy and coworkers, it is known that the interconversion of phytic acid from the equatorial form to the axial form occurs between the pH 9 and 9.5. Therefore, Co$^{2+}$ ions were chelated to phytic acid at pH 9.01 to ensure that the phytic acid could concomitantly adsorb to zirconium phosphonate surfaces and form strong interactions with Co$^{2+}$ ions for histagged protein binding. Preliminary fluorescence results showed that the amount of protein binding to the CoIP$_6$ slides was comparable to protein immobilized on Ni(II)-phytic acid surfaces.

Because different amounts of phytic acid were used in the protein binding experiments of the phytic acid surfaces in the equatorial form, a direct comparison to the fluorescence experiments of axial bound phytic acid cannot be done. However, XPS experiments showed that even after buffer rinse of a surface treated with 10 mM phytic acid (equatorial form), phytic acid was not removed (data not shown). Therefore, it can be assumed that more protein binds to the metal-phytic acid surface when the phytic acid is bound to the surface in the axial form. Though there is more protein bound to the surface, the amount of protein on the metal-phytic acid surface is significantly less than the amount of bound protein on commercially available slides (Figure 5-4).

The specificity of protein binding was determined by washing the Ni-phytic acid slide with imidazole. Imidazole is a strong unidentate ligand/ligates displacer. The purpose of the imidazole is to act as a competitive chelator of the Ni ions. The
imidazole displaces the histagged proteins bound through the Ni ions. At low concentrations, imidazole can be used to pretreat the column or surface to remove weakly bound metal ions, which will prevent metal ion leakage during purification methods (concentrations = 1mM - 10 mM).\textsuperscript{144} It will not scavenge strongly bound ions from the surface. It can also be used to elute proteins from a surface (concentrations = 1mM - 100mM).\textsuperscript{58} In theory, any protein left on the surface is bound non-specifically.\textsuperscript{140,145}

For the 10 mM surface, there is significantly less fluorescence after washing with the imidazole which suggests that most of the fluorescence seen on the surface is due to specifically bound protein. The fluorescence intensity of the specifically bound protein is about 6900 fluorescence units. There is a similar amount of fluorescence on the surface after imidazole washings on both the 10 mM and 50 mM IP\textsubscript{6} surface suggesting that there is consistent amount of non-specific protein binding on the phytic acid modified surfaces. The amount of specifically bound protein on the 50 mM surface cannot be accurately determined from these results because the calculated amount of specifically bound protein is within experimental error of the amount of protein remaining on the surface after imidazole treatment.

**Exploiting the Properties of Cobalt for Irreversible Protein Binding**

It is a common practice to bind divalent cobalt ions to the metal chelator iminodiacetic acid (IDA) for histag protein purification (Figure 1-6A where M\textsuperscript{n+} = Co\textsuperscript{2+}). Imidazole side chains of histagged proteins are immobilized to the Co(II)-IDA surface by displacing water molecules on the Co(II) coordination sites. An advantage of using Co\textsuperscript{2+} ions over other histag chelating metal ions (Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}) is that Co\textsuperscript{2+} is more specific for histagged proteins then Ni\textsuperscript{2+}, which results in less non-specific protein binding when
using divalent cobalt ions.\textsuperscript{145} Although cobalt ions are more specific for histagged proteins, nickel ions have a greater affinity for histags.\textsuperscript{145} For this reason, some researchers who use Co-IDA columns to purify histagged proteins have taken advantage of the fact that cobalt can form irreversible bonds with the imidazole side chains of histagged proteins. Hale published a report that showed that Co\textsuperscript{2+} can be oxidized to Co\textsuperscript{3+} to form “exchange inert” or irreversible complexes with bound histagged proteins.\textsuperscript{143} Proteins were bound to Co\textsuperscript{2+}-IDA in a reversible manner and after binding, the divalent cobalt were oxidized with dilute hydrogen peroxide to form an trivalent cobalt complex with the bound histagged proteins. Another report by Zatloukalová and coworkers stated that Co\textsuperscript{2+} ions could be oxidized to Co\textsuperscript{3+} before histagged protein binding, which would keep protein from being exposed to the oxidant hydrogen peroxide.\textsuperscript{146} However, this theory seems unlikely because in order for protein immobilization to occur on these surfaces, histagged proteins would have to cause a reduction of Co\textsuperscript{3+} to Co\textsuperscript{2+}. Histagged proteins are not a strong enough oxidant to facilitate this reaction. Presumably, the Co-phytic acid surface will retain more protein on the surface if the adsorbed Co\textsuperscript{2+} ions can be oxidized to the +3 state after protein immobilization. To test this hypothesis, Co-phytic acid slides were, first, analyzed with XPS before and after water rinses to determine if divalent cobalt ions could be oxidized. Secondly, the surfaces were washed with TBS rinses to simulate rinsing to remove non-specifically bound proteins. This experiment was called a blank rinse because no protein molecules were analyzed using XPS experiments.

Co\textsuperscript{2+} oxidation on the phytic acid surface was done on a ColP\textsubscript{6} surface that was deposited into a solution of 0.03% H\textsubscript{2}O\textsubscript{2} in TBS for 90 minutes. XPS shows that there is
phytic acid and cobalt on the slide before and after the slide was treated with the dilute peroxide (data not shown). A closer investigation of the cobalt on each spectra showed that Co (2p) peak before buffer wash is at 785 eV and the Co (2p) peak is at 783.5 eV. The Co peak is shifted towards a lower binding energy after oxidation. This suggests the presence of Co$^{3+}$ on the surface after oxidation with peroxide.$^{147}$

XPS analyses of the elemental ratios of Co-phytic acid surfaces before and after TBS rinses and after oxidation of cobalt are shown in Table 5-2. CoIP$_6$ surfaces that were washed with water show a P/Zr ratio of 2, which is similar to the results seen with original metal-phytic acid surfaces discussed in the previous chapter. After washing the CoIP$_6$ slides with TBS, the P/Zr ratio only decreases slightly to 1.8. However, the amount of cobalt on the surfaces decreases significantly. This suggests that the buffer is removing the cobalt ions from the surface as seen in previous studies.

After the peroxide oxidation treatment CoIP$_6$ slides, the results are similar to the elemental values after the slide was washed with TBS. The P/Zr ratio is about 1.7 and the Co (2p) peak has an area of about 9% of the total peak area. This data suggests that although TBS removes much of the cobalt, the cobalt ions that remain on the surface are in the +3 oxidation state. CLSM was used determine whether histagged proteins can remain immobilized on this surface after cobalt is in +3 oxidation state. Although there are cobalt ions on the surface, CLSM experiments of protein bound to Co$^{3+}$-phytic acid surfaces yielded little to no fluorescence intensity. This result was not entirely unexpected because due to the low percentage of cobalt present on the surfaces.
Bisphosphonate Linke vs. Phytic Acid Linker for Histag Protein Binding

Because modification with phytic acid for histagged protein binding was not successful, our colleagues at the Université of Nantes suggested an alternative approach for histagged binding to zirconium phosphonate surfaces. A mono-NTA and bis-NTA functional adaptor was synthesized for modification of zirconium phosphonate surfaces (Figure 5-5). The adaptors contained a multivalent phosphonic acid anchor at one extremity and either a mono-NTA or bis-NTA moiety at the other. The phosphonate groups provide a stable bond to the zirconium interface by multipoint attachment. The NTA moieties could be complexed with Ni$^{2+}$ ions for histagged protein capture. In Figure 5-6, XPS spectra of mono-NTA and bis-NTA coated slides are compared to the bare zirconium phosphonate surface, after exposure to Ni$^{2+}$ ions. Binding to the NTA-coated slides is evident, with more Ni$^{2+}$ complexed to the bis-NTA coated slide. Upon reaction with the mono-NTA adaptor, a significant increase of the P/Zr ratio was observed (P/Zr = 1.4) which confirms the binding of bisphosphonate on the surface. Knowing the surface density of the zirconium ions within the monolayer, 4.2 x $10^{14}$ atoms/cm$^2$, the mono-NTA adaptor coverage was estimated to be 6.3 x $10^{13}$ molecules/cm$^2$. With the bis-NTA adapter, a P/Zr of 1.18 was determined, which indicates half the surface coverage for the mono NTA adaptor. However, as this adapter has two NTAs per molecule, the NTA density on the surface is roughly similar to that of the mono-NTA adaptor. Comparatively, after desorbing 10 mM phytic acid on zirconium phosphonate slides, a P/Zr of 1.2 is obtained (Table 5-1), which gives a similar surface density to that of the bis-NTA linker. However, after attempts to bind proteins, the metal ions are removed from phytic acid modified surface. Fluorescence results of the protein binding to mono-NTA and bis-NTA linker show histag proteins can
be immobilized on the NTA-modified surfaces and the histag proteins can bind other proteins after immobilization indicating that little to no loss of histagged protein structure and/or function after immobilization of the surface. More information about the mono-NTA and bis-NTA linker, including fluorescence measurements of protein binding on NTA-adaptor modified zirconium phosphonate surfaces, can be found in Appendix A.

Discussion

Protein binding on Ni- and Co-phytic acid complexes formed with phytic acid in the axial form yielded higher fluorescence than when the phytic acid is in the equatorial form (Chapter 4). However, fluorescence of bound protein on commercially available slides was significantly greater than of metal-phytic acid modified surfaces. Specific binding of protein on a Ni-phytic acid formed from 10 mM phytic acid surface was much higher than on surfaces prepared with 50 mM IP₆. Comparing this data to the XPS analysis implies that there may not have been any Ni-phytic acid complexes on the surface when prepared from 50 mM IP₆ and the Ni peak shown is due to nickel ions adsorption to terminal oxides and hydroxides on zirconium phosphonate film. Attempts to increase protein binding on Co-phytic acid surfaces by oxidation of the cobalt ions proved unsuccessful. The most likely cause of this is that exposure of these slides to tris-buffered saline disrupts the bonds of the CoIP₆ complex, removing the cobalt ions but leaving phytic acid immobilized to the surface.

Conclusion

Ni-phytic acid and Co-phytic acid complexes bind zirconium phosphonate when the phytic acid is bound in the axial form. XPS results show that phytic acid remains bound to the surface even after rinsing with buffer. Although there was some histagged binding to these surfaces, it did not rival the amount of binding on commercially
available Ni-NTA surfaces. Further exploration into this problem revealed that most of the metal ions are washed off the surface after these rinses due to the weak bonds of the metal-phytic acid complexes. Whereas, common chelators such as NTA, EDTA, and IDA interact with metals covalently, metal binding to phytic acid is dominated with ionic/electrostatic interactions. Attempts to covalently bind Co ion to the surface by the oxidation of Co(II) to the more stable Co(III) proved unsuccessful. This system, therefore, is not adequate for histagged protein binding studies.

Bisphosphonate adapters were synthesized by Cinier et al. and surface analysis of those linkers was carried out in our lab. These linkers provide specific anchoring of histagged proteins to zirconium phosphonate surfaces. The bifunctional adapter contains a multivalent phosphonic acid anchor at one extremity and a NTA group at the other. The phosphonate groups provide a stable bond to the zirconium interface and the affinity tag strategy provides a uniform orientation of proteins on the surface with high-density coverage allowing for high efficiency protein binding.
Figure 5-1. The conformation inversion of phytic acid between the 1ax/5eq and 5ax/1eq from between pH 9.0 - 9.5. Below pH 9.0 - 9.5, phosphates on carbon 1, 3, 4, 5, and 6 are in positioned equatorially to the ring. Above this pH, the phosphates are positioned axially to the ring. The phosphate groups in the axial form of phytic may be better positioned to bind the zirconium phosphonate surface. In its axial form, phytic acid should be able bind to the zirconium phosphonate surface through phosphates on carbons 1, 3, and 5 or carbons 4 and 6. In contrast, in its equatorial form, phytic acid will only bind to the zirconium phosphonate surface through one phosphate. Binding it in the axial form on the zirconated surface should lead to higher stability of immobilized phytic acid.

Table 5-1. Summary of the elements of interests on the Ni-phytic acid surfaces.

<table>
<thead>
<tr>
<th></th>
<th>Zr</th>
<th>P</th>
<th>Ni</th>
<th>P/Zr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM IP₆</td>
<td>50%</td>
<td>50%</td>
<td>--</td>
<td>1 / 1</td>
</tr>
<tr>
<td>10mM IP₆</td>
<td>33%</td>
<td>38%</td>
<td>29%</td>
<td>1.2 / 1</td>
</tr>
<tr>
<td>50mM IP₆</td>
<td>37%</td>
<td>40%</td>
<td>21%</td>
<td>1.1 / 1</td>
</tr>
</tbody>
</table>
Figure 5-2. Summary of the Ni(2p3), Zr (3d), and P(2p3) of 1mM (A), 10mM (B) and 50mM (C) phytic acid (pH 10.82) on zirconium phosphonate monolayer slides. There is no Ni on the surface deposited in 1mM phytic acid. However, there is a considerable amount of metal ions on the 10 mM and 50 mM phytic acid surface.
Figure 5-3. Fluorescence intensity of protein bound on Ni-phytic acid and Co-phytic acid surfaces. Protein binding to metal-phytic acid surfaces with phytic acid in the axial form does produce more protein binding, however, the amount of bound protein is significantly less than observed with the commercially available slides.
Figure 5-4. Fluorescence intensity of protein bound to Co-IP₆, Ni-IP₆, and commercially available Ni-NTA slides (Xenopore). Protein binds to the metal-phytic acid surface when the phytic acid is bound to the surface in the axial form. The amount of protein on the metal-phytic acid surface is significantly less than the amount of bound protein on commercially available slides.

Table 5-2. XPS Results of peak areas of Co (2p), P (2p3) and Zr (3d) peaks of a CoIP₆ slides after being rinsed with water, TBS buffer and dilute peroxide oxidation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Co</th>
<th>P</th>
<th>Zr</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Water Rinse</td>
<td>42%</td>
<td>39%</td>
<td>19%</td>
</tr>
<tr>
<td>After TBS Rinse</td>
<td>9%</td>
<td>59%</td>
<td>32%</td>
</tr>
<tr>
<td>After Dilute Peroxide</td>
<td>9%</td>
<td>58%</td>
<td>33%</td>
</tr>
</tbody>
</table>

Figure 5-5. Mono-NTA (top) and bis-NTA (bottom). Mathieu Cinier in Nantes, France synthesized both molecules. Zirconium phosphonate can be modified with these molecules for binding of histagged proteins on surfaces.
Figure 5-6. Peaks of Ni(2p3), Zr(3d), and P(2p3) obtained from XPS analysis for (a) zirconium phosphonate monolayer surface, (b) mono-NTA modified surface and (c) bis-NTA modified surface. No Ni(2p3) peak is seen on the bare zirconium phosphonate surface.
CHAPTER 6
QUARTZ CRYSTAL MICROBALANCE AS A TOOL FOR DETERMINATION OF
SSDNA COVERAGE ON ZIRCONIUM PHOSPHONATE SURFACES

Introduction

In contrast to organic microarray surfaces, the Talham group found that terminal phosphate-modified DNA oligonucleotides bind specifically to reactive inorganic zirconium phosphonate surfaces. Phosphate terminated probes attach specifically via coordinate covalent bonding to a zirconium phosphonate modified surface. An advantage to using phosphate terminated DNA is it is a naturally occurring process; thus, phosphorylation does not alter the nature of the DNA probe. Microarrays using zirconated substrates have been studied previously to determine if phosphorylated probe molecules can hybridize with its complementary sequence. Molecular fluorescence was used to empirically determine under what conditions optimal specific target binding occurred.\(^{38}\)

Although, we know that phosphate-modified DNA oligonucleotides bind to zirconium phosphonate surfaces, there were still questions about this interaction that remain unanswered. Questions such as:

1. How do DNA probes orient themselves on the monolayer?
2. How are the probes spaced and organized on the monolayer?
3. How can probe binding be controlled on the monolayer?

To answer these questions, we first need to know the surface density of probe DNA on the zirconium phosphonate surfaces. The surface density of phosphate-modified oligonucleotides on the surface was determined using quartz crystal microbalance (QCM) analysis. First, the zirconium phosphonate monolayer film was transferred onto the QCM resonator crystal. Subsequently, the maximum amount of
immobilized DNA probe on the surface could be determined using probe spotting conditions that were optimized for zirconium phosphonate microarray applications. A probe surface coverage of DNA 1.95 x 10^{11} ± 0.62 x 10^{11} molecules/cm^2 was obtained by QCM measurements. These results are quite similar to the results of the same system obtained by XPS.

Probe density values were compared to published reports of oligonucleotide probe on gold surfaces. Our comparisons led us to believe that with the amount of oligonucleotide probe coverage on the zirconium phosphonate, a large percentage of target DNA molecules should hybridized to the immobilized probe DNA. However, upon further investigation by Sarah Lane in the Talham group, it was shown that low surface coverage of the DNA indicates that although phosphodiester backbone cannot hold the DNA on zirconium throughout rinsing, the interaction of the backbone and surface causes the probe DNA to lay down on the surface preventing more covalent binding of DNA.

**Experimental Section**

**Materials**

Nine megahertz QCM resonators (Part # 131218-9) were purchased from International Crystal Manufacturing (Oklahoma City, OK). The quartz crystal was 0.538" in diameter. The electrode consisted of a layer of gold (1000 Å) with a chromium underlayer (100 Å). The electrode diameter was 0.201". Probe (5’- PO_4^-GGGGGGGGGGCCGCCGGAGGTTAAGATCGAGATCCA -3’) and target (5’-TGGATCTCGATCTTAACCTCCGGCGG-3’) DNA was HPLC-purified from Invitrogen (Carlsbad, CA) and used as received. Probe and target oligonucleotides were resuspended in enough water to make 100 μM stock solutions and then the
oligonucleotides were aliquoted into one-time-use volumes. All other chemicals were purchased from Sigma (St. Louis, MO) and used without further modification.

**Zirconium Phosphonate Film on QCM Resonator**

Before determination of the surface density of oligonucleotides on the zirconium phosphonate monolayers, the monolayer had to be transferred onto the QCM resonator. QCM resonators were cleaned following the RCA cleaning method (2 minutes, each step). The gold portion of the resonator was made hydrophobic by immersing the resonator in a 1mM octadecylmercaptan for 24 hours and then washed extensively with ethanol. Next, the surrounding quartz was made hydrophobic by immersing the resonators in a solution of 5mM OTS in bicyclohexyl for 2 minutes. An LB layer of octadecylphosphonic acid (ODPA) from a calcium chloride subphase was transferred onto the hydrophobic substrate as described in the Chapter 4. These monolayers are prepared using a step-wise deposition procedure\(^{32}\) that is shown in Figure 4-3.

**DNA Immobilization and Hybridization**

Before DNA immobilization, the resonance frequency of the zirconated quartz crystal was measured. Probe DNA was then immobilized to the zirconated resonator using a protocol already established to be the optimal conditions to immobilize DNA on the zirconated surface. Briefly, 40 μM DNA in 1X sodium saline citrate (SSC) buffer (pH 6) was immobilized on the QCM resonator overnight. The DNA was rinsed for two minutes in 2X SSC buffer with 0.1% SDS, two minutes in 1X SSC buffer, and two-minute immersion in 0.2X SSC buffer twice.

After rinsing, the resonance frequency was measured again. A decrease in frequency means that the resonator has acquired more mass. This mass is due only to
the amount of immobilized probe DNA bound to the zirconium phosphonate modified substrate.

**Instrumental Techniques**

QCM is a mass-sensing technique based upon the piezoelectric effect. By applying an alternating current through a quartz surface, a mechanical oscillation of characteristic frequency is produced by the crystal. The QCM resonator consists of a shear mode AT-cut crystal sandwiched between two gold active electrodes. Although, gold electrodes were used in this experiment, any conducting metal can be used for the electrodes. The activity of the resonator comes from a voltage that is applied across the electrodes. When voltage is applied, the crystal is deformed in a unique shear pattern. For the AT-cut crystal, the displacement maxima occur at the crystal faces which is why the QCM resonator is sensitive to the accumulation of surface mass. Only mass deposited on the gold electrodes corresponds to frequency/mass changes.

QCM was first used in a sensing mode when Sauerbrey reported proportional change with the frequency of an oscillating crystal and the mass changes on the metal electrodes. The frequency of the QCM resonator modified with a zirconium phosphonate film was measured using a QCA 922 quartz crystal microbalance (Princeton Applied Research) and frequency was recorded using the WinEChem software (SEIKO EG&G). Frequency of the resonator was recorded after deposition of zirconium phosphonate monolayer surface. Probe DNA was immobilized to the quartz resonator surface (as described above). Afterwards, the resonance frequency was recorded again. The change in frequency was used to find the mass that correlated to the amount of immobilized probe DNA using the Sauerbrey equation. The Sauerbrey Equation is:
\[ \Delta F = -\frac{2F_0^2}{A(\mu_q \rho_q)^{1/2}} \Delta m \]  

where \( \Delta F \) is the measured resonant frequency change (Hz), \( F \) is the intrinsic crystal frequency, \( \Delta m \) is the mass change (g), \( A \) is the area of the electrode (0.204 cm\(^2\) in these experiments), \( \rho_q \) is the density of quartz (2.65 g/cm\(^3\)), and \( \mu \) is the shear modulus of quartz (2.95 x 10\(^{11}\) g/cm·s\(^2\)).

XPS was performed using a UHV XPS/ESCA PHI 5100 system. Survey scans of the surfaces were taken with a Mg K\(\alpha\) x-ray source and analyzed as described in Chapter 4.

**Results**

**Zirconium Phosphonate Monolayer on QCM Resonators**

Monolayer transfer has previously been done on glass, silica and quartz slides, all which can be coated with OTS to make the surface initially hydrophobic. One of the challenges that incurred was determining whether the zirconium phosphonate monolayer would transfer onto the QCM resonator, which consists of gold and quartz portions. Monolayer transfer on the edge of substrates is not complete or reproducible. In order to ensure complete transfer of the zirconium phosphonate monolayer on the gold electrode of the resonator, the entire surface of the QCM was made hydrophobic. Zirconium phosphonate monolayer transfer would begin on the hydrophobic quartz and fully transfer onto the gold electrode of the resonator. X-ray photoelectron spectroscopy was used to determine if a monolayer of zirconium phosphonate had been transferred onto the resonator. It has been shown previously that zirconium phosphonate
monolayers have a P/Zr \(= 1\).\textsuperscript{37} Figure 6-1 shows that there is the correct ratio of zirconium to phosphorus on a QCM resonator after transfer of the monolayer.

**DNA Probe Density**

QCM was used to determine mass changes due to immobilized probe oligonucleotides on the zirconium phosphonate surface. The immobilized mass on the crystal directly correlates to the mass per area, the unit in which surface density is reported. Knowing the frequency change (and, subsequently, the mass change) on the QCM, molecular weight of the DNA and the surface area of the gold electrode of the QCM resonator (0.204 cm\(^2\)) allows for the determination of the surface density of probe and target DNA. Molecular weight of the probe DNA is 14,260.8 g/mol.

In addition to a phosphate modification, the oligonucleotide probes were modified with 11 guanine nucleotides at the 5’ end; this is called a polyG spacer. In the presence of sodium ions (like the buffer used in these experiments), the polyG spacer can form stable, four stranded helices (Figure 6-2).\textsuperscript{84} DNA exhibits higher hybridization efficiency with the polyG spacer than without it,\textsuperscript{38,148,150} and the polyG spacer is positioned the probe DNA in the correct orientation to undergo hybridization with its complement strand. Previous investigations have shown that the use of other homonucleotide spacers [poly(dA), poly(dT), poly(dC)] do not improve DNA hybridization on the zirconium phosphonate surfaces.\textsuperscript{148} Experiments have shown that binding DNA with poly(dA) modification on zirconated films shows the highest surface coverage but rinses off the surface with more stringent rinses whereas, the surface coverage of DNA with the poly(dG) probes remains constant on the surface after stringent rinsing.\textsuperscript{148}

Figure 6-3 is an example of the frequency changes before and after DNA probe immobilization. In this particular experiment, a decrease in frequency of 32 Hz is seen
on the resonator after DNA probe immobilization. This is equal to a mass change of 3.84 ng on the QCM surface. A 3.8 ng mass change corresponds to a maximum surface density of $7.75 \times 10^{11}$ molecules/cm$^2$. An average of five experiments indicated a probe density of $1.95 \times 10^{11} \pm 0.62 \times 10^{11}$ molecules/cm$^2$. These results are quite similar to the results of the same system obtained by XPS. Lane et al. showed a probe density of DNA on the zirconated surface of $2.8 \times 10^{11}$ molecules/cm$^2$. This surface coverage is lower than what other researchers have measured for DNA immobilized on gold surfaces however, the maximum amount of DNA coverage on a surface does not give the optimal hybridization efficiency.

Georgiadis and coworkers studied the influence of probe density on DNA hybridization. They found that the maximum density of oligonucleotide on gold surface was $1.2 \times 10^{13}$ molecules/cm$^2$. Petrovykh et al. found a maximum surface coverage of DNA on gold to be $3.7 \times 10^{13}$ molecules/cm$^2$. This is assumed to be a condensed monolayer of DNA on the surface. However, with a full monolayer of DNA, the percentage of complementary target DNA molecules that bound immobilized probe was very low (< 20%). In fact, the amount of probe DNA on the surface that gave the highest hybridization efficiency was $2.0 \times 10^{12}$ molecules/cm$^2$ (hybridization efficiency ≈ 70%). This value is comparable to the maximum amount of DNA probe on the zirconium phosphonate surface. We attempted similar experiments of target DNA hybridization to the bound immobilized probe on the zirconium phosphonate surfaces. Probe hybridization was attempted on QCM resonators, however, due to the modification chemistry involved with the hybridization, accurate experiments of hybridization efficiency could not be completed with the QCM technique.
Discussion

DNA probe binding was seen on zirconium phosphonate and analyzed with the QCM technique. Single-stranded DNA probe density measured with QCM was similar to the probe density calculated by XPS. There is a much smaller amount of DNA on the zirconium phosphonate surface as compared to thiolated DNA on gold. This is best explained by the fact that oligonucleotides covalently attached to the zirconium surface through a 5’ phosphate will physisorb to the surface through the phosphodiester backbone preventing further DNA attachments.\textsuperscript{148} The probe density of DNA on the surface is important because it is a controlling factor for the efficiency of target capture.\textsuperscript{151} Due to the chemistry involved in functionalizing quartz crystals with complementary DNA, the hybridization data obtained by QCM was not reproducible.

Conclusion

The surface coverage of ssDNA on a zirconium phosphonate monolayer was calculated with QCM. The coverage is lower than reported value for thiolated-DNA on gold. Surfaces were modified with DNA using spotting conditions optimized by fluorescence microscopy. Low surface coverage of the DNA indicates that although phosphodiester backbone cannot hold the DNA on zirconium throughout rinsings, the interaction of the backbone and surface causes the probe DNA to lay down on the surface preventing more covalent binding of DNA.
Figure 6-1. XPS of zirconium phosphonate modified QCM 9 MHz crystal. Both the gold and quartz portions of the crystal were made hydrophobic before monolayer transfer. The slide shows a zirconium to phosphorus ratio of about 1/1, which is characteristic of the surface.
Figure 6-2. Possible tetraplex formation from polyG aggregates. In the presence of potassium or sodium ions, like in the sodium citrate buffer, strands of polyG can form stable four-stranded helices.
Figure 6-3. Sample experiment of the resonator frequency before and after probe immobilization on a zirconium phosphonate modified QCM crystal. Probe binding causes a decrease in the resonator frequency; this value can be used in the Sauerbrey Equation to find the DNA mass increase on the surface.
In order to provide specific anchoring of oligo-histagged proteins to the zirconium phosphonate surface, Mathieu Cinier at the Université of Nantes designed a bifunctional adaptor containing a multivalent phosphonic acid anchor at one extremity and a NTA group at the other. The phosphonate groups provide a stable bond to the zirconium interface by multipoint attachment. This affinity tag strategy provides a uniform orientation of proteins on the surface and high-density coverage without the need to perform complicated chemistry on the protein targets or on the solid support. Stable binding of the bifunctional adaptor is demonstrated, allowing reversible capture of histagged proteins. This technology is applied to a new class of small and stable capture proteins, the affitins, which are shown to keep their binding properties when immobilized on the zirconium phosphonate surface and to exhibit a high signal to noise ratio relative to arrays prepared from other NTA functionalized supports.
Figure A-1. Affitin immobilization on a microarray format. The nickel-loaded NTA functionalized slides were spotted with affitin at fixed concentrations prior to a blocking step with α-casein, which due to its high phosphate content provides efficient saturation of the nonspotted areas, hindering nonspecific protein binding. The activity of the immobilized affitin was investigated upon incubation of the surface with AlexaFluor 647 labeled lysozyme overnight.

Figure A-2. Fluorescence intensity versus concentration of spotted affitin on zirconium phosphonate slides functionalized with mono Ni-NTA (dark grey) and bis Ni-NTA (light grey) groups. Fluorescence data values correspond to the mean and the e-cart type range for 3 replicates within 1 slide (inset) image obtained at 60% laser power and 60% photomultiplier gain.
Figure A-3. Compared performances for lysozyme capture of H4 affitin immobilized on different substrates, slide E: epoxide surface from Nexterion®, slide E-NTA: Ni-NTA functionalized epoxide surface, mono NTA: mono Ni-NTA functionalized ODPA/Zr surface, FAST slide. *Fluorescence intensities were measured at lower laser power (30) and gain value (40) with the FAST slide.
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Monique Nicole Williams was born in Washington D.C. in the summer of 1980. Raised mainly in North Carolina, she graduated from the NC School of Science and Mathematics in Durham, NC in 1998. After attending North Carolina Central University for one year, she transferred North Carolina State University and graduated with a degree in Chemistry and minor in Biotechnology in December 2002. Two months after her college graduation, she felt free to quit her job as a hostess at Chili’s when she secured a job at Merck where she did stability testing on finished pharmaceutical products. After a year at Merck, she decided to pursue a Ph.D. in chemistry at the University of Florida. She defended her dissertation in November 2009.