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Exploring Zirconia as a Column Packing Material

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EXPLORING ZIRCONIA AS A COLUMN PACKING MATERIAL

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Tushar Ghugare
August 2010

EXPLORING ZIRCONIA AS A COLUMN PACKING MATERIAL

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EXPLORING ZIRCONIA AS A COLUMN PACKING MATERIAL.

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Zirconia is one of the most promising column packing materials for High Performance Liquid Chromatography (HPLC). The perfect HPLC support material should be energetically homogenous, have a high surface area on which different chemical species can reversibly attach and be physically and chemically stable over a wide range of pH, temperature and solvent conditions. Most existing supports do not have all of these properties. This project is also focused on a proteomics study. Zirconia, hafnium oxide and titanium oxide which are some of the more promising materials currently available, can be used for the separation and analysis of phosphorylated proteins. Adenosine triphosphate, Adenosine diphosphate and Adenosine monophosphate were used as prototypes for phosphorylated proteins. Separation, absorption, fluorescence and SEM studies were performed to determine the adsorption of Adenosine phosphates species at a particular pH on Zirconia. Zirconia was also used for the purification of Fibrinogen Growth Factor (FGF) protein, which are a family of growth factors involved in angiogenesis, wound healing, and embryonic development. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to analyze the off-column purification and separation of this protein. This research suggests that, at acidic conditions, adenosine monophosphate has more favorable absorption on the Zirconia surface. On the other hand, the separation study suggests that basic conditions are more

favorable for the absorption of ATP, ADP and AMP when mixed together on Zirconia 500. Furthermore, it was found that Zirconia is a very promising material for the purification of FGF protein.

INTRODUCTION

Zirconium dioxide (ZrO_2 , Zirconia) is an oxide of Zirconium. Scientists are interested in this material because of its wide range of industrial applications. Some of the industrial applications of Zirconia include hot metal extrusion dies, powder compacting dies, oxygen sensors, fuel cell membranes, jewelry, subframes, semiconductors, cutting tools, seals valves, pump repellers, refractory applications and orthopedic implants. There are many physical and chemical properties which make Zirconia promising [3]. Some of the important physical and chemical properties of Zirconia are a high dielectric constant, high density, low thermal conductivity, and chemical inertness, resistance to molten metals, ionic electrical conduction, wear resistance, high fracture toughness and high hardness [4].

Crystallinity:

Zirconia is available in four forms: amorphous, tetragonal, cubic and monoclinic. Figure 1 and Figure 2 show the appearance of both the amorphous and cubic forms of Zirconia, respectively. Zirconia's optical, thermal and electrical properties depend on the structure. The crystallographic form can change from one form to another in following way [7]:





Figure 1: Amorphous Zirconia



Figure 2: Cubic Zirconia

Other general properties of Zirconia:

The general properties of Zirconia are listed in Table 1 [3].

No	Property	Description
1	Molecular formula	ZrO ₂
2	Molar mass	123.218 g/mol
3	Appearance	White powder
4	Density	5.68 g/cm ³
5	Melting point	2715 °C
6	Boiling point	4300 °C
7	Solubility in water	Negligible
8	Solubility	Soluble in HF, and hot HSO ₄ , HNO ₃ , HCl
9	Refractive index	2.13

Table 1: General Properties of Zirconia

Monoclinic Zirconia and the origin of surface activity:

Monoclinic Zirconia is a crystalline substance in which all Zirconia atoms are heptacoordinated to oxygen atom [7].

Two different kinds of oxygen atoms are present in monoclinic Zirconia:

- A. Tricoordinated oxygen O(1)
- B. Tetracoordinated oxygen O(2)

As can be seen in Figure 3, planes perpendicular to the x crystallographic axis are composed of sequential layers of oxygen O(1), then a Zirconium atom and then oxygen O(2) and again O(1) [7].

In Case A, the oxygen O(1) atom as shown in Figure 5, is coordinated by two bonds with two surface zirconium atom with every second type 1 oxygen O(1) coordinated with a single zirconium atom. It is observed that some of the valences of both Zirconia and oxygen remain unsatisfied. The Zirconium atom bears a positive charge and the oxygen atom bears negative charge. This is the origin of the unique Lewis acid and base properties of Zirconium oxide.

In Case B, as shown in Figure 6, the O(2) remains at top layer. In this case four coordination valences of the zirconium atoms are projected towards the top layer of oxygen. Thus, it can be said that three coordination bonds of Zirconium are satisfied by the subsequent layer of type one oxygen O(1). Only two of the zirconium valences are satisfied by the type two O(2) oxygen. Consequently there is positive charge on the Zirconium atom which acts as a Lewis acid site. The negative charge on the oxygen atom means there is a corresponding Lewis base site on the oxygen. There are $5 \mu\text{mol}$ of Lewis acid sites/ m^2 and about $4 \mu\text{mol}$ of Lewis base sites/ m^2 on the surface of Zirconia.

Zirconia is also known to ion exchange for both cation and anion, as it is amphoteric in nature [7].

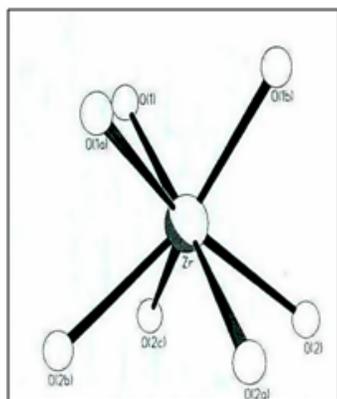


Figure 3: Coordination Sphere Zirconium atom in Monoclinic Zirconia. There are three oxygen of O1 type and four oxygen of O2 type.

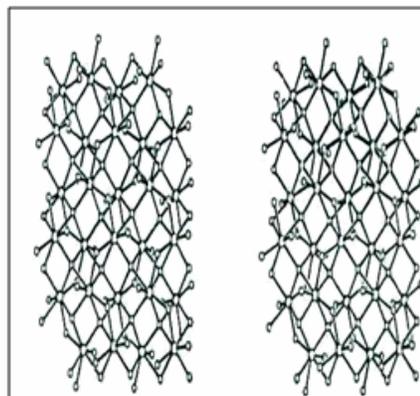


Figure 4: Stereoscopic view of monoclinic Zirconia crystal.

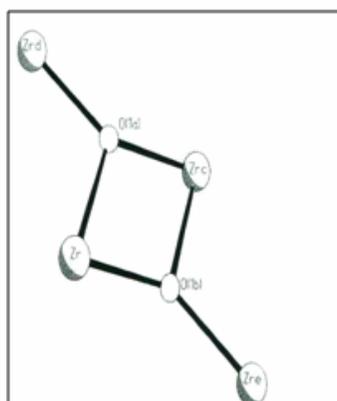


Figure 5: Coordination sphere of O1 type Zirconia atom. Each O1 type oxygen is coordinated with three Zirconium atoms.

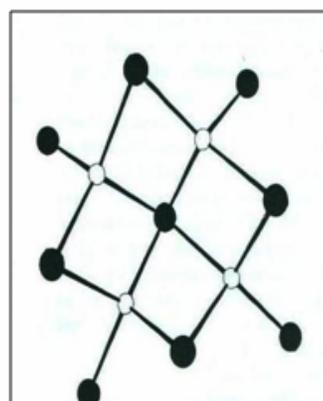


Figure 6: Coordination sphere of O2 type oxygen atom. Each O2 type oxygen is coordinated with four Zirconium atoms. Black dot = Zirconia, White dot = O(2)

Surface Area:

Surface area is a very important property for chromatography. Surface area of Zirconia depends upon the thermal history of the sample. It has been found that surface area decreases sharply between 300 °C and 500°C. It has also been found that particles heated above 500 °C will have specific surface area below 100m² /g. Two processes are responsible for decreasing the surface area: Microcrystallite growth and Intercrystallite sintering. This means that Zirconia treated at higher temperature has a lower surface area. It has also been found that the condition of the micropores is different at different temperatures. A Zirconia sample which is treated at 150-300 °C has transitional micropores, at 300-600 °C has transitional macropores and at 700-900 °C has macroporous absorbents. It clearly shows that Zirconia treated below 350-450 °C contains large number of micropores. Pore size also increases with temperature. Surface area also depends on the crystallinity of the substance. The tetragonal material has a surface area of 90m²/g after treatment at 800 °C. This area is due to micro porosity. The surface area of monoclinic Zirconia is more stable than tetragonal Zirconia [7].

Density of Zirconia:

Cubic Zirconia has highest density near about 6.27 g/cm³. Silica is reported to be 2.2 g/cm³. The density of the tetragonal form of Zirconia is higher near about 6.10 g/cm³. The apparent density of porous Zirconia depends on the degree of hydration and can vary from 0.48 g/cm³ to 2.33 g/cm³. The value of density strongly depends on the sample's thermal history. At higher temperatures, the sample has higher apparent volume. It is noteworthy that zirconia's apparent density is 3 to 4 times higher than silica. Due to the higher packing density it can be concluded that 30 m² /g Zirconia has a surface area equivalent to 90-120 m² /g of silica [7].

Pore Volume and porosity:

Sample history affects the pore volume. The volume of sample decreases after thermal treatment at 200 °C. The pore volume of Zirconia is generally much lower than that of silica. Pore volume of Zirconia depends on the size of microcrystallites. It can be concluded that Zirconia with the same porosity as silica will always have a lower pore volume than silica [7].

Pore size distribution and shape of pores:

Pore size distribution reflects the structure of the particle surface. Zirconia often shows a bimodal pore size distribution. IUPAC has classified isotherms in six types and hysteresis loops in four types. Figure 7 gives a brief summary of the classification of different isotherms and hysteresis loop. The type IV isotherm and H1 hysteresis loop are the most favorable for chromatographic purposes and Zirconia satisfies this condition. This is due to the capillary condensation in mesopores [7].

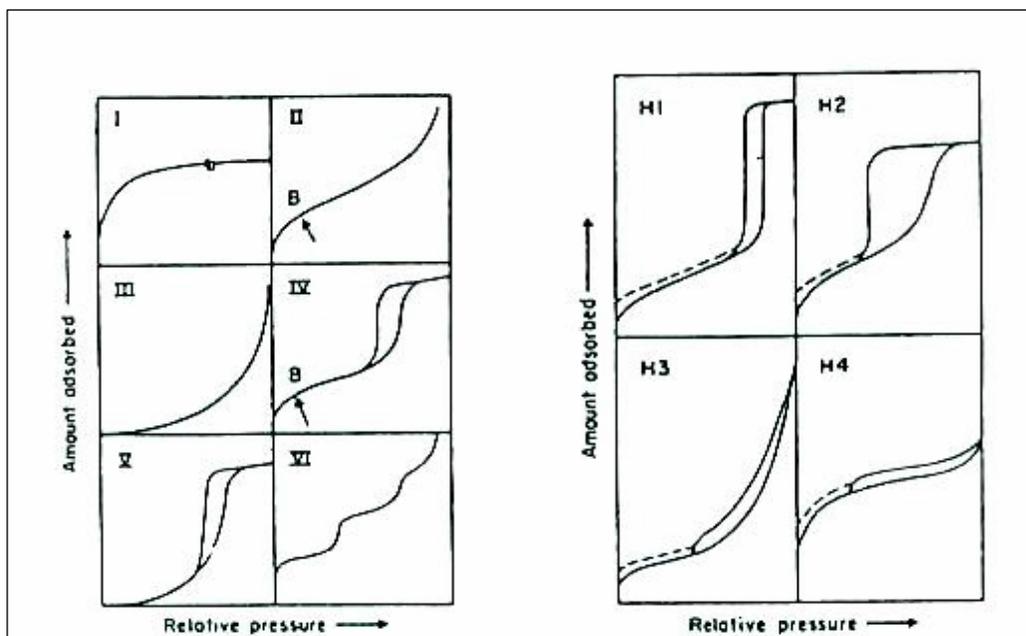


Figure 7: Different types of isotherms and hysteresis loop

Mechanical Strength:

It is found that the mechanical strength of Zirconia depends on microparticle sintering during thermal treatment. Its mechanical strength derives from microparticle sintering during thermal treatment [7].

Purity of Zirconia:

The presence of minute metallic impurities can create catalytic properties in Zirconia [7]. Incorporation of sulfate ions can generate super acid sites on Zirconia. Moreover, addition of SiO_2 , TiO_2 , Al_2O_3 , SnO_2 , CdO and ZnO can considerably increase the acidity of surface. Even small amounts of silicon can create catalytic properties in Zirconia. Many Zirconia naturally contain Hf, Fe, Ti, Si, Cu, Fe, K, Al, Ca, Mg, Mn, Th, V. It is very important to pay attention to this when synthesizing Zirconia [7].

Chemical Stability of Zirconia:

The strength of the Zirconia-oxygen bond is almost the same as the silicon oxygen bond. In monoclinic material, the zirconium atom is bonded to seven oxygen atoms. In silica, the silicon atom is bonded with four oxygen atoms. This makes Zirconia more stable to the extreme acidic and basic condition with some exceptions. Zirconia dissolves in HF, concentrated H₂SO₄ and hot concentrated H₃PO₄ and very concentrated HNO₃ [7].

Comparison between Zirconia, Silica and Polymeric phases:

Table 2 helps to compare Zirconia with Silica and other polymeric phases. As Zirconia is better in each case, there is a need to develop this material to overcome the disadvantages of other supporting materials [7].

Substance Properties	Zirconia	Silica	Polymeric phases
Mechanical Stability	++	++	+
High surface area	++	++	++
Control of particle diameter	++	++	++
Swelling	++	++	-
Chemical Stability	++	-	++
Thermal Stability	++	+	-
Control of Average pore diameter	++	++	+

Table 2: Comparison between Zirconia, Silica and Polymeric phases

++ = Very good performance.

+ = Good performance.

- = Fair performance.

Why Adenosine Phosphates?

Protein phosphorylation is one of the important biochemical processes in the body. It regulates many functions in the body. Phosphorylated protein is analyzed by traditional methods such as labeling protein with ^{32}P to monitor phosphorylation. Another method is the Edman degradation on phosphopeptides to localize the site of phosphorylation. There are many disadvantages to these methods such as the radioactivity of ^{32}P as well as the methods are time consuming and laborious. Furthermore, Edman degradation produces no data from proteins/peptides with blocked N-termini (i.e., N-terminal acetylation). This results in a loss of sample. An alternate option is Mass Spectroscopy (MS). MS is an emerging trend of analysis for post translational modification such as the phosphorylation. There are also some other difficulties such as the stoichiometric level of phosphorylation for a given protein is very low and, hence, difficult to identify. It has also been found that the intensity of the signals due to phosphorylated peptides in MS is lower than those for the non-phosphorylated species. It is therefore advantageous to separate/isolate the phosphorylated peptides/proteins from non-phosphorylated species in a given sample to facilitate the detection and identification of phosphorylation events via MS. One aim of our research is to study the absorption of different phosphorylated proteins on the surface of Zirconia. As it is very difficult and costly to obtain the phosphorylated protein, ATP, ADP and AMP were used as a prototype of phosphorylated protein. We hope to be able to correlate our results with phosphorylated proteins in the future. Figure 8 illustrates our research. Zirconia is in the tip. Zirconia should absorb phosphorylated proteins and then analysis should be done either by HPLC or by MS [1][8].

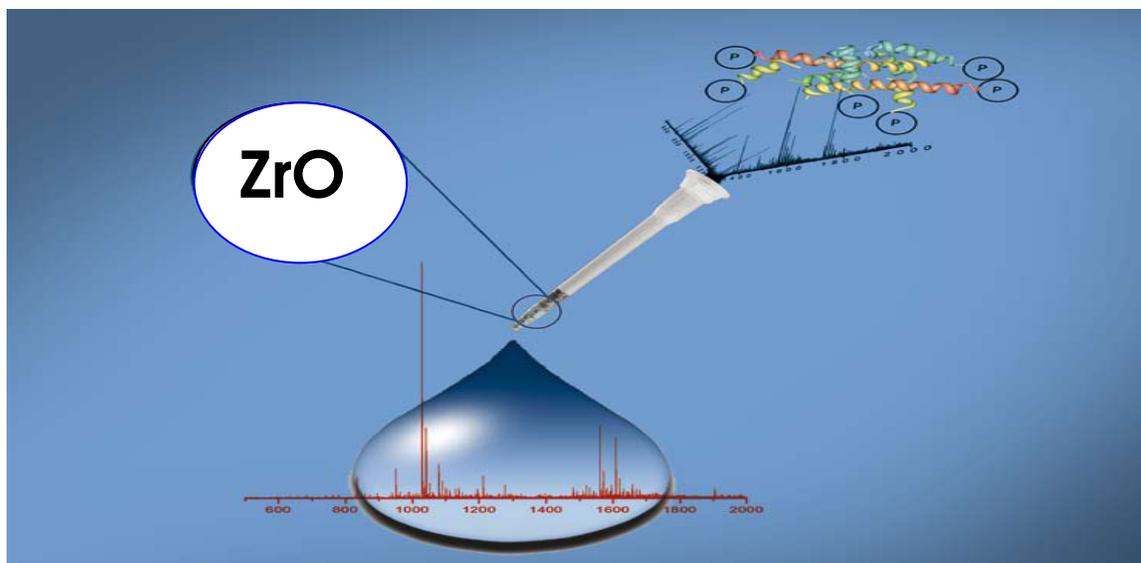


Figure 8: Brief idea of research

Purification of FGF protein:

Members of the fibroblast growth factor (FGF) family have been associated with a variety of important functions including angiogenesis and wound repair, survival of neurons, and pathogenesis of various diseases [2]. Figure 10 gives an idea about the structure of the FGF protein. It can be seen in Figure 9 that FGFs interact with specific FGF receptors and heparin sulfate proteoglycans on the surfaces of cells. Conventionally, synthetic heparin sepharose column chromatography method was used to purify FGF. While this method is very efficient, it has several disadvantages including long experimental times, high cost, and maintenance of the heparin column. Therefore, in the present study we devised an efficient off-column purification method that minimizes the disadvantages associated with heparin column purification. In this newer method, we used Zirconia which has both Lewis acid and Lewis base sites. Zirconia is inexpensive and it can be used over a wide pH range. In this purification technique, phosphate and tris buffer systems were used. Two parameters (i.e. pH and ionic strength) of the buffer

system were changed. Results of SDS-PAGE analysis indicated the presence of FGF [9][10][11].

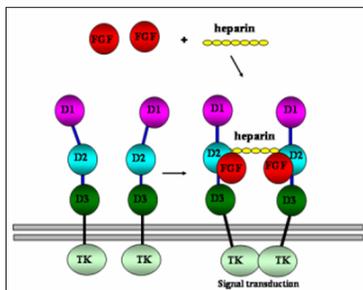


Figure 9: Schematic representation of some of the structural events involved in the interaction of FGF with its receptor.

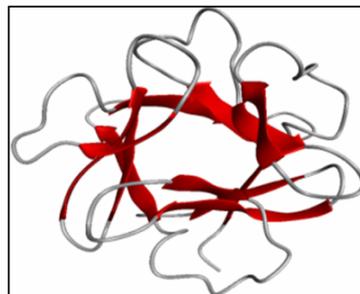
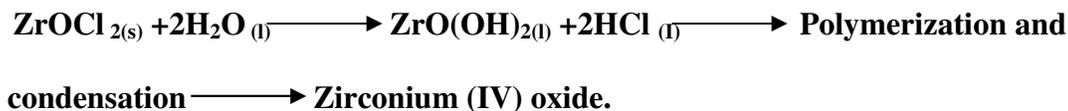


Figure 10: The structure of FGF-1 consist of 12 β -strands arranged into a β -barrel architecture

I. SYNTHESIS OF ZIRCONIA



Actual Synthesis of Zirconia:

1. An appropriate amount of ZrCl_4 was dissolved in H_2O to create a 1.28 M ZrCl_4 aqueous solution. Water was added drop wise, as ZrCl_4 reacted vigorously. The undissolved solid was vortexed.
2. The solution was allowed to cool to room temperature and 6.5 mole equivalent of N-Methyl Formamide (NMF) was added. Then this mixture was vortexed. In this reaction N-Methylformamide provides pore formation.
3. The solution was kept for 3 days.
4. After 3 days 7.4 mol equivalent of the gelling agent Propylene Oxide (PO), one mole of ZrCl_4 was added. Solution was vortexed. Gelling took place within a minute.
5. Tightly capped vessels containing gelled monoliths were aged at 50°C for 3 days.
6. After that, aged monoliths were subjected to 1 day of water exchanges (twice a day) and 1 day of isopropyl alcohol exchange (twice a day).
7. Exchanges monoliths were then air dried typically for a day.
8. Dried monoliths were heated to $80\text{-}120\text{-}150^\circ\text{C}$ (6hrs at each temperature), followed by heat treatment to 300°C , 500°C and 700°C (minimum of 12 hrs at each temperature). The ramp rate was $1^\circ\text{C} / \text{minutes}$.

9. Thus, according to final heat treatment Zirconia is classified in Zr700 and Zr500.

Analysis of Zirconia:

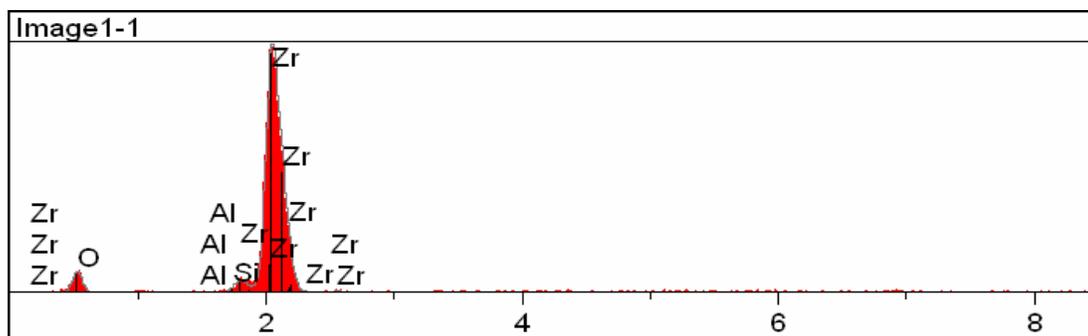


Figure 11: Elemental composition of Zirconia

Elt.	Line	Intensity	Error	Atomic	Conc	Units	
C	Kα	0.00	0.000	0.000	0.000	wt.%	
O	Kα	13.97	1.365	66.227	25.641	wt.%	
Al	Kα	0.36	0.220	0.127	0.083	wt.%	
Si	Kα	0.00	0.000	0.000	0.000	wt.%	
Zr	Lα	178.32	4.876	33.646	74.276	wt.%	
				100.000	100.000	wt.%	Total

Table 3: Elemental composition of Zirconia

Analysis of the synthesized Zirconia was done using a JEOL 5400LV scanning electron microscope. From Figure 11 as well as from Table 3, it can be concluded that Zirconia was synthesized successfully but the presence of Aluminum is an impurity.

II. EXPERIMENTAL

Absorption (Kinetics) Experiment:

Absorption spectra were collected of all Adenosine phosphates on the surface of Zirconia at different pHs, using a Perkin Elmer Lambda-35 UV- VIS spectrophotometer. Firstly, Tris buffer was prepared and Adenosine Phosphates were added at different pHs. All samples were weighed on a Denver M-220D balance.

- a. Preparation of Tris Buffer is as follows:

Table 4 details the preparation of Tris buffer.

pH	pH4.00	pH7.0	pH10.00
Description	6.07g of Tris Acid+500 mL water then pH was adjusted to 4.0	7.55 of Tris Acid+500 mL water then pH was adjusted to pH 7.0	7.855 of Tris Base +500 mL water then pH was adjusted to pH 10.0

Table 4: Preparation of Tris Buffer

b. Preparation of Different Adenosine Phosphate samples is as follows:

Table 5 details the different Adenosine Phosphate samples.

No	Type of Adenosine Phosphate.	Procedure
1	ATP at pH 4	0.0054 g of ATP + 100 mL pH 4.00 Tris buffer
2	ATP at pH 7	0.0053 g of ATP + 100 mL pH 7.00Tris buffer
3	ATP at pH 10	0.0051 g of ATP + 100 mL pH 10.00Tris buffer
4	ADP at pH 4	0.0041 g of ATP + 100 mL pH 4.00 Tris buffer
5	ADP at pH 7	0.0040 g of ATP + 100 mL pH 7.00 Tris buffer
6	ADP at pH 10	0.0042 g of ATP + 100 mL pH 10.00 Tris buffer
7	AMP at pH 4	0.0034 g of ATP + 100 mL pH 4.00 Tris buffer
8	AMP at pH 7	0.0033 g of ATP + 100 mL pH 7.00Tris buffer
9	AMP at pH 10	0.0035 g of ATP + 100 mL pH 10.00Tris buffer

Table 5: Preparation of Different Adenosine Phosphate samples

c. Procedure for Absorption (Kinetics) Experiment:

First, about 0.4000 g of Zirconia was measured directly in a cuvette and water was added to it and centrifuged in ICE Centrifuge at 3000 rpm for 15 minutes to settle the suspended particles. After centrifugation, supernatant was taken out and care was taken that the bed of Zirconia was not disturbed. After that the desired Adenosine phosphate at a desired pH was added to the cuvette and centrifuged again to settle the suspended particles. Before putting the sample in the UV- VIS spectrophotometer, it was allowed to warm up for 20 minutes and desired conditions were set followed by an auto zero. Finally, the sample was placed in a proper cuvette holder and analysis was started.

d. Conditions for Absorption analysis are as follows:

Table 6 provides Conditions for Absorption analysis.

NO	Condition	Description
1	λ max	254 nm
2	Slit width	2 nm
3	Time span	24 Hours.
4	Take reading after each	2 sec

Table 6: Conditions for Absorption analysis

Fluorescence Study:

Fluorescence was done to examine the absorption behavior of all Adenosine phosphate on the surface of Zirconia at different pH.

a. Procedure for Fluorescence Study:

Approximately 0.1000g of Zirconia was placed in a weighing boat. Exactly 1.00 mL of 0.0001 M at the desired Adenosine phosphate species at a desired pH was added to the boat. The same solutions, as previously given in Table 5, were again used for this Fluorescence study. The mixture was mixed using a stirrer and allowed to air dry at room temperature for three days. Solid-state Fluorescence analysis was done using a Perkin Elmer Fluorometer-Lambda 35. The glass of the solid-state sample holder was washed with both water and acetone. The sample was placed in the sample holder and the holder was oriented at the proper position in the fluorometer. The fluorometer was allowed to warm up for 15 minutes. Appropriate conditions for emission and excitation spectra were selected and analysis was done.

b. Typical conditions for Fluorescence Analysis:

Table 7 illustrates a typical set of conditions.

Emission Spectra		Excitation Spectra	
Excitation Wavelength	254 nm	Emission Wavelength	410 nm
Range	270 nm-470 nm	Range	230 nm-370 nm
Slit Width for both Excitation and Emission region	10 nm	Slit Width for both Excitation and Emission region	10 nm
Scan Speed	500 nm/ minutes	Scan Speed	500 nm/ minutes

Table 7: Conditions for Fluorescence Analysis

Separation Studies:

The aim of this experiment was to study the effect of Zirconia on the mixture of ATP, ADP and AMP at 3 different pHs (i.e. 4, 7 and 10). Separation was done with High performance liquid chromatography (HPLC) in which Varian 9012 pump was used for the separation and a Varian photo diode array UV-Vis detector was used for the detection of separated sample.

a. Principle of Separation:

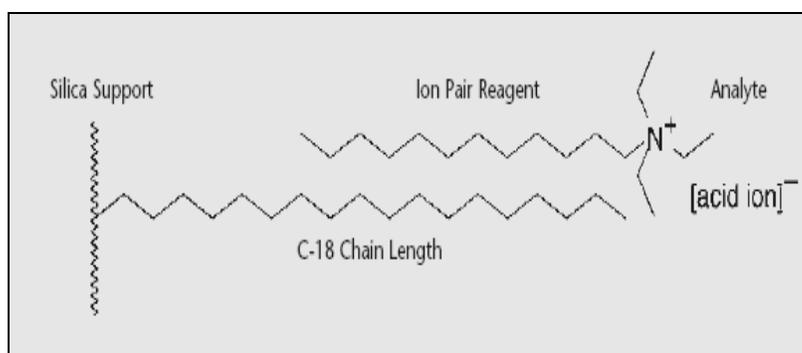


Figure 12: Theory of Separation

Ion pair reversed phase liquid chromatography was used for the separation of the three Adenosine phosphates. As shown in Figure 12, C-18 chains attach to silica support and ion pair reagent (i.e. Tetra butyl ammonium hydrogen sulphate) attaches to the C-18 chain. The analyte, Adenosine Phosphate, attaches to ion pair reagent and, accordingly, the composition of mobile phase, pH and flow rate separation and resolution of peaks can be decided.

b. Experimental Plot:

A half mL of 0.0001 M ATP +0.5 mL of 0.0001M ADP +0.5 mL of 0.0001M AMP in pH 4 buffer , 0.5 mL of 0.0001 M ATP +0.5 mL of 0.0001M ADP +0.5 mL of 0.0001M AMP in pH 7 buffer, 0.5 mL of 0.0001 M ATP +0.5 mL of 0.0001M ADP +0.5 mL of 0.0001M AMP in pH 10 buffer were added to Zr500 and Zr700. Thus kinetics of ATP, ADP and AMP was examined with Zr500 and Zr700 from 1 hour to 24 hours. Appendorfs tubes were put on rotor for proper mixing. Then on each hour appendorfs were removed out from the rotator and then these appendorfs were centrifuged for 10 minutes at 10000 rpm. Then the supernatant was taken out and the precipitate containing Zirconia was discarded. Using the same process, the rest of the samples were treated at different times e.g. 2,3,4,5,10,15,20 and 24. Finally, 54 samples were ready for analysis. This whole experiment was repeated three times to test for repeatability. After analysis, peak areas of each peak were noted and with the aid of a calibration curve, the amount of the analyte was determined.

c. Mobile Phase:

Mobile Phase A: 60mmol/L of Dipotassium Phospahte and 0.45mmol/L Tetra butyl ammonium hydrogen sulphate was dissolved in water and, with the help of phosphoric acid, pH was adjusted to 3.5. Mobile Phase A was prepared in Nano pure water form Bio tech centre and pH was adjusted using Fisher Scientific AB-15 pH meter. Mobile Phase B: Acetonitrile.

d. Other Parameters:

The flow rate of mobile phase was 0.80 mL / minute. The relative ratio of mobile phase A to mobile phase B was 98 % A and 2 % B. The run time was 40 minutes. The pressure of pump was 400 Psi. The column was Reversed Phase C-18 and λ_{max} was 254 nm.

e. Peaks:

Figure 13 shows the separated 0.0001 MAMP, 0.0001M ADP and 0.0001 ATP peaks respectively.

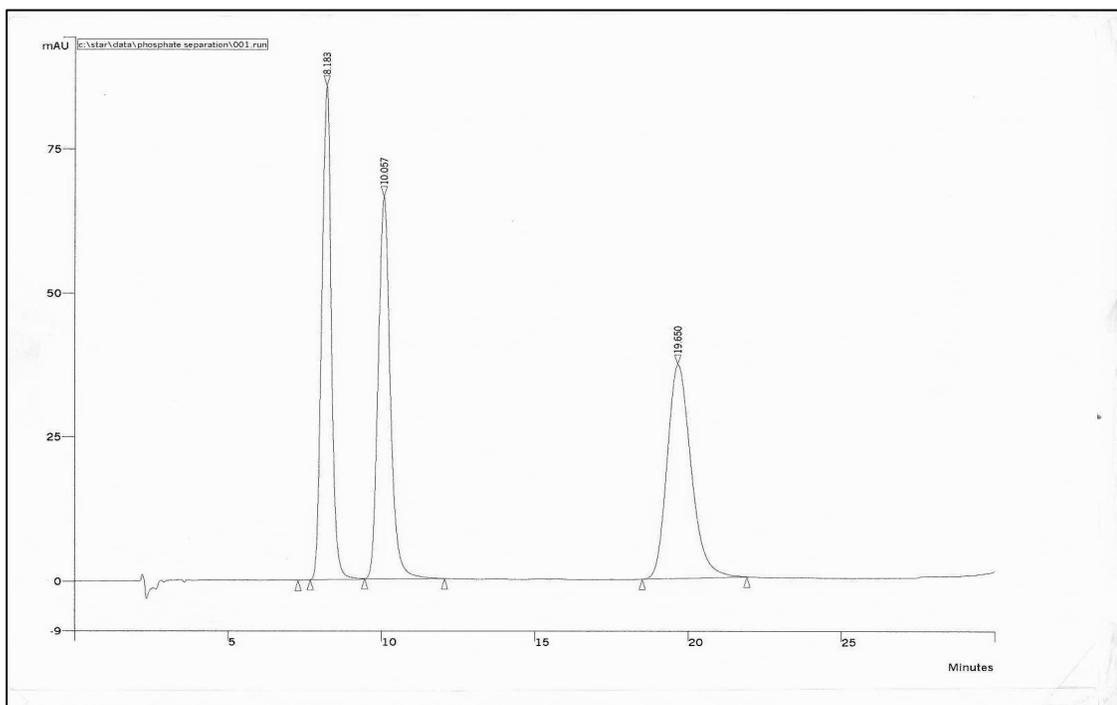


Figure 13: Separated AMP (Peak1), ADP (Peak2) and ATP (Peak3) peaks respectively

f. Unknown Peak.

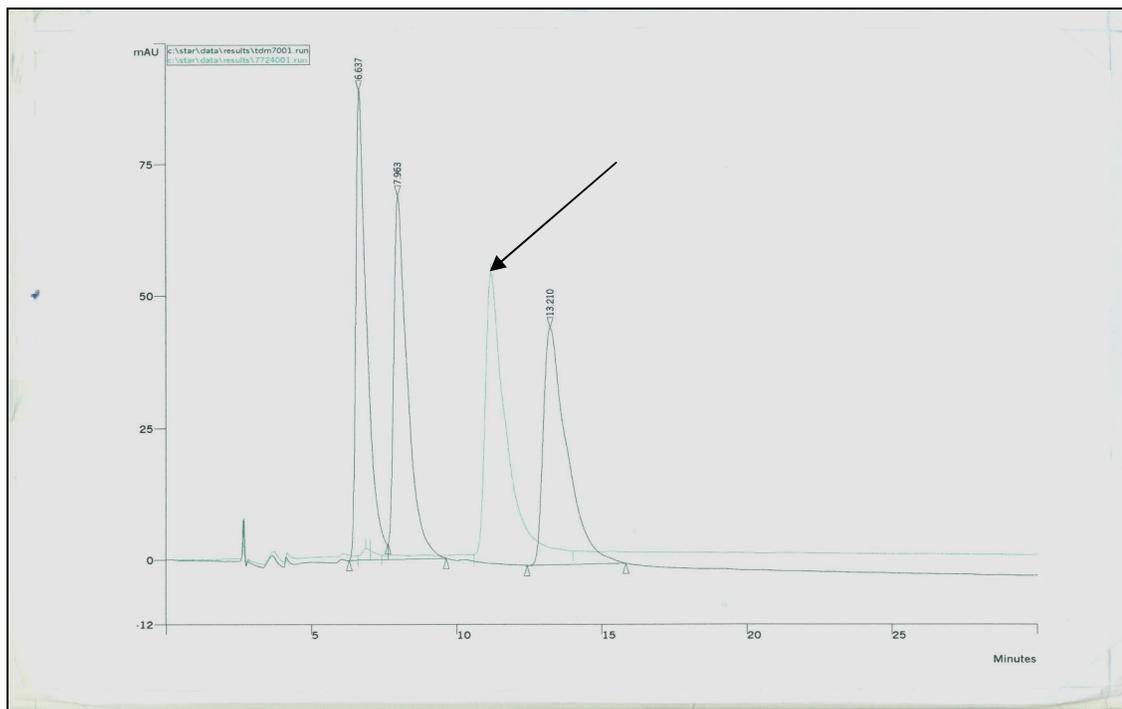


Figure 14: Superimposed image of normal AMP, ADP and ATP with unknown peak

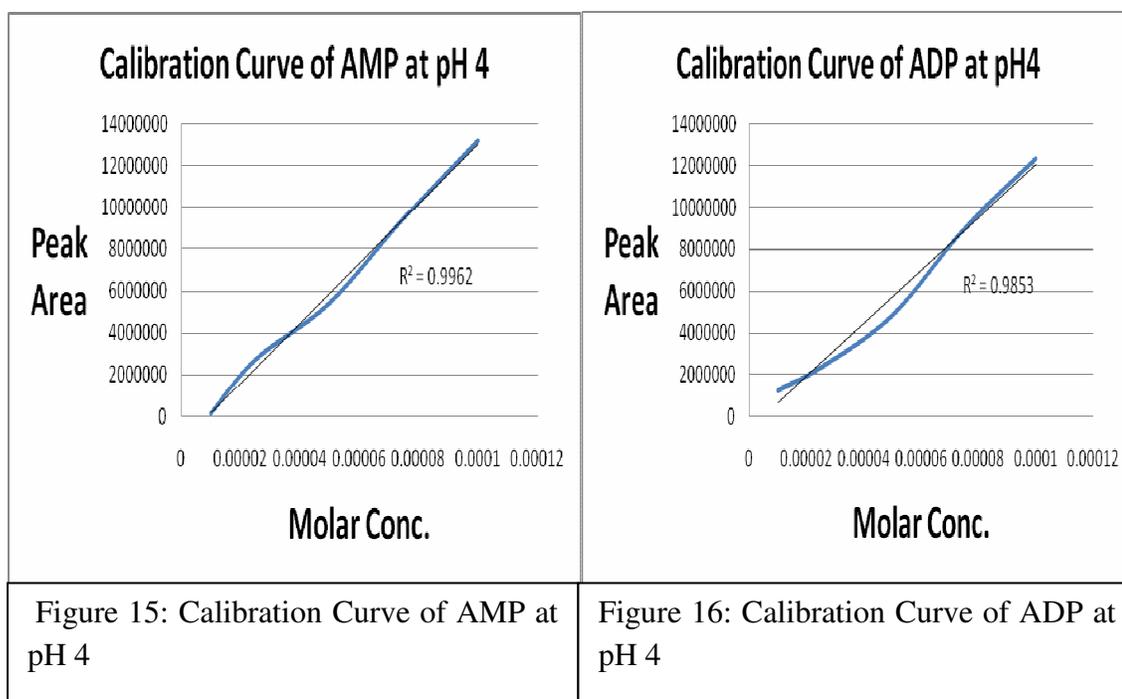
After treatment of ATP, ADP and AMP together with Zirconia at particular pH some unknown peak was found. In Figure 14 there is the unknown peak which is shown by an arrow and all other peaks are of AMP, ADP and ATP.

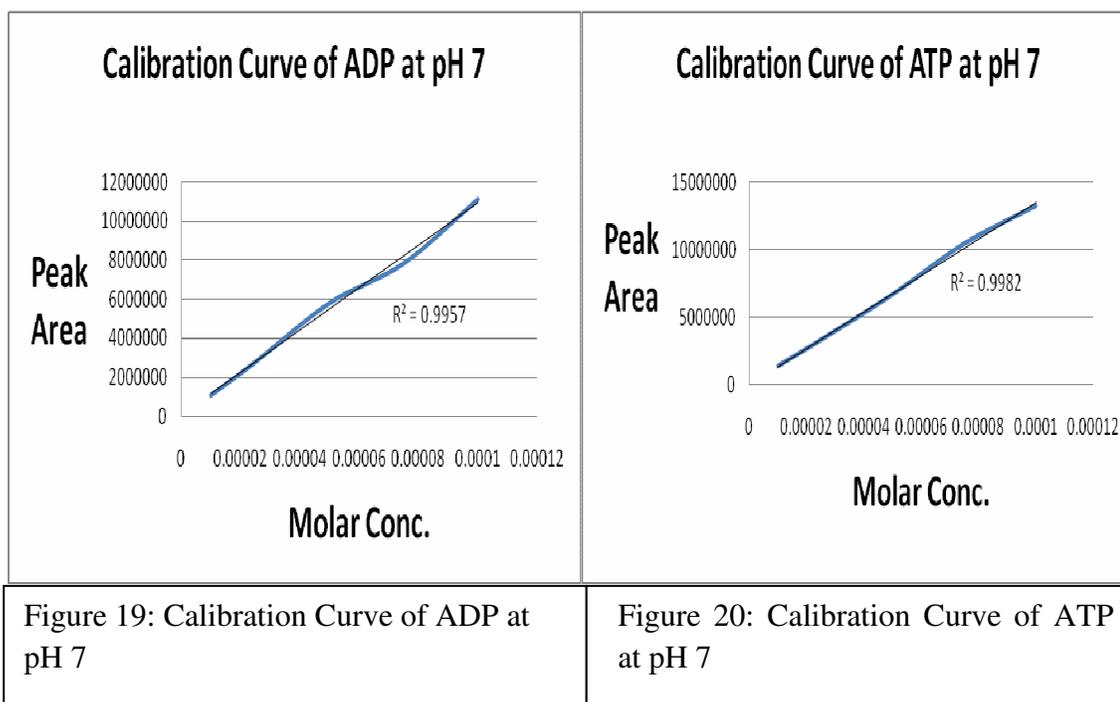
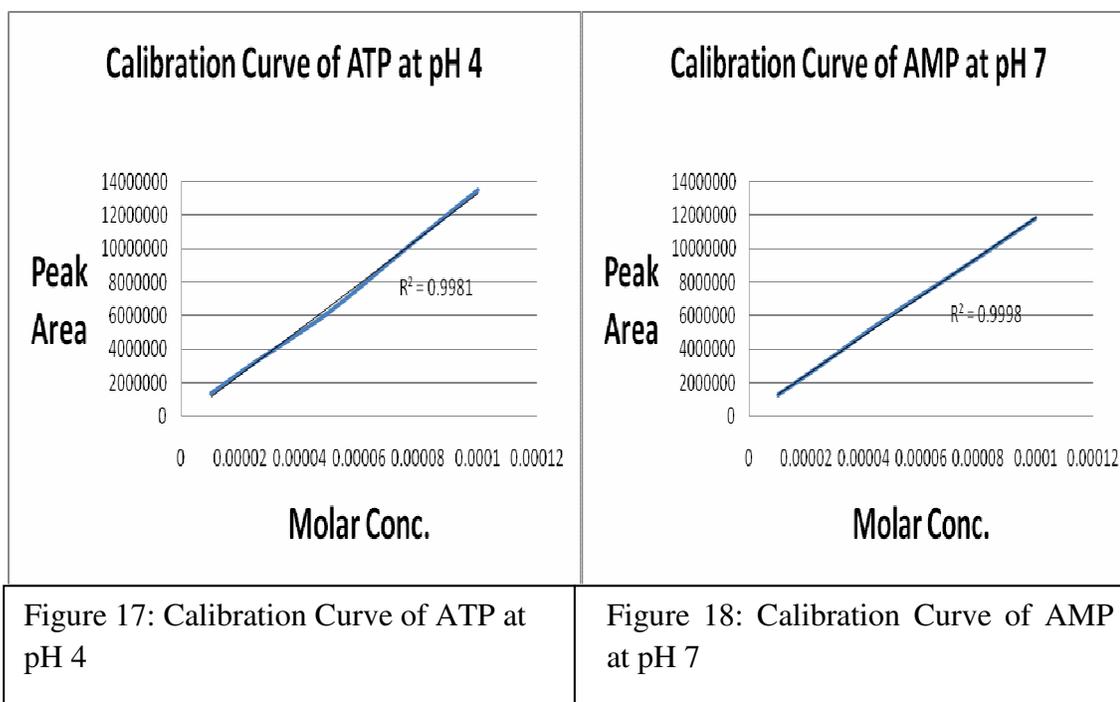
g. Calibration:

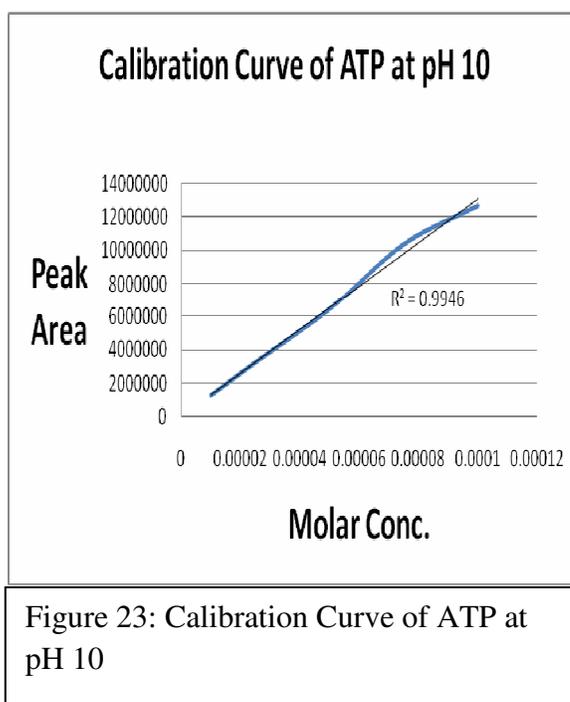
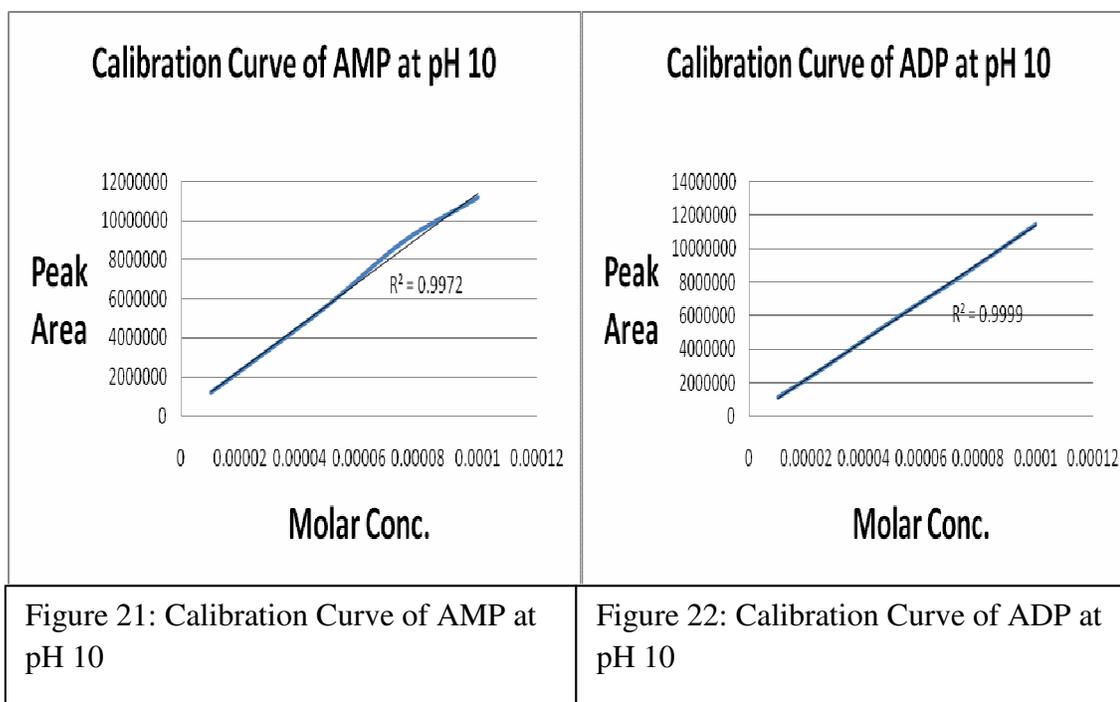
Following solutions with given concentrations were used for setting the Calibration Curve.

1. 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ATP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ADP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of AMP at pH 4 buffer.
2. 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ATP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ADP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of AMP at pH 7 buffer.
3. 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ATP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of ADP + 0.00001M, 0.000025M, 0.00005M, 0.000075M, 0.0001M of AMP at pH 10 buffer.

h. Calibration Curves:







Solid Phase Extraction:

The aim of this experiment is to confirm the absorbance of the Adenosine Phosphates species on the surface of Zirconia 500 at basic pH.

a. Experimental Procedure:

One and a half mL volume of Zirconia 500 was packed in the solid phase extraction column. The Zirconia 500 was cleaned with 10 mL of 1.0 M of NH_4OH followed by 10 mL of 1% acetic acid and finally by 10 mL of nano pure water. Flow rate was 150 mL /Hr. After cleaning the analysis was started by passing a solution of 3.3 mL of 0.0001 M AMP, 3.3 mL of 0.0001M ADP and 3.3 mL of 0.0001 M ATP in pH 10 buffer and was collected and preserved for analysis in a falcon tube. Finally, 2 mL of 0.1 M of NH_4OH was passed through the column to collect the absorbed and unreacted Adenosine Phosphate species and was collected and preserved for analysis in a small vial. This whole experiment was repeated three times to test for repeatability. After collection, all samples were centrifuged at 10000 rpm for 10 minutes to settle the unwanted substance and supernatant was collected for analysis.

Zirconia for purification of FGF:

a. Pre-Culture:

Five mL of Lysogeny broth (LB) medium was poured. Five μ L of Ampicillin and Chloramphenicol was added to avoid the growth of unwanted microbes. Then, 1000 μ L of *E. coli* glycerol stock was added. The sample was placed in the incubator shaker at 37 °C at 170 rpm for 14 hours. After 14 hours, this preculture was added to one liter of LB broth medium and followed by addition of 1000 μ L of Ampicillin and Chloramphenicol. This culture was kept in the incubator at 37 °C at 170 rpm. After three hours, one mL of 0.8 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added. This culture was grown for a total of nine hours to reach the maximum population of *E. coli*. After incubation, the culture was centrifuged in a 500 mL centrifuge bottle for seven minutes at 6000 rpm. After centrifugation, the supernatant was discarded and the remaining pellet was resuspended in 25 mL of Phosphate Buffer (pH 7.2) and stored at -80 °C.

b. Sonification:

FGF, which was stored at -80 °C, was sonified with 80 strokes per three minutes with a break of five minutes after 80 strokes. Amplitude was 60 and pulse was on (20 sec) and off (5 sec). This whole process was done in ice and the sonicator was cleaned with alcohol and nanowater before use.

c. Purification:

After the sonification sample was centrifuged, 1 mL of supernatant and some part of the pellet (precipitated substance) was preserved for gel analysis with the remaining supernatant and pellet collected in two different falcon tubes. The supernatant was added to 1.000 g of Zirconia and it was kept in ice for 30 minutes to absorb FGF to the Zirconia.

The samples were then centrifuged 12000 rpm for seven minutes without breaks. The same conditions were used for all centrifugation operation. The supernatants were placed in falcon tubes and to the precipitate (Zirconia + FGF and other protein) 10 mL of 10 mM tris HCl + 50mM of NaCl at pH 7.0 was added (as per experiment, concentrations of buffer and salt were changed). Again, this sample was kept in ice for 30 minutes. The purpose of the salt and buffer was to take out the unwanted protein as well as FGF protein which is attached to Zirconia. After 30 minutes, the Falcon tube was taken out of ice and centrifuged to separate the buffer salt solution from Zirconia + Zirconia attached FGF. Again, a different concentration of buffer containing varying amount of salt were added to the precipitate and same process was repeated. The concentration of salt and buffer is explained with each gel. After the collection of different salt buffer solution, whole falcon tubes were centrifuged and one mL of supernatant was collected for gel analysis. Figure 14 illustrates the purification process.

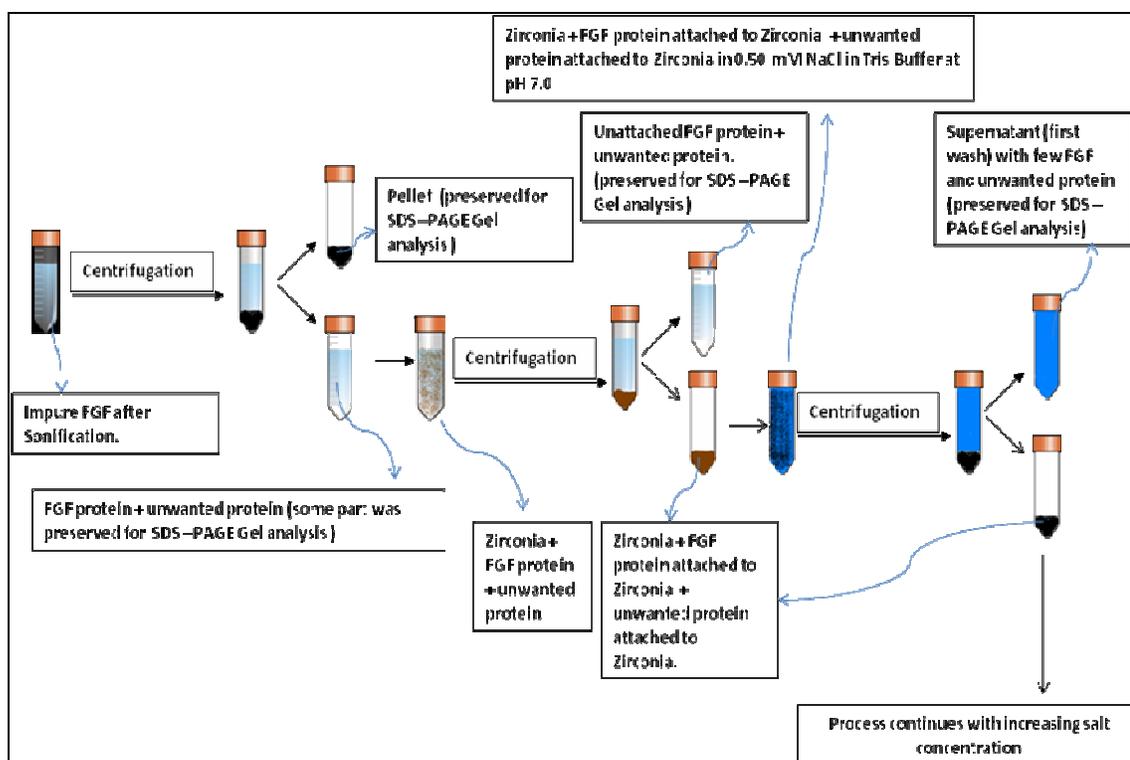


Figure 24: Schematic representation of Purification process

c. Sample Preparation before Gel Analysis.

To the collected supernatant, 100 μ L Trichloro Acetic Acid (TCA) was added to precipitate the dissolved proteins. The sample was kept in ice for five minutes and centrifuged at 11000 rpm for four minutes. The supernatant was discarded and to the pellet, 200 μ L of acetone was added and kept for five minutes to wash the precipitate protein and then vortexed and centrifuged at 11000 rpm for four minutes. The supernatant was discarded and to the pellet, 15 μ L of eight M urea was added to denature the protein. Then, five μ L of loading dye was added to the denatured protein to stain the protein. The appendorf (which is a small centrifuge tube) containing the stained protein was vortexed and centrifuged for four minutes at 11000 rpm. Then the appendorfs were heated at 80 $^{\circ}$ C to 90 $^{\circ}$ C for one minute and centrifuged for one minute at 11000 rpm. After this the sample was ready for analysis.

Chemicals in sample preparation:

Chemicals	Method of preparation	Use
Loading Dye	5.4 mL of Nano pure water + 0.6 mL of 0.5 M Tris pH 6.8+ 1.0 mL of glycerol+ 2.0 mL of 10 % SDS + 0.5 mL of 0.1% Bromophenol Blue	For staining precipitated protein
0.1% Bromophenol Blue in 50 mL	50 mg in 50 mL of nanopure water	For preparation of loading dye about 2X dye.
2X dye	475 μ L of Loading Dye to microcentrifuge tube + 25 μ L of Mercaptoethanol.	To stain the prepared gel which is having separated protein
Running Buffer for 1 liter	30.8 g Tris Base + 144 g Glycine + SDS 10 g	To maintain the pH of system this is having running gel.
Distaining Buffer for 1 liter	75 mL of Glacial Acetic Acid + 150 mL of Methanol + 775 mL of Nano Pure water.	To distain the prepared gel.(which do distain protein)

Table 8: Chemicals in sample preparation

d. Preparation of GEL and Sample Running:

Chemicals	Method of preparation	SDS-PAGE 15 % Separation gel	SDS-PAGE 5 % Stacking Gel	Function
Nano Pure water	From Biotech center	1.175 mL	1.97 mL	As a solvent
1 M Tris Buffer	121.14 g in 1000 mL water and pH was adjusted as per required.	1.87 mL (pH 8.8)	310 μ L (pH 6.8)	Buffer to maintain respective pH.
10% SDS (Sodium lauryl sulfate)	To 100 g of SDS sufficient water was added to make 1000 mL of solution.	80 μ L	25 μ L	Gives negative charge to precipitated protein.
40 % Acrylamide	To 40 g of Acrylamide sufficient water was added to make 100 mL of solution.	1.875 mL	312 μ L	Forms mesh like structure in gel.
10 %APS (Ammonium persulphate)	To 10 g of APS sufficient water was added to make 100 mL of solution.	25 μ L	12.5 μ L	APS is an initiator for gel formation
TEMED (N, N, N', N'-tetramethylethylenediamine)	Commercially available.	5 μ L	3 μ L	Decides rate of polymerization

Table 9: Chemicals in Gel Preparation

In SDS-PAGE, there are two parts: The upper part is called the stacking gel and lower part is the separating gel. In Table 8 and Table 9, the amount of chemicals which are required for the synthesis of these Gels is given. The separating gel should be prepared and poured into a gel cassette. After formation of the solid separating gel, the stacking gel should be prepared and poured over the separating gel. To make the ten wells, a comb should be fixed in stacking gel. After formation of the solid stacking gel, the gel cassette should be placed in the negative electrode chamber and the whole assembly should be placed in a tank. Running buffer should then be poured in the tank and the prepared sample placed in the wells. The proper electrode should be placed at the proper position and voltage, ampere and time should be set in such a way that the gel should not over run. Ideal conditions are 170 voltage, 40 ampere and 75 minutes. Figure 25 gives a brief idea about preparation of gel and running of sample.

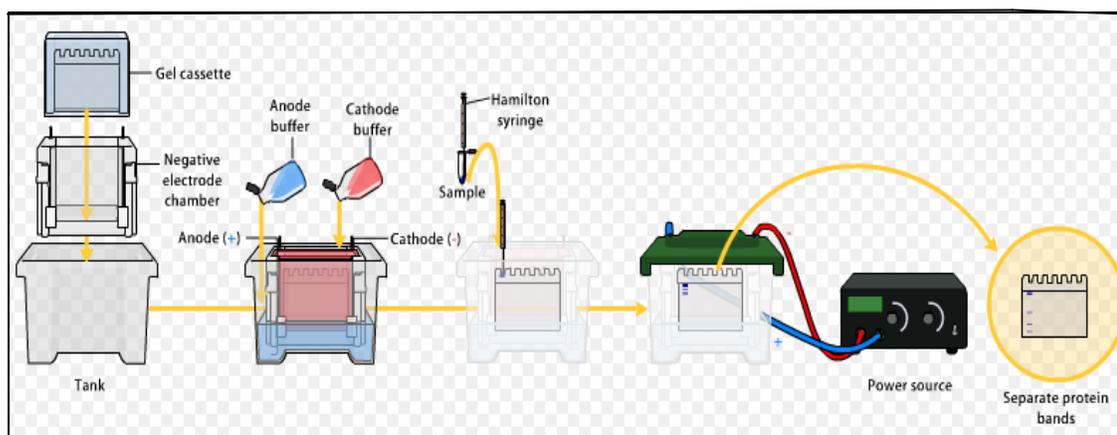


Figure 25: Preparation of Gels

SEM Study

The SEM studies were done using the Ogden College JEOL 5400LV Scanning Electron Microscope. The surface characteristic of Zirconia after synthesis were examined as well as the effect of various Adenosine phosphate species on the surface of Zirconia after both the kinetics and florescence study was done. The effect of the FGF protein on the surface of Zirconia was also examined.

Sample preparation:

Elemental Analysis: Zirconia was mounted on sticky black electrical tape and then examined at low pressure mode for elemental analysis.

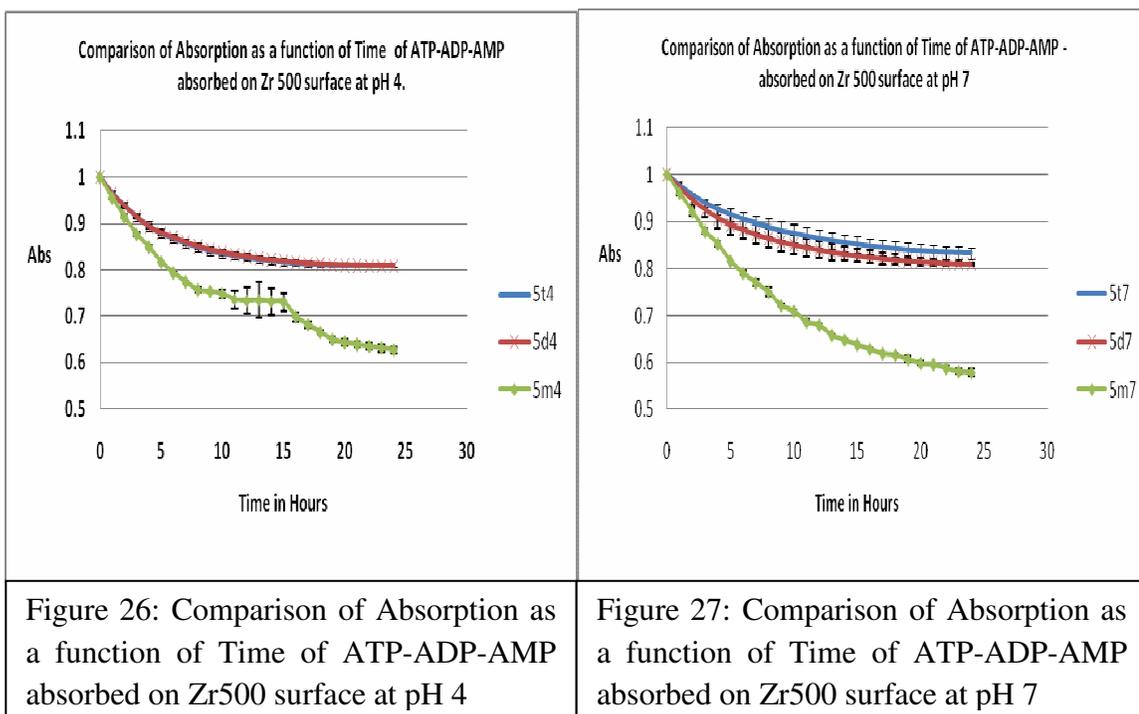
SEM Images: As Zirconia is a non-conductive material, it was first gold coated and then examined at resolutions of 2000 and 10000 for the SEM images.

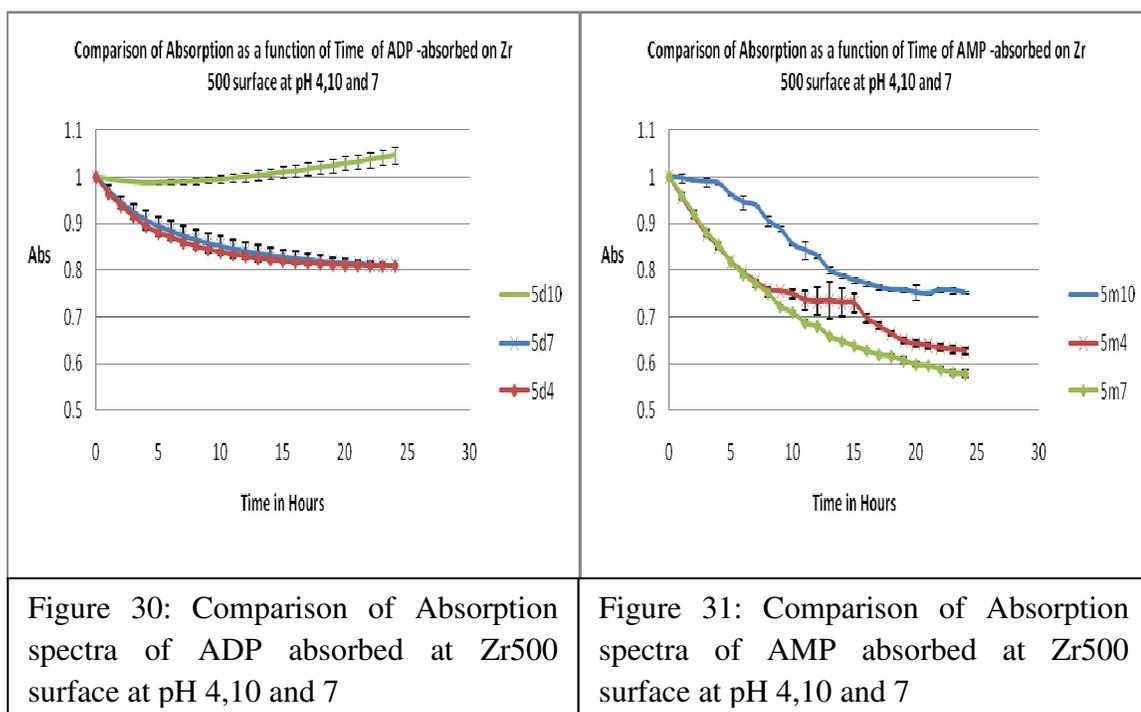
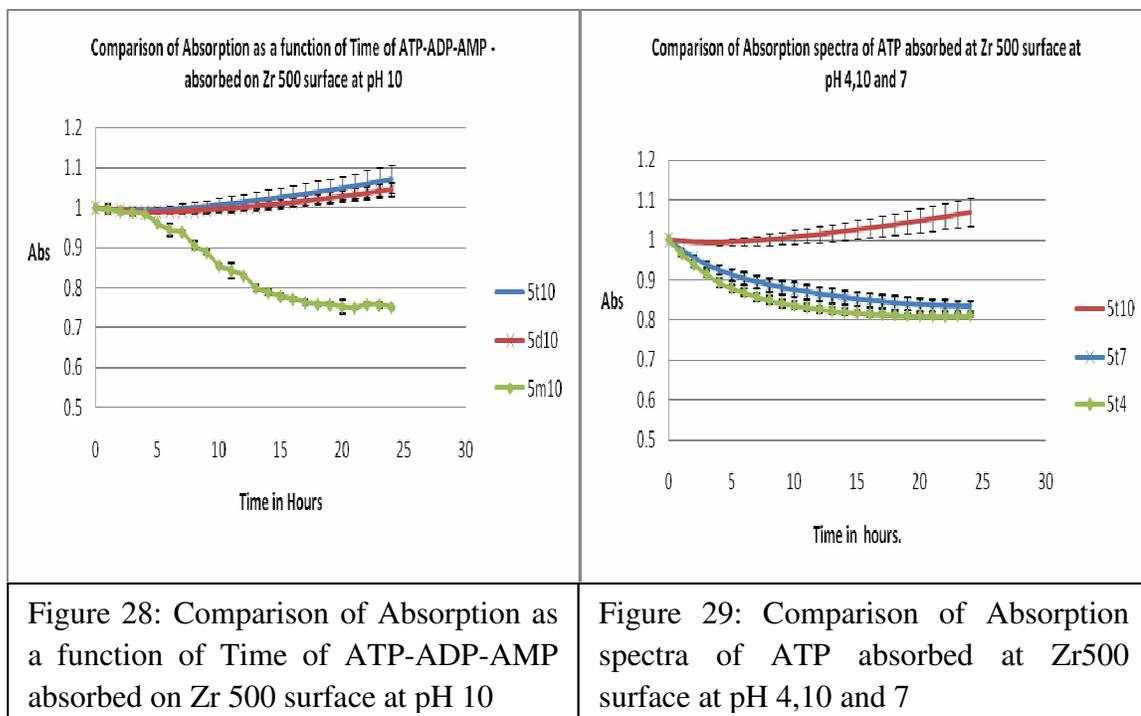
III. RESULT AND DISCUSSION.

Absorption Experiment:

Graphs:

Figures of different Adenosine Phosphate absorbed on Zirconia 500 surfaces at different pH.





Where:

5m4: AMP absorbed on Zr500 at pH 4, 5m7: AMP absorbed on Zr500 at pH 7, 5m10: AMP absorbed on Zr500 at pH 10, 5d4: ADP absorbed on Zr500 at pH 4, 5d7: ADP absorbed on Zr500 at pH 7, 5d10: ADP absorbed on Zr500 at pH 10, 5t4: ATP absorbed on Zr500 at pH 4, 5t7: ATP absorbed on Zr500 at pH 4, 5t10: ATP absorbed on Zr500 at pH 10.

Figures of different Adenosine Phosphate absorbed on Zirconia 700 surface at different pH.

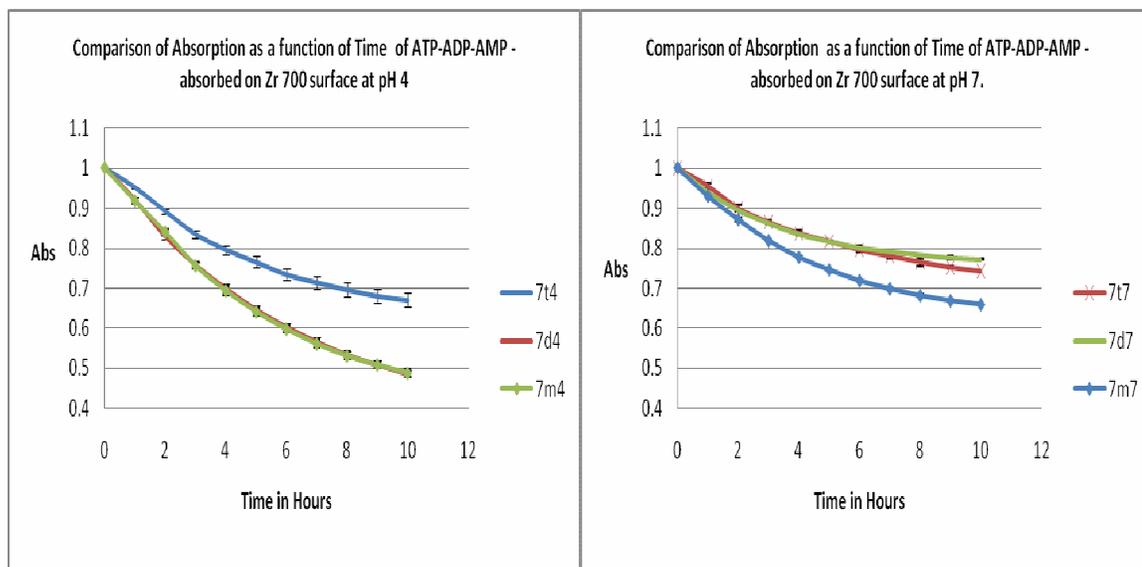


Figure 32: Comparison of Absorption as a function of Time of ATP-ADP-AMP absorbed on Zr700 surface at pH 4

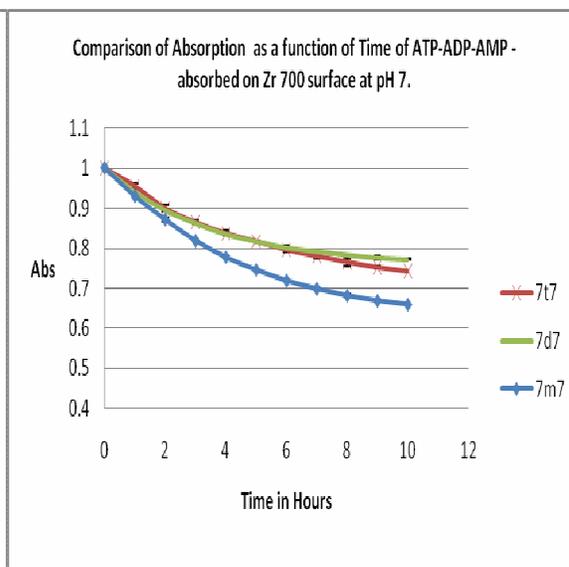


Figure 33: Comparison of Absorption as a function of Time of ATP-ADP-AMP absorbed on Zr700 surface at pH 7

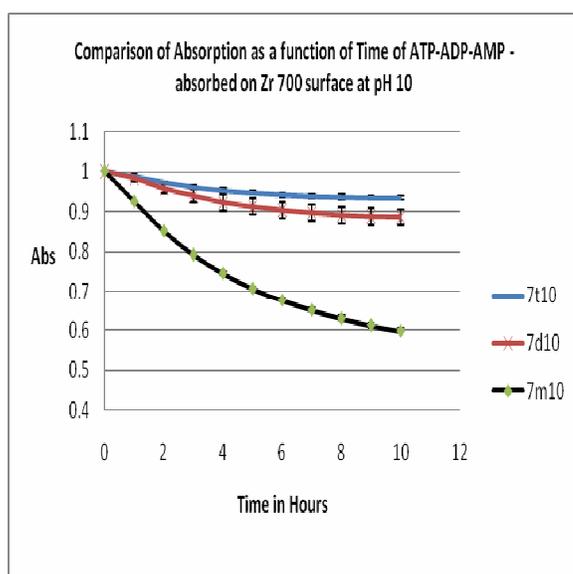


Figure 34: Comparison of Absorption as a function of Time of ATP-ADP-AMP absorbed on Zr700 surface at pH 10

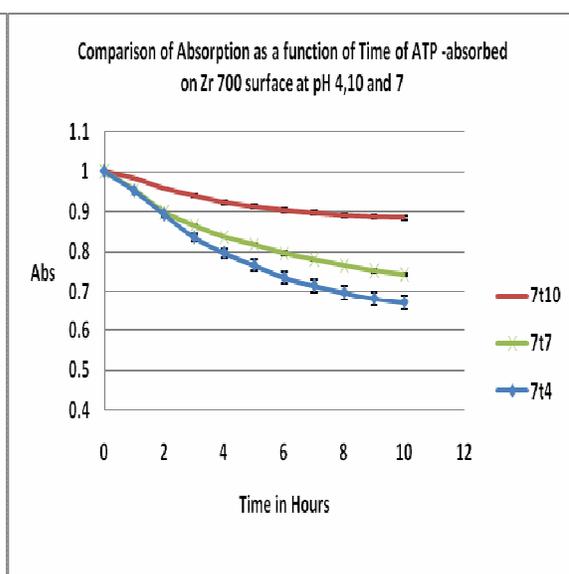
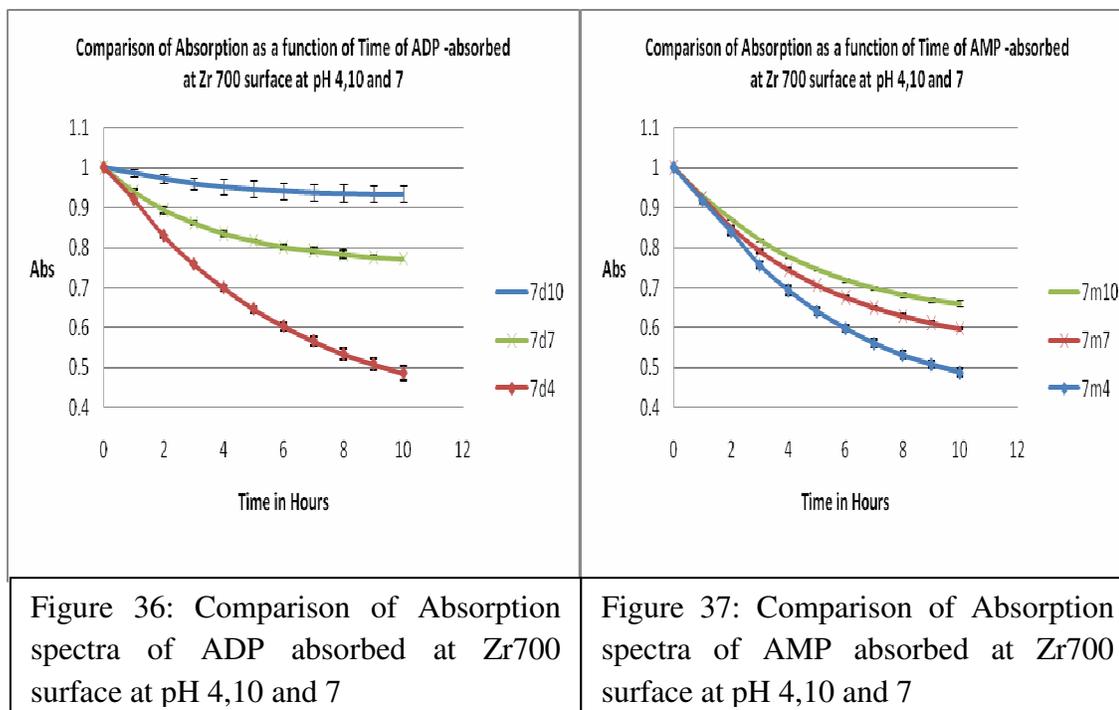


Figure 35: Comparison of Absorption spectra of ATP absorbed at Zr700 surface at pH 4,10 and 7



Where:

7m4: AMP absorbed on Zr700 at pH 4, 7m7: AMP absorbed on Zr700 at pH 7, 7m10: AMP absorbed on Zr700 at pH 10, 7d4: ADP absorbed on Zr700 at pH 4, 7d7: ADP absorbed on Zr700 at pH 7, 7d10: ADP absorbed on Zr700 at pH 10, 7t4: ATP absorbed on Zr700 at pH 4, 7t7: ATP absorbed on Zr700 at pH 4, 7t10: ATP absorbed on Zr700 at pH 10.

Results:

Comparison of Absorption as a function of time was done. Figure 26 to Figure 37 clearly shows that for both Zr500 and Zr700 the surface absorption trend is AMP >ADP>ATP. In the same manner where pH is concerned, on both Zr500 and Zr700 surface absorption sequence is pH4>pH 7>pH10. Zirconia surface may get positively charged due to the acidic pH and thus it may attract negatively charged phosphate group more effectively than neutral and basic pH.

Fluorescence Experiment:

Graphs:

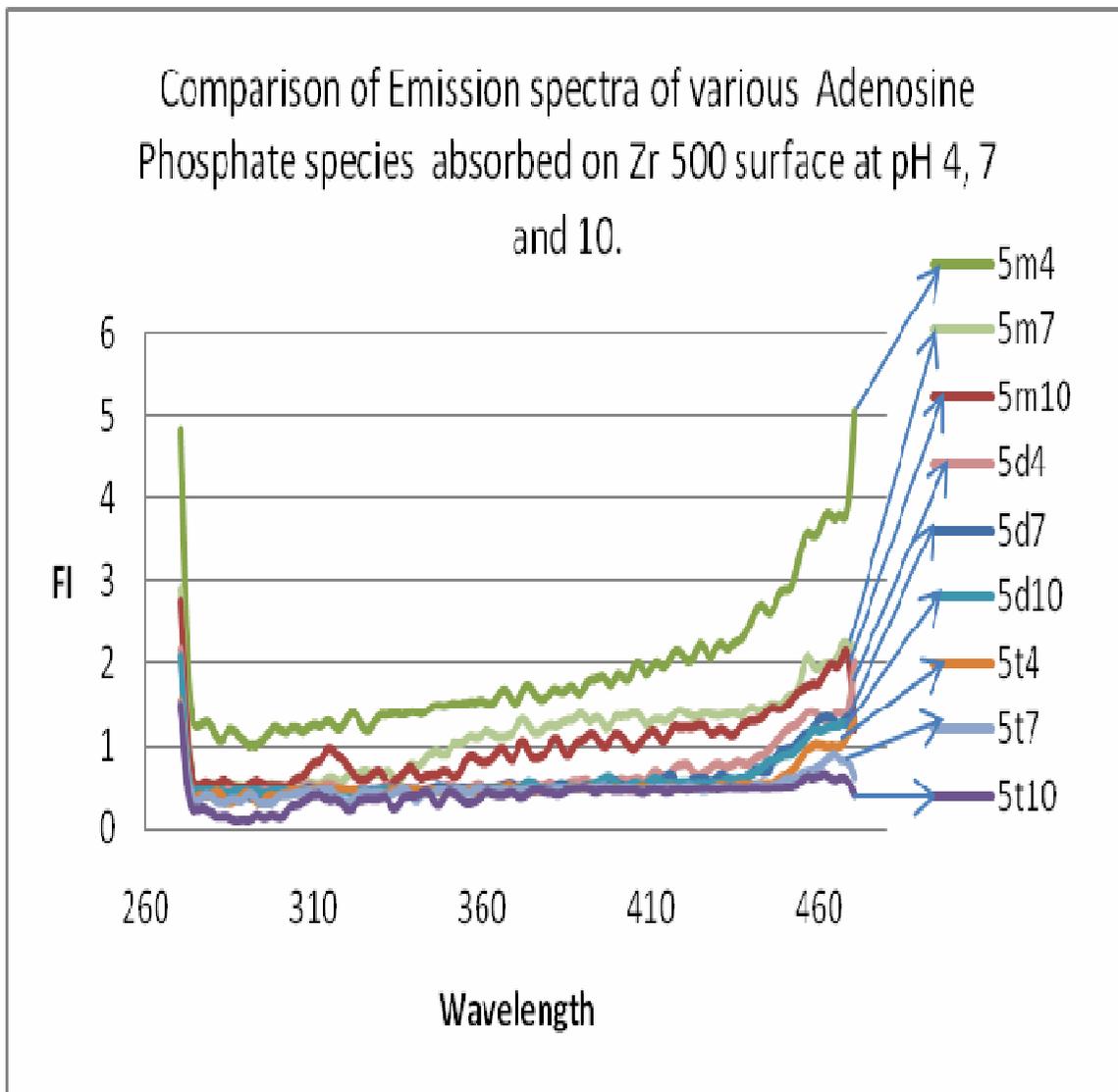


Figure 38: Comparison of Emission spectra of various Adenosine Phosphate species absorbed on Zr500 surface at pH 4, 7 and 10.

Where:

5m4: AMP absorbed on Zr500 at pH 4, 5m7: AMP absorbed on Zr500 at pH 7,
 5m10: AMP absorbed on Zr500 at pH 10, 5d4: ADP absorbed on Zr500 at pH 4,
 5d7: ADP absorbed on Zr500 at pH 7, 5d10: ADP absorbed on Zr500 at pH 10,
 5t4: ATP absorbed on Zr500 at pH 4, 5t7: ATP absorbed on Zr500 at pH 4, 5t10:
 ATP absorbed on Zr500 at pH 10.

Observation: The spectra clearly shows that the surface absorption trend is
 5m4>5m7>5m10>5d4>5d7>5d10>5t4>5t7>5t10.

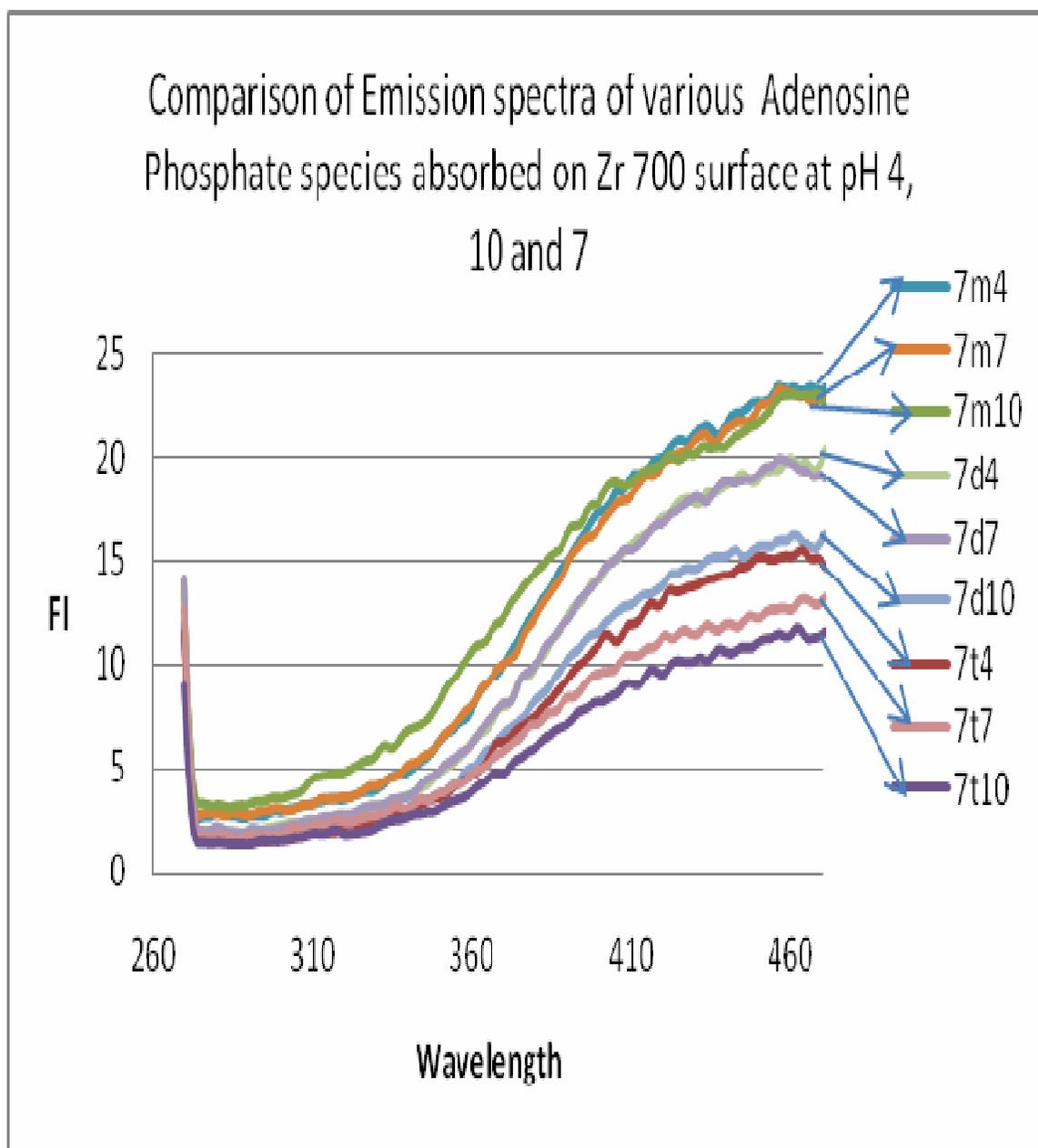


Figure 39: Comparison of Emission spectra of various Adenosine Phosphate species absorbed on Zr 700 surface at pH 4, 7 and 10.

Where:

7m4: AMP absorbed on Zr700 at pH 4, 7m7: AMP absorbed on Zr700 at pH 7,
 7m10: AMP absorbed on Zr700 at pH 10, 7d4: ADP absorbed on Zr700 at pH 4,
 7d7: ADP absorbed on Zr700 at pH 7, 7d10: ADP absorbed on Zr700 at pH 10,
 7t4: ATP absorbed on Zr700 at pH 4, 7t7: ATP absorbed on Zr700 at pH 4, 7t10:
 ATP absorbed on Zr700 at pH 10.

Observation: The spectra clearly shows that the surface absorption trend is
 $7m4 > 7m7 > 7m10 > 7d4 > 7d7 > 7d10 > 7t4 > 7t7 > 7t10$.

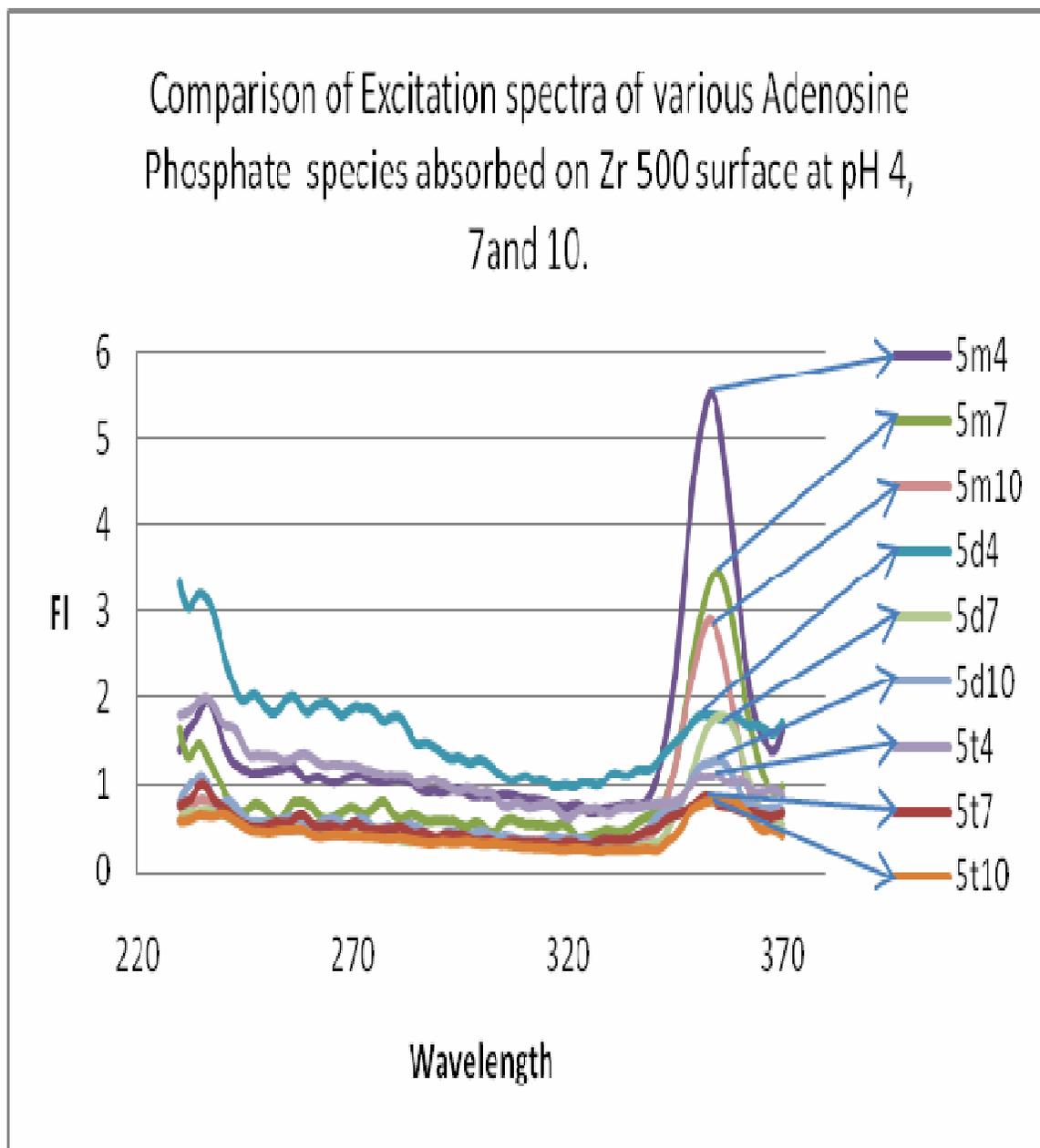


Figure 40: Comparison of Excitation spectra of various Adenosine Phosphate species absorbed on Zr500 surface at pH 4,7 and 10.

Where:

5m4: AMP absorbed on Zr500 at pH 4, 5m7: AMP absorbed on Zr500 at pH 7,
 5m10: AMP absorbed on Zr500 at pH 10, 5d4: ADP absorbed on Zr500 at pH 4,
 5d7: ADP absorbed on Zr500 at pH 7, 5d10: ADP absorbed on Zr500 at pH 10,
 5t4: ATP absorbed on Zr500 at pH 4, 5t7: ATP absorbed on Zr500 at pH 4, 5t10:
 ATP absorbed on Zr500 at pH 10.

Observation: The spectra clearly shows that the surface absorption trend is
 5m4>5m7>5m10>5d4>5d7>5d10>5t4>5t7>5t10.

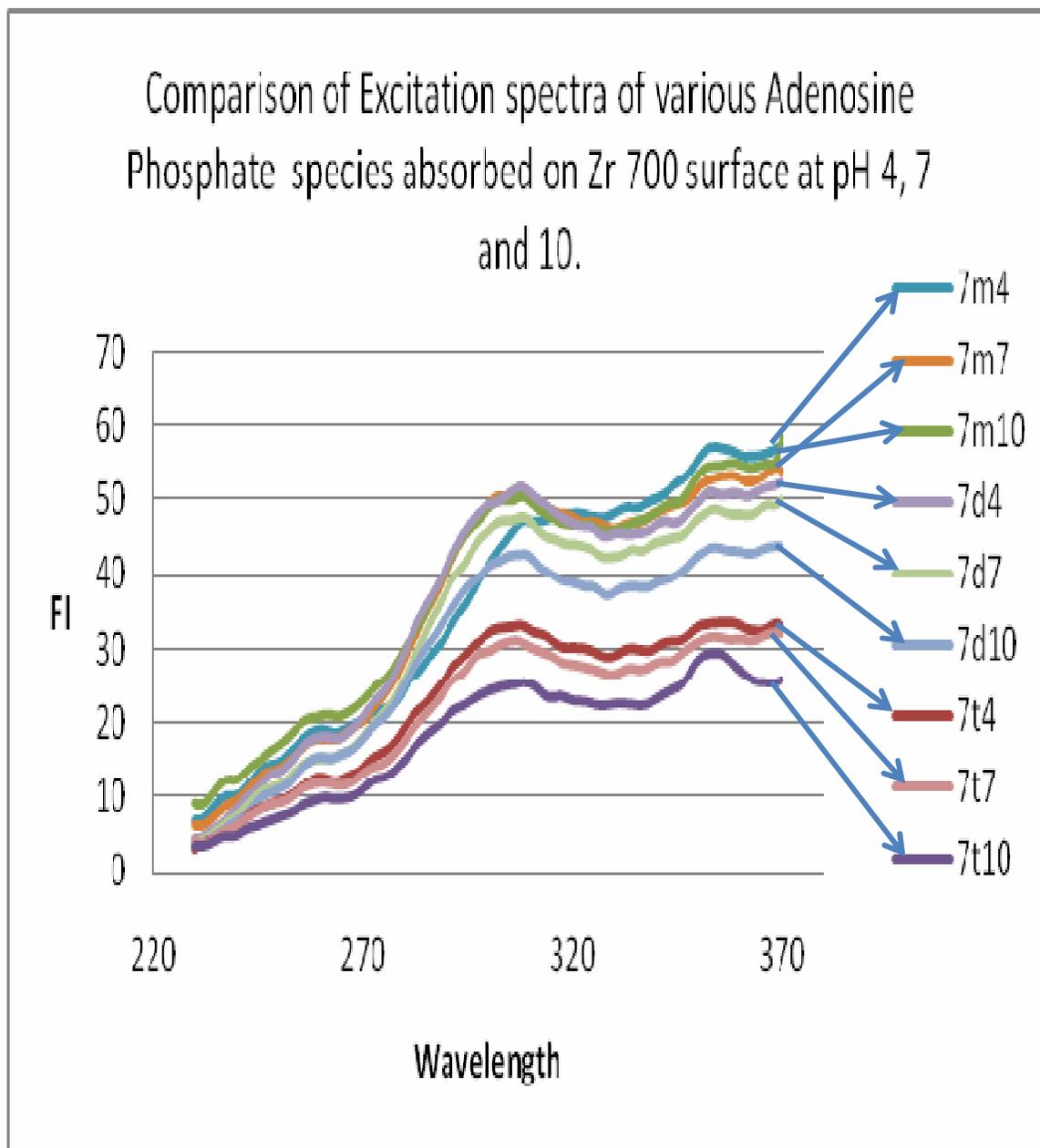


Figure 41: Comparison of Excitation spectra of various Adenosine Phosphate species absorbed on Zr700 surface at pH 4,7and 10.

Where:

7m4: AMP absorbed on Zr700 at pH 4, 7m7: AMP absorbed on Zr700 at pH 7,
 7m10: AMP absorbed on Zr700 at pH 10, 7d4: ADP absorbed on Zr700 at pH 4,
 7d7: ADP absorbed on Zr700 at pH 7, 7d10: ADP absorbed on Zr700 at pH 10,
 7t4: ATP absorbed on Zr700 at pH 4, 7t7: ATP absorbed on Zr700 at pH 4, 7t10:
 ATP absorbed on Zr700 at pH 10.

Observation: The spectra clearly shows that the surface absorption trend is
 $7m4 > 7m7 > 7m10 > 7d4 > 7d7 > 7d10 > 7t4 > 7t7 > 7t10$.

Results:

The following Excitation and Emission spectra were collected:

a. Emission spectra of various Adenosine Phosphate species absorbed on Zr500 species surface at pH 4, 7 and 10:

AMP, ADP, ATP at pH 4; AMP, ADP, ATP at pH 7; AMP, ADP, ATP at pH 10.

b. Emission spectra of various Adenosine Phosphate species absorbed on Zr700 species surface at pH 4, 7 and 10:

AMP, ADP, ATP at pH 4; AMP, ADP, ATP at pH 7; AMP, ADP, ATP at pH 10.

c. Excitation spectra of various Adenosine Phosphate species absorbed on Zr500 species surface at pH 4, 7 and 10:

AMP, ADP, ATP at pH 4; AMP, ADP, ATP at pH 7; AMP, ADP, ATP at pH 10.

d. Excitation spectra of various Adenosine Phosphate species absorbed on Zr700 species surface at pH 4, 7 and 10:

AMP, ADP, ATP at pH 4; AMP, ADP, ATP at pH 7; AMP, ADP, ATP at pH 10.

Comparison of Florescence spectra was done. Figure 38 to Figure 41 clearly shows that for both Zr500 and Zr700 the surface absorption trend is AMP >ADP>ATP. In the same manner where pH is concerned, on both Zr500 and Zr700 surface absorption sequence is pH4>pH7>pH10. Zirconia surface may get positively charged due to the acidic pH and, thus, it may attract negatively charged phosphate group more effectively than at neutral and basic pH.

Separation Study:

- i. Results of different solutions treated with Zirconia 500.
1. Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 4) treated with Zirconia 500.

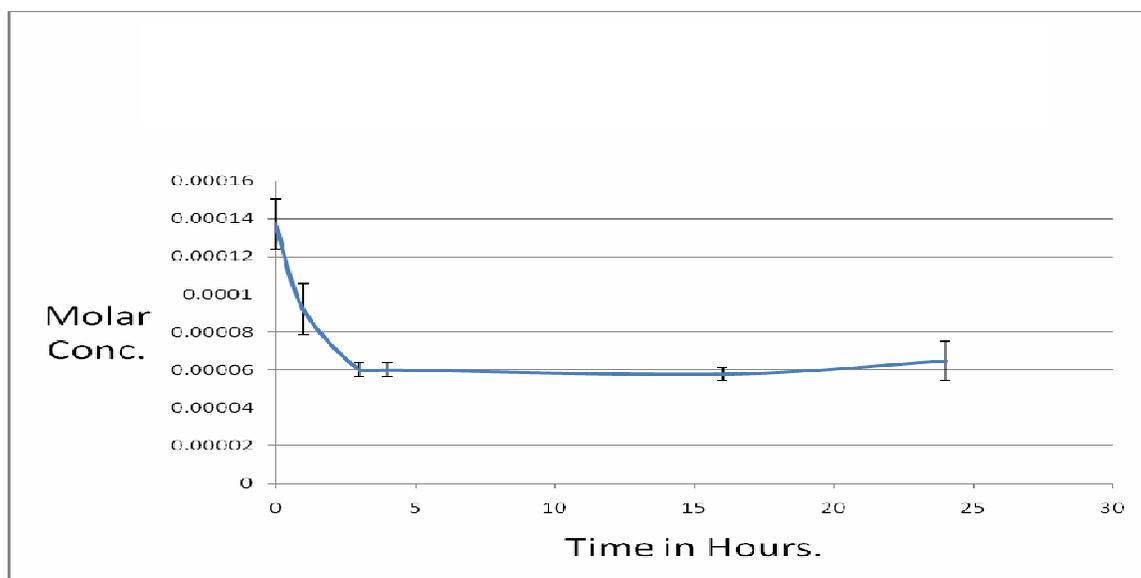


Figure 42: Absorption as function of time – AMP (Peak 1) of Solution No. A (ATP+ADP+AMP at pH 4) treated with Zr500

2. Absorption as function of time –Unknown peak of Solution (ATP+ADP+AMP at pH 4) treated with Zirconia 500.

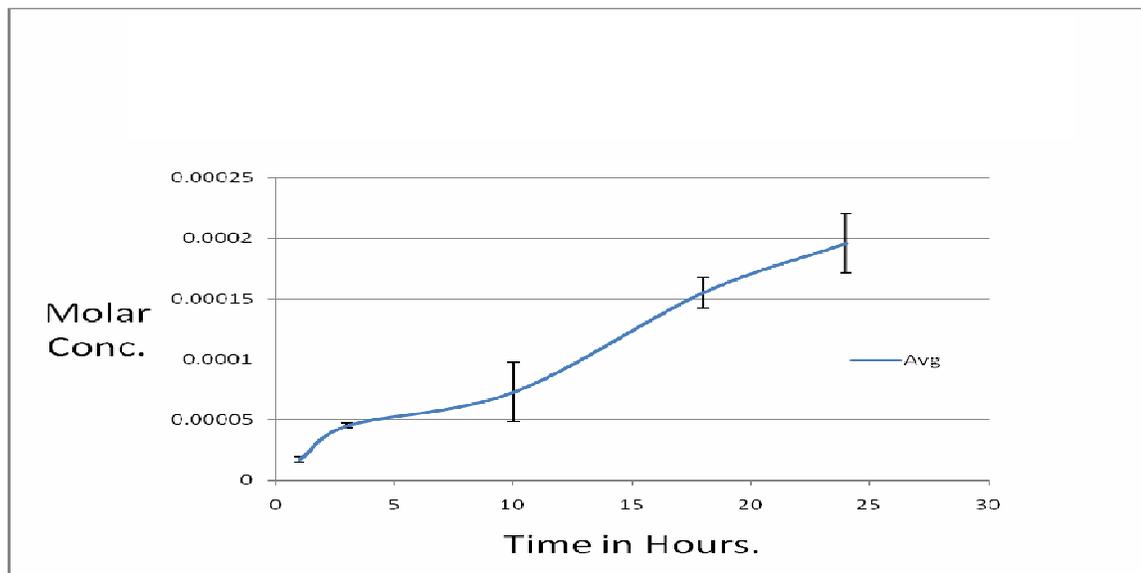


Figure 43: Absorption as function of time –Unknown peak of Solution (ATP+ADP+AMP at pH 4) treated with Zr500

3. Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 7) treated with Zirconia 500.

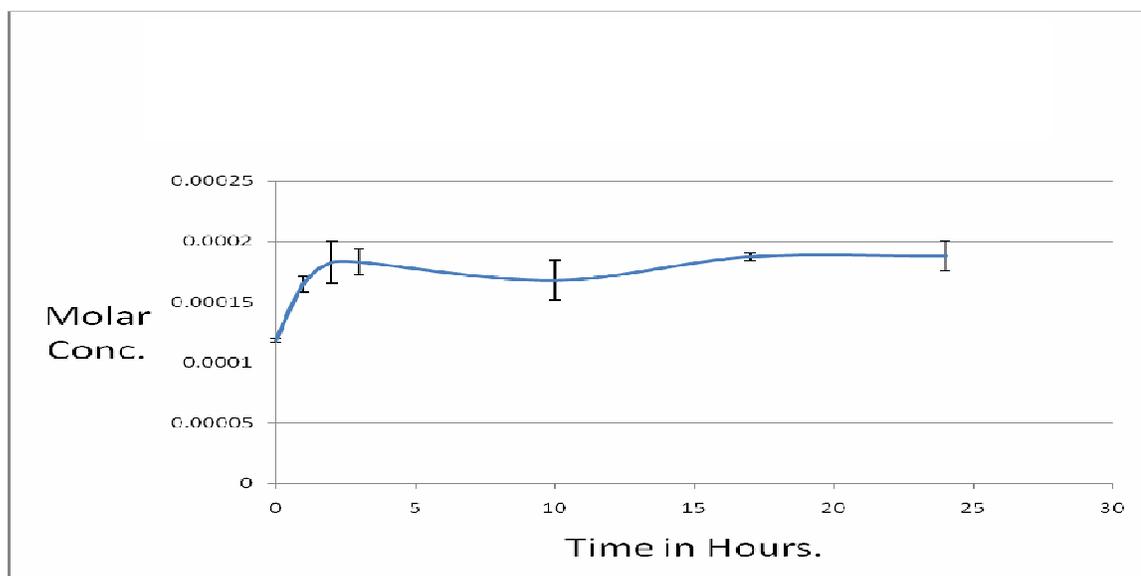


Figure 44: Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 7) treated with Zr500

1. Absorption as function of time – Unknown Peak of Solution No. B (ATP+ADP+AMP at pH 7) treated with Zirconia 500.

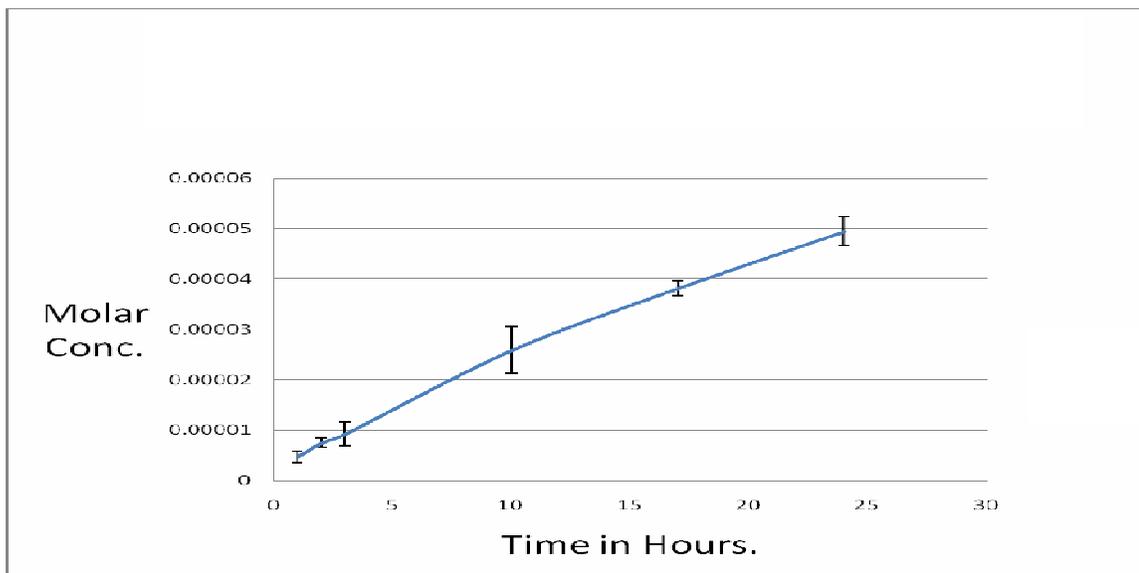


Figure 45: Absorption as function of time – Unknown Peak of Solution No. B (ATP+ADP+AMP at pH 7) treated with Zr500

2. Absorption as function of time –AMP of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 500.

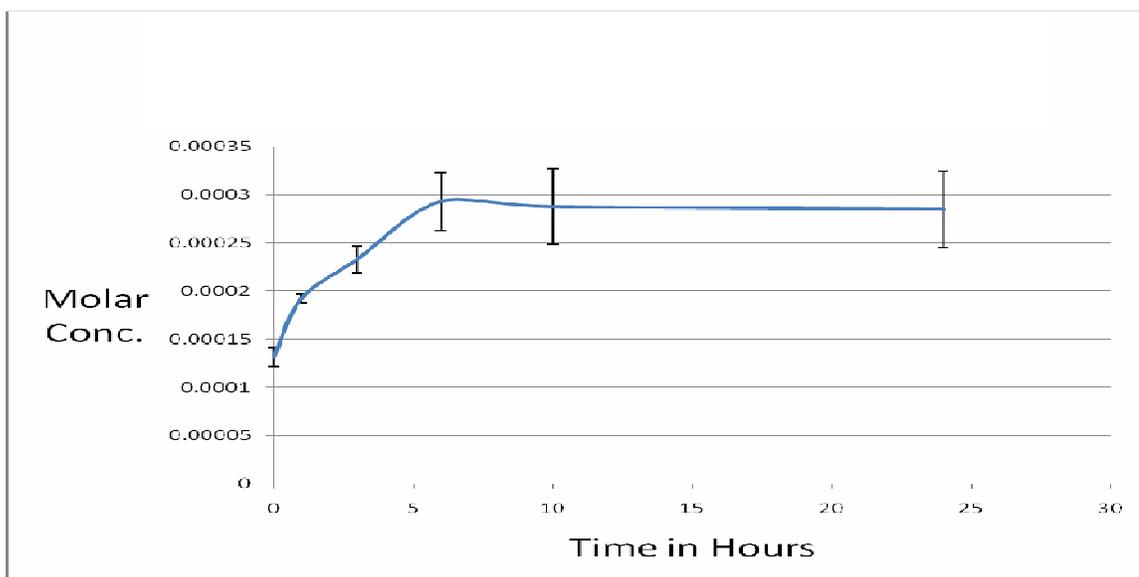


Figure 46: Absorption as function of time –AMP of Solution (ATP+ADP+AMP at pH 10) treated with Zr500

3. Absorption as function of time –ADP of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 500.

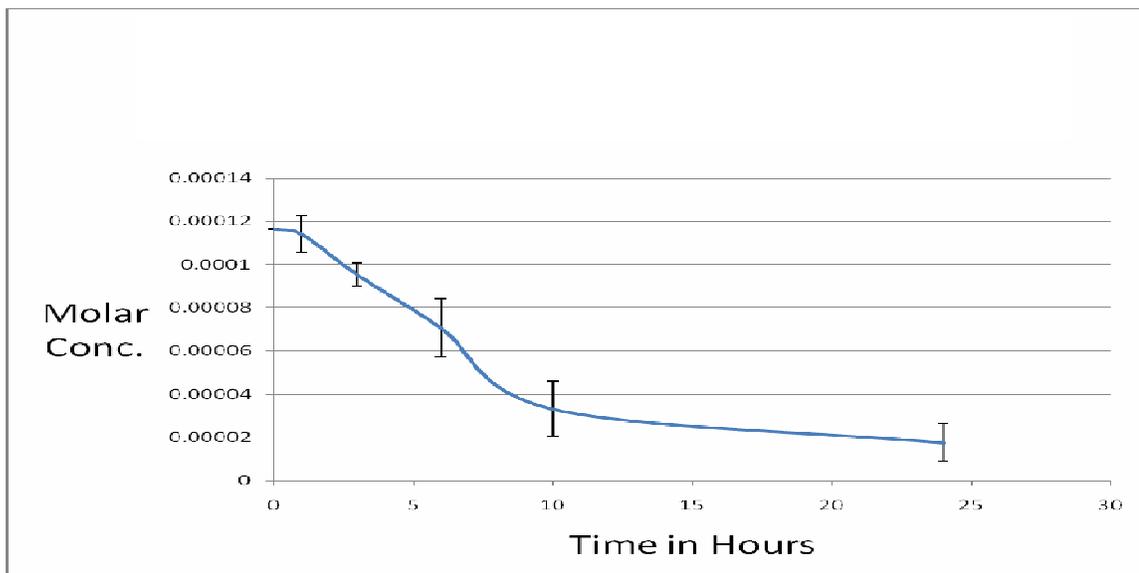


Figure 47: Absorption as function of time –ADP of Solution (ATP+ADP+AMP at pH 10) treated with Zr500

4. Absorption as function of time –ATP of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 500.

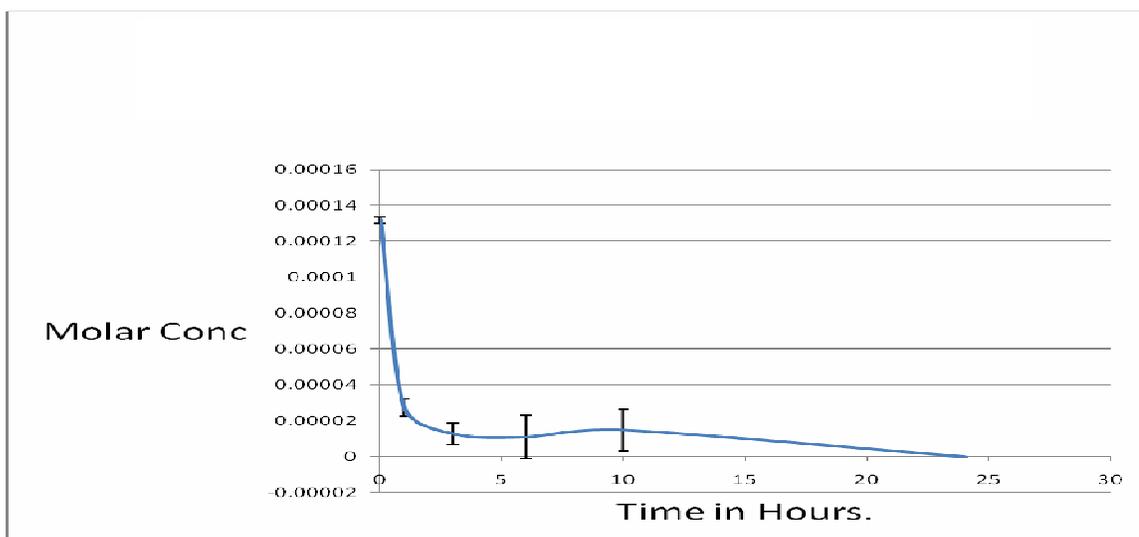


Figure 48: Absorption as function of time –ATP of Solution (ATP+ADP+AMP at pH 10) treated with Zr500

- ii. Result of different solution treated with Zirconia 700.
1. Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 4) treated with Zirconia 700.

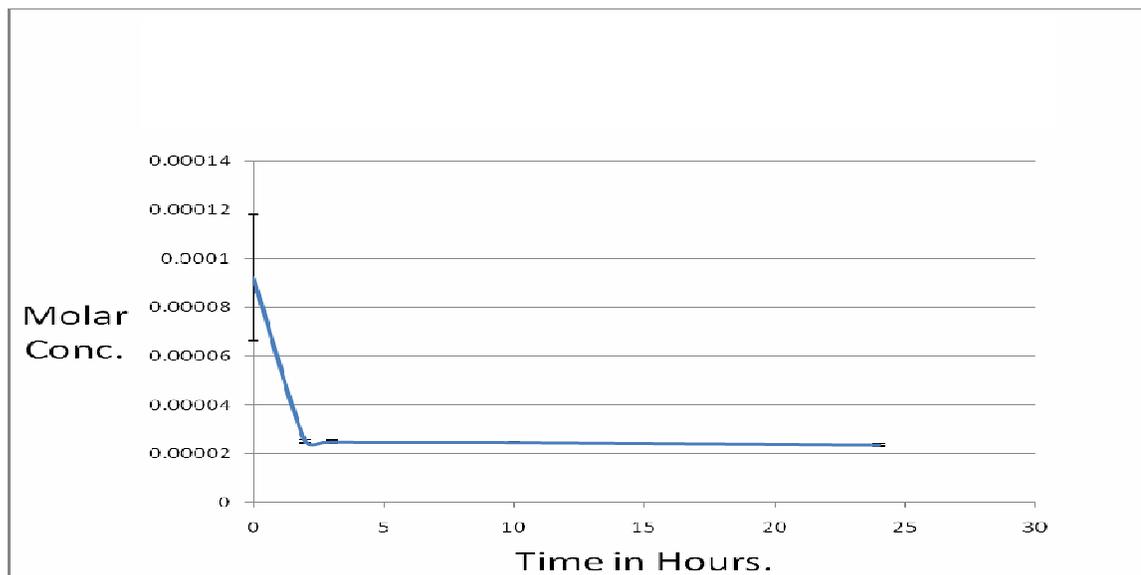


Figure 49: Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 4) treated with Zr700.

2. Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 4) treated with Zirconia 700.

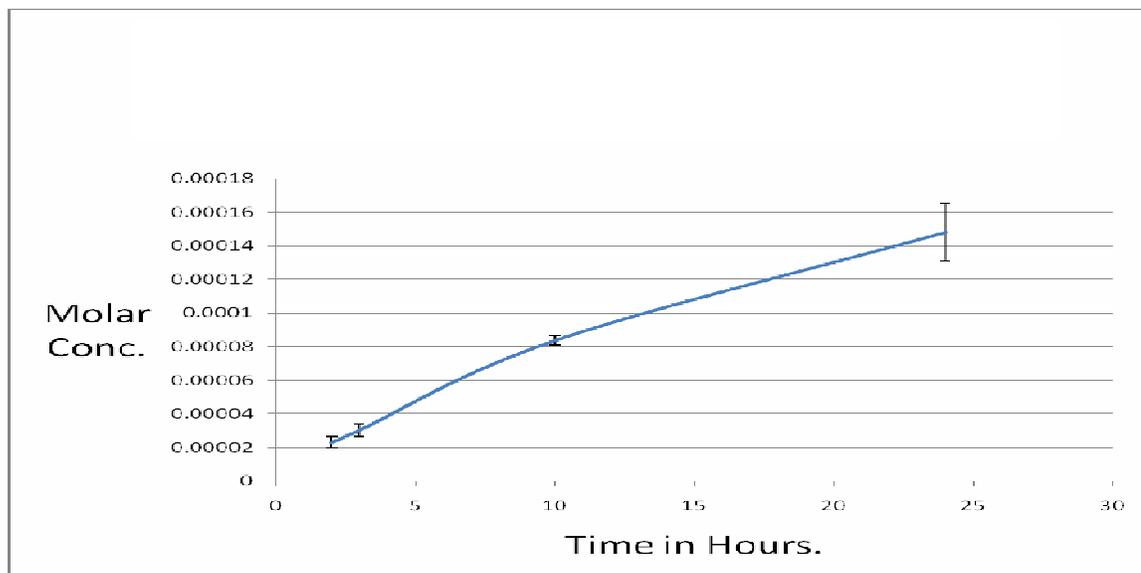


Figure 50: Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 4) treated with Zr 700

3. Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 7) treated with Zirconia 700.

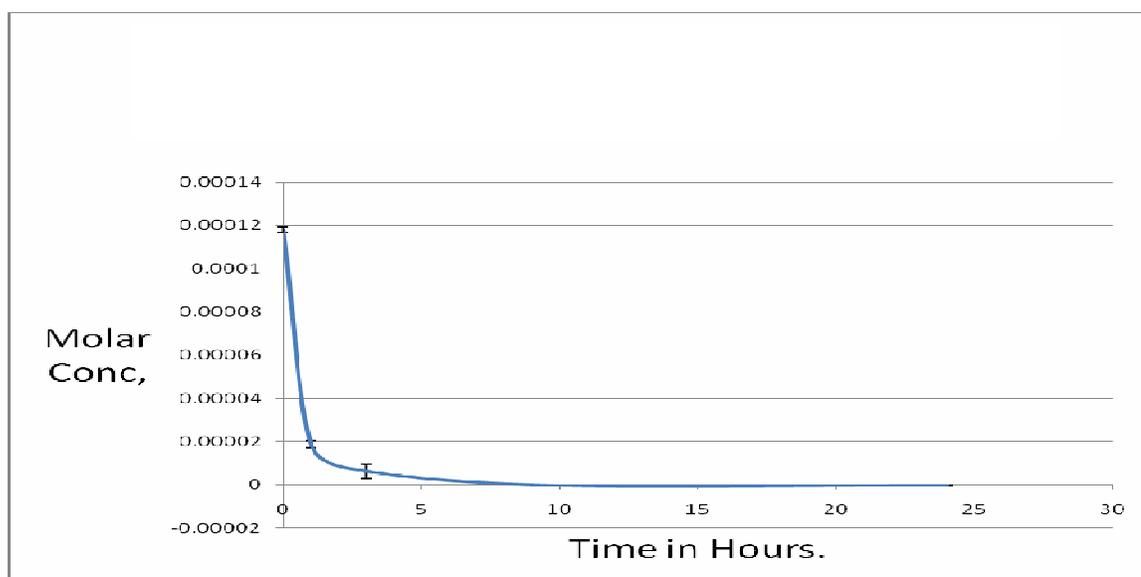


Figure 51: Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 7) treated with Zr700

4. Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 7) treated with Zirconia 700.

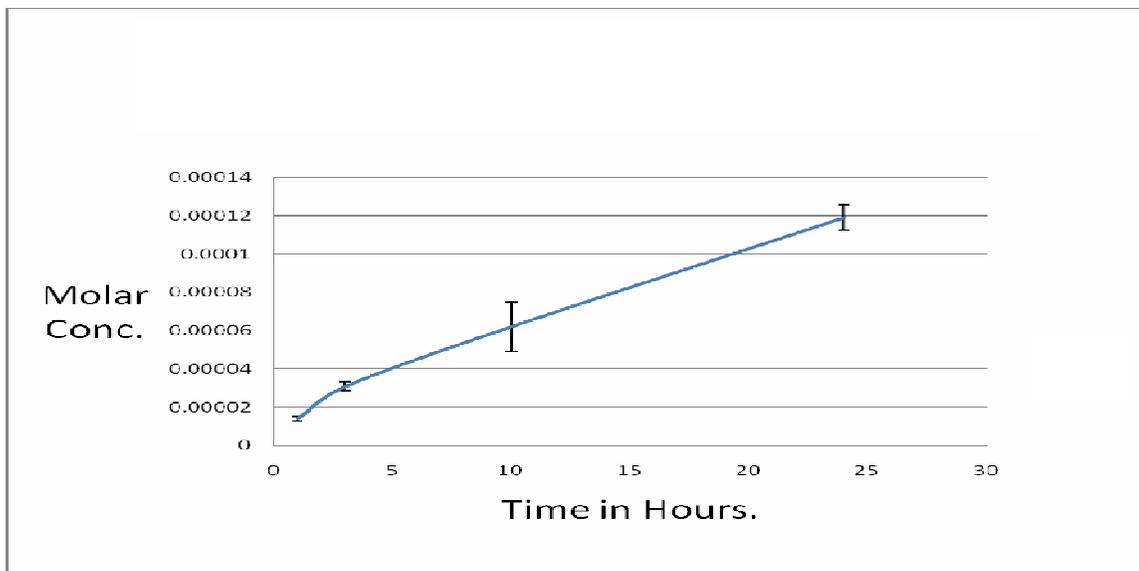


Figure 52: Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 7) treated with Zr700

5. Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 700.

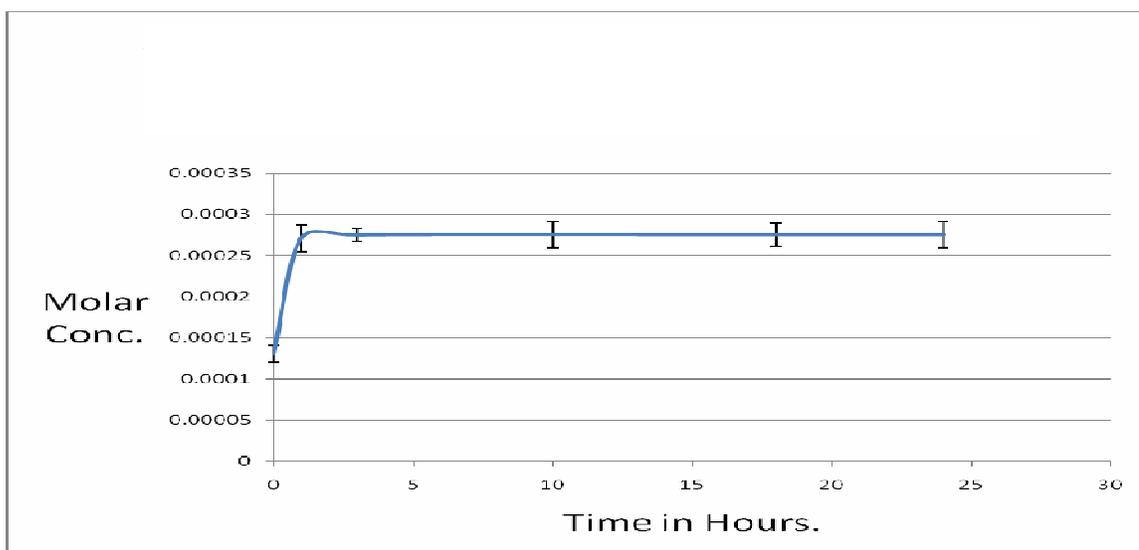


Figure 53: Absorption as function of time – AMP of Solution (ATP+ADP+AMP at pH 10) treated with Zr700

6. Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 700.

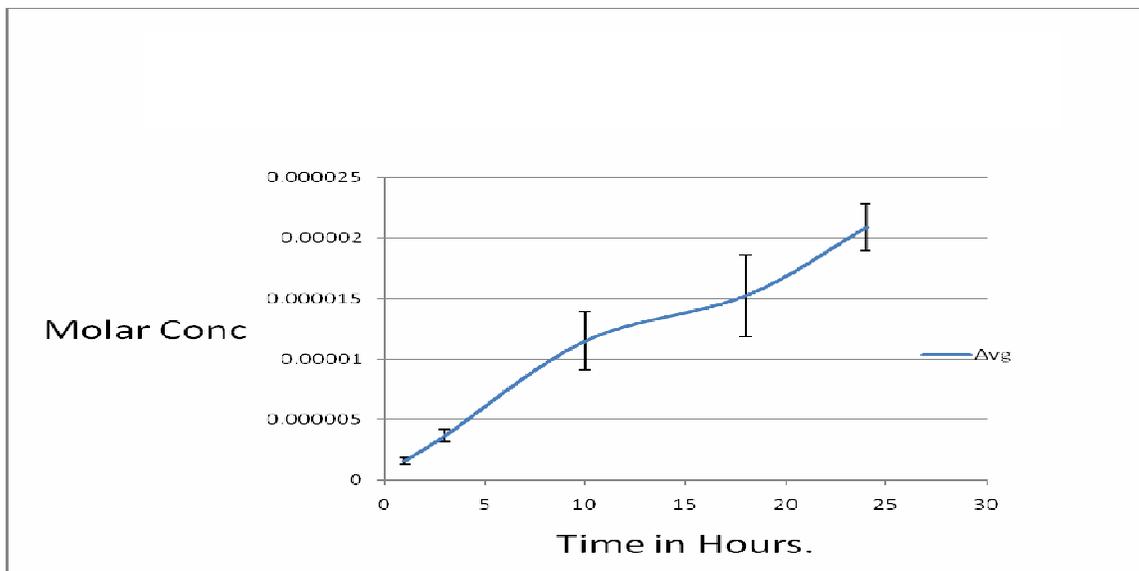


Figure 54: Absorption as function of time –Unknown Peak of Solution (ATP+ADP+AMP at pH 10) treated with Zr700

Results:

1. After the HPLC analysis of the different Adenosine Phosphates at different pHs (solution) treated with Zirconia, some unexpected results were encountered. We expected to get all three peaks (i.e. ATP, ADP and AMP) with the decreasing amount per time, but instead of getting three different peaks, two peaks sometimes resulted with increasing or decreasing amount of analyte. It was also difficult to judge about the actual analyte because the retention times of the peaks were different than expected. The reason behind this result may be due to the catalytic property of Zirconia.

2. When Zirconia 500 was treated with ATP, ADP and AMP at pH 10 (Solution C), it was found that this is the only condition where all 3 peaks could be detected, but the amount of AMP was increasing and ADP and ATP was decreasing.

Solid Phase Extraction:

Graphs:

- AMP, ADP, ATP peaks of Solution (ATP+ADP+AMP at pH 10) treated with Zirconia 500.

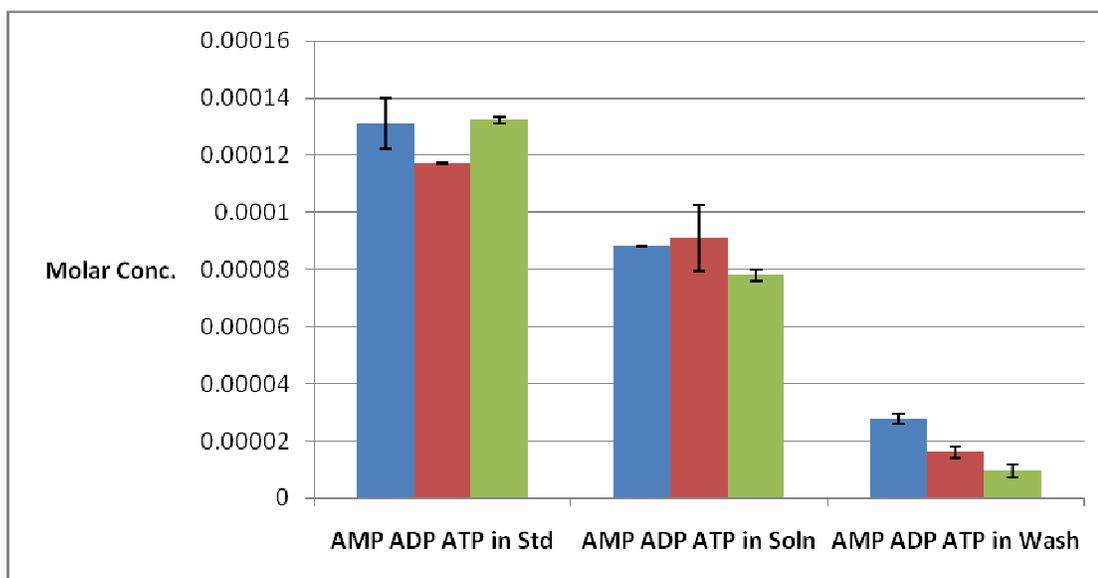


Figure 55: AMP, ADP, ATP peaks of Solution of (ATP+ADP+AMP at pH 10) treated with Zr500

In Figure 55, all three peaks are present in both conditions after treatment of 0.0001M ATP+0.0001M ADP+0.0001M AMP at pH 10 buffer solution with Zirconia 500 and after washing the Zirconia 500 with 0.1M NH₄OH.

Results:

After solid phase extraction it is confirmed that at pH 10.00 all three Adenosine Phosphates species get absorbed on the Zirconia 500 surface. Further, rinsing Zirconia 500 with 0.1 M of NH₄OH could recover all three Adenosine Phosphates species. Exact values are given in Table 10 which shows that approximately 32 %, 22 % and 41 % of

AMP, ADP and ATP respectively get absorbed on the Zirconia surface and, in the wash, approximately 21 %, 13 % and 7 % of AMP, ADP and ATP respectively came out.

Species Condition	AMP Percentage	ADP Percentage	ATP Percentage
Std	100.00	100.00	100.00
Amount absorbed on Zirconia Surface.	32.48	22.48	41.11
Amount in wash	21.74	13.62	7.08

Table 60: SPE results.

Purification of FGF protein:

- A. Use of Silica for FGF purification: As most of FGF protein is coming out in first wash (S1), silica is not a good material for FGF purification.

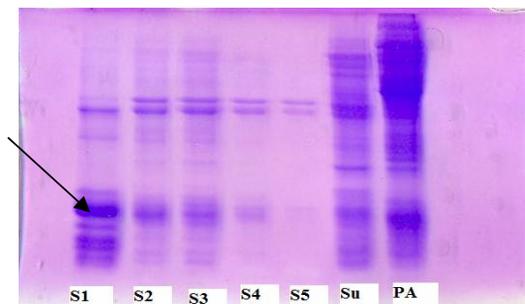


Figure 56: Silica for FGF purification.

Su=Supernatant,

PA=Protein Marker,

S1=Silica+FGF supernatant (30 minutes incubation),

S2=10 mL of 10 mM Tris HCl buffer+50mM NaCl with pH 7.0 (1st 30 minutes incubation),

S3=10 mL of 10 mM Tris HCl buffer+50mM NaCl with pH 7.0 (2nd 30 minutes incubation),

S4=10 mL of 10 mM Tris HCl buffer+50mM NaCl with pH 7.0 (3rd 30 minutes incubation),

S5=10 mL of 10 mM Tris HCl buffer+50mM NaCl with pH 7.0 (4th 30 minutes incubation).

- B. Comparison of Zirconia 700 and Zirconia 500: Zirconia 500 can be considered as better material for purification as compared to Zirconia 700. It can be seen that in the eighth wash Zirconia 500 can purify more FGF protein than Zirconia 700.

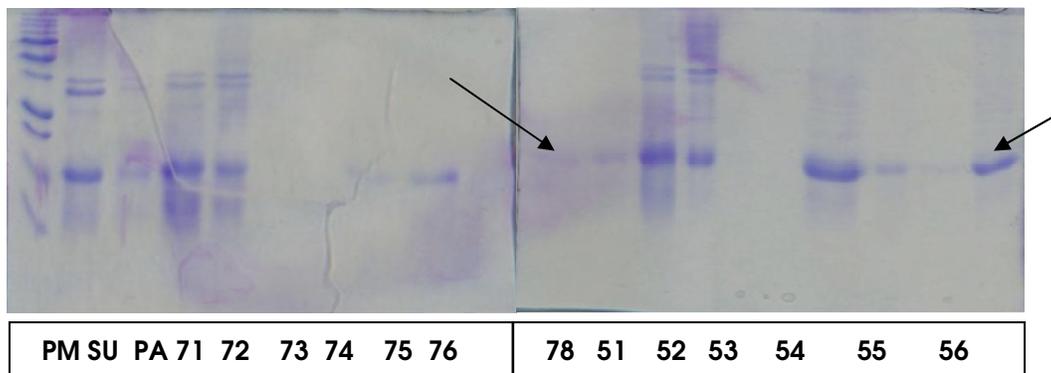


Figure 57: Zr700 for FGF purification. Figure 58: Zr500 for FGF purification.

PM=Protein Marker,

SU=Supernatant,

PA=Pellet,

7=Zirconia prepared at 700 °C,

5=Zirconia prepared at 500 °C,

71/51=Zirconia+FGF supernatant(30 minutes incubation),

72/52=Zirconia+10 mM Tris HCl buffer & 50 mM NaCl pH 7.0 (1st 30 minutes incubation),

73/53=Zirconia+10 mM Tris HCl buffer & 50 mM NaCl pH 7.0 (2nd 30 minutes incubation),

74/54=Zirconia+10 mM Tris HCl buffer & 50 mM NaCl pH 7.0 (3rd 30 minutes incubation),

75/55=Zirconia+1.5 mM NaCl & 10 mM phosphate buffer with pH 7.2 (1st 30 minutes incubation),

76/56=Zirconia+1.5 mM NaCl & 10 mM phosphate buffer with pH 7.2 (2nd 30 minutes incubation),

77/57=Zirconia+1.5 mM NaCl & 10 mM phosphate buffer pH 7.2 (3rd 30 minutes incubation),

78/58=Zirconia+1.5 mM NaCl & 10 mM phosphate buffer pH 7.2 (4th 40 hours incubation).

C. With Phosphate buffer and NaCl Salt :

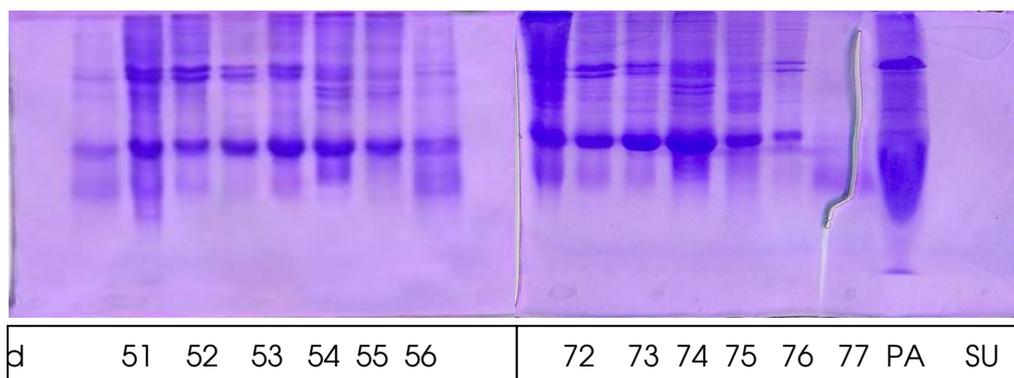


Figure 59: Zr500 for FGF purification. Figure 60: Zr700 for FGF purification.

SU=Supernatant,

PA=Pellet,

7=Zirconia prepared at 700 °C,

5=Zirconia prepared at 500 °C,

71/51=Zirconia+ FGF supernatant (30 minutes incubation),

72/52=Zirconia+10 mM phosphate buffer & 50 mM NaCl pH 7.0 (1st 30 minutes incubation),

73/53=Zirconia+10 mM phosphate buffer & 50 mM NaCl pH 7.0 (2nd 30 minutes incubation),

74/54=Zirconia+10 mM phosphate buffer & 250 mM NaCl pH 7.0 (1st 30 minutes incubation),

75/55=Zirconia+10 mM phosphate buffer & 500 mM NaCl pH 7.0 (1st 30 minutes incubation),

76/56=Zirconia+10 mM phosphate buffer & 1.5M NaCl pH 7.0 (1st 30 minutes incubation),

77/57=Zirconia+10 mM phosphate buffer & 1.5M NaCl pH 7.0 (2st 30 minutes incubation).

- D. Comparison of Tris and HEPES buffer: Tris buffer can provide better condition for the purification of FGF protein. It can be seen that the sixth wash with Tris buffer gives more FGF protein than the sixth wash with HEPES buffer.

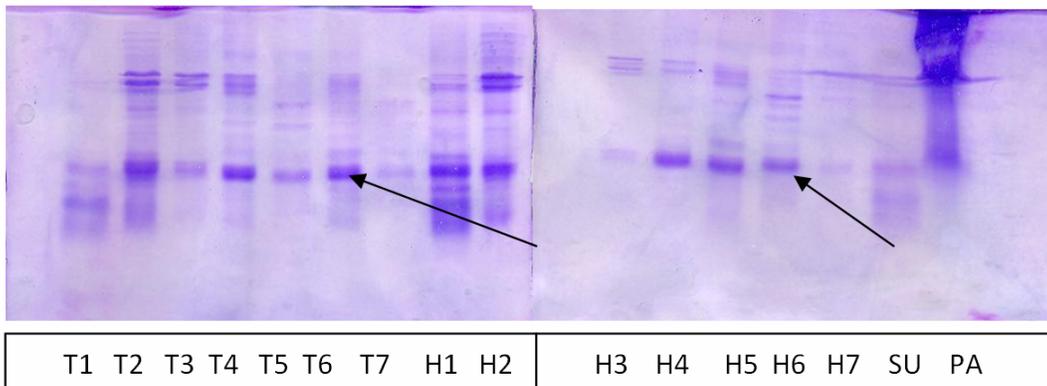


Figure 61: Zirconia 500 with Tris buffer for FGF purification.

Figure 62: Zirconia 500 with HEPES buffer for FGF purification.

SU=Supernatant,

PA=Pellet,

T1/H1=Zirconia+FGF supernatant (30 minutes incubation),

T2/H2=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 50 mM NaCl pH 7.5 (1st 30 minutes incubation),

T3/H3=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 50 mM NaCl pH 7.5 (2nd 30 minutes incubation),

T4/H4=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 250 mM NaCl pH 7.5 (1st 30 minutes incubation),

T5/H5=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 500 mM NaCl pH 7.5 (1st 30 minutes incubation),

T6/H6=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 1.50 M NaCl pH 7.5 (1st 30 minutes incubation),

T7/H7=Zirconia+10 mM Tris HCl buffer/HEPES buffer & 1.50 M NaCl pH 7.5 (2nd 30 minutes incubation).

Results:

After running several gels some conclusion can be drawn. First, it was found that silica cannot be used for the purification of FGF protein. Further, Zirconia is very promising for purification of FGF protein, Zirconia 500 proved to be very efficient for

the purification of FGF. Finally, Tris and Phosphate buffers can provide better conditions than the HEPES buffer for the purification of FGF protein.

SEM Study:

a. Figure 63 and Figure 64 show SEM Image of Zirconia 500.

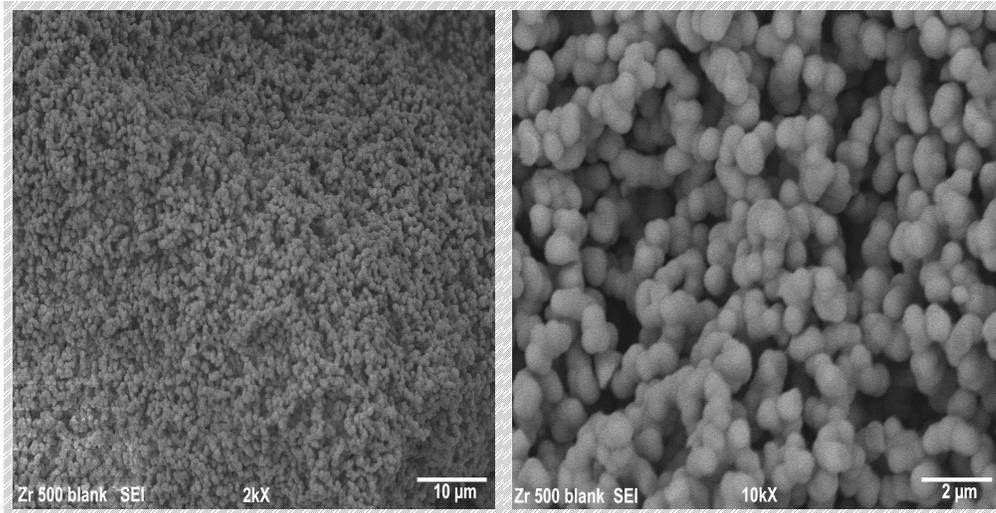


Figure 63: With 2000 x resolution. Figure 64: With 10 000 x resolution.

Observation: Each monolith has rounder or circular shape.

b. Figure 65 and Figure 66 show SEM Image of Zirconia 700.

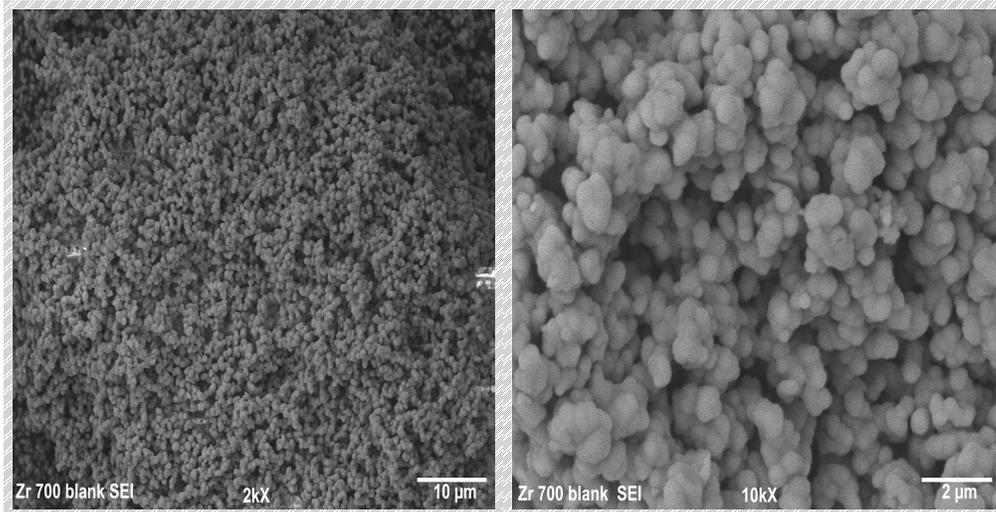


Figure 65: With 2000X resolution. Figure 66: With 10000X resolution.

Observation: Each monolith has rounder or circular shape and number of pores in

Zirconia 500 is more than Zirconia 700.

- c. Figure 67 and Figure 68 show SEM Image of Zirconia after Fluorescence Experiment.

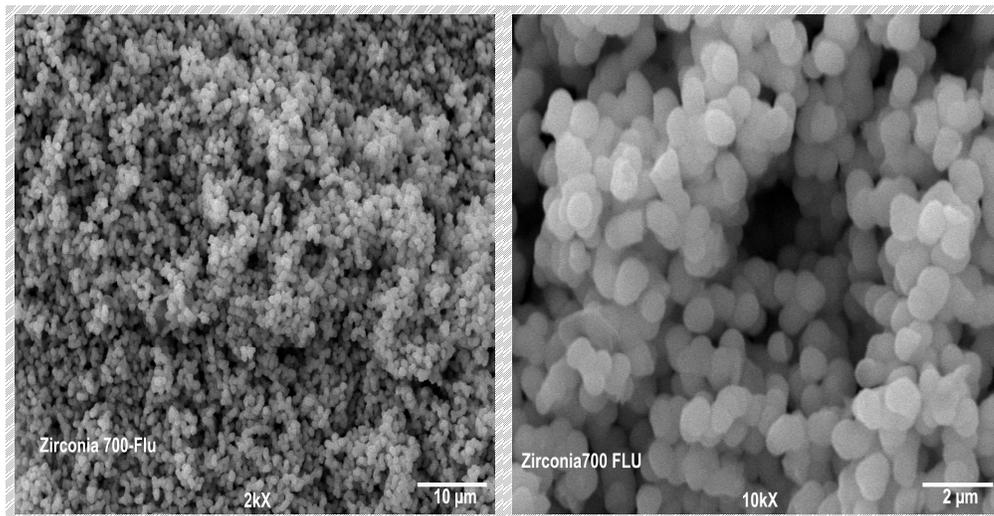


Figure 67: With 2000X resolution. Figure 68: With 10 000X resolution.

Observation: Adenosine Phosphate species changed the size of Zirconia monolith. It can also be seen that the Zirconia became more porous.

- d. Figure 69 and Figure 70 show SEM Image of Zirconia after FGF Purification.

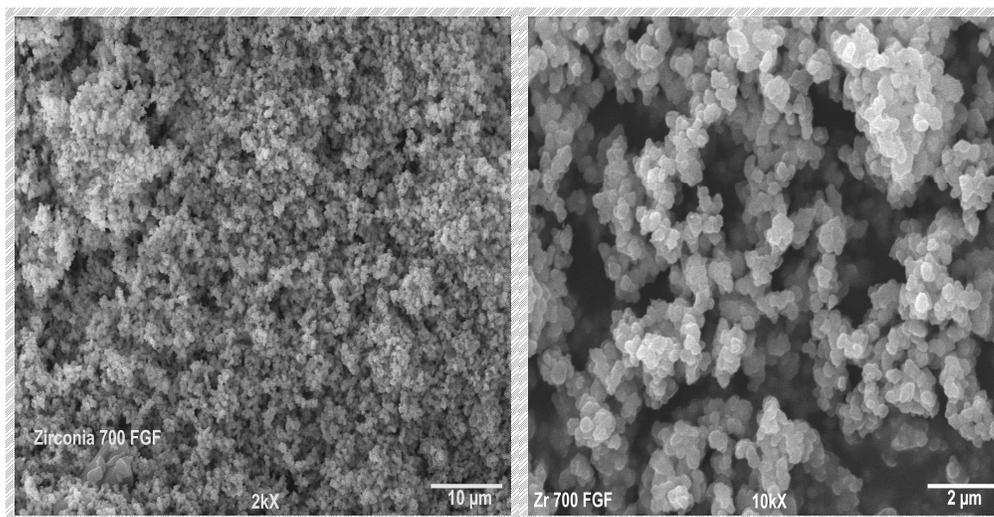


Figure 69: With 2000X resolution. Figure 70: With 10000X resolution.

Observation: Buffer and FGF protein changed the shape of Zirconia monolith.

- e. Figure 71 and Figure 72 show SEM Image of Zirconia after Absorption Experiment.

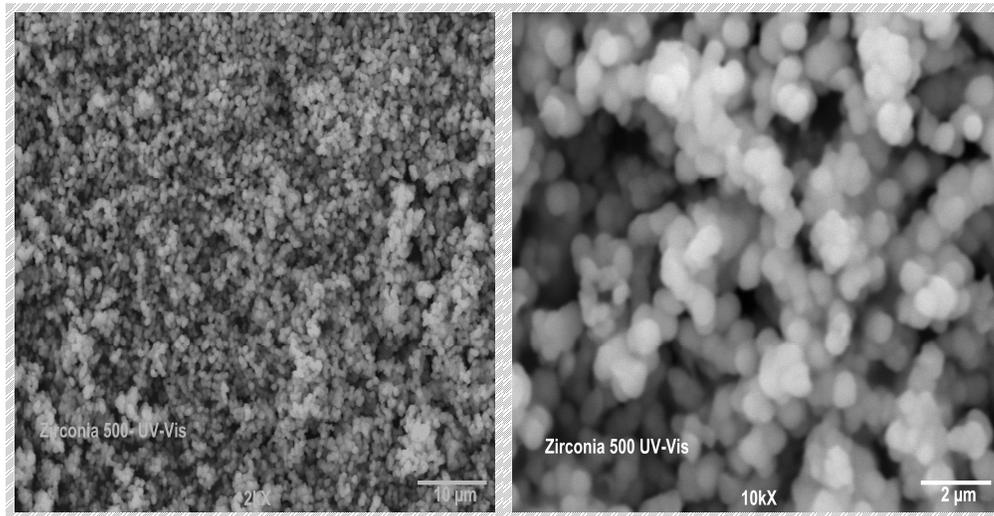


Figure 71: With 2000X resolution. Figure 72: With 10000X resolution.

Observation: Adenosine Phosphate species changed the size of Zirconia monolith. It can also be seen that the Zirconia became more porous.

- f. Figure 73 and Figure 74 show SEM Image of Zirconia after washing.

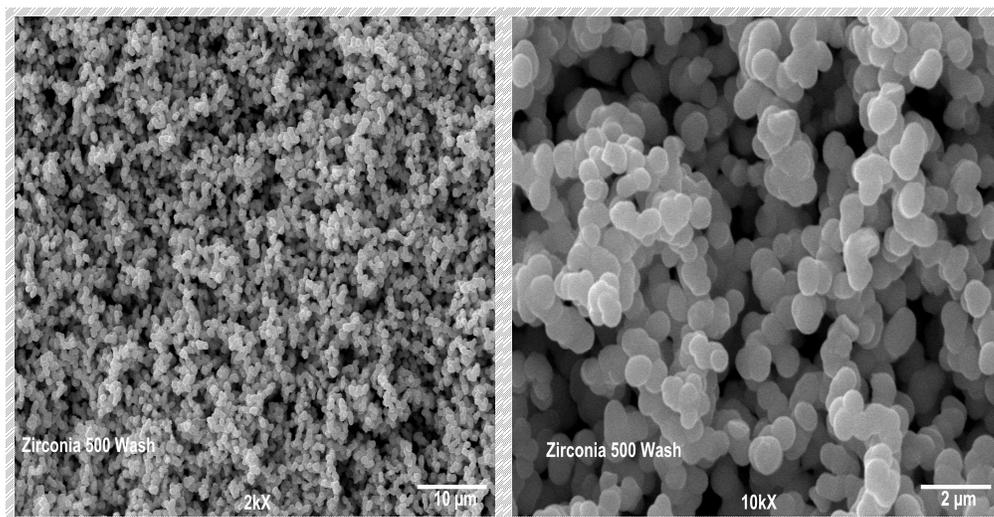


Figure 73: With 2000X resolution. Figure 74: With 10000X resolution.

Observation: It can be seen that after washing, Zirconia regained its original size and shape.

IV. CONCLUSIONS

Absorption Experiment:

Comparison of Absorption as a function of time was done. Figure 26 to Figure 37 clearly shows that for both Zr500 and Zr700 the surface absorption trend is AMP >ADP>ATP. In the same manner where pH is concerned, on both Zr500 and Zr700 surface absorption sequence is pH4>pH 7>pH10.

Fluorescence Experiment:

Comparison of Absorption as a function of time was done. Figure 38 to Figure 41 clearly shows that for both Zr500 and Zr700 the surface absorption trend is AMP >ADP>ATP. In the same manner where pH is concerned, on both Zr500 and Zr700 surface absorption sequence is pH4>pH 7>pH10.

Separation Experiment:

- i. After the HPLC analysis of the different Adenosine Phosphates at different pHs (solution) treated with Zirconia, some unexpected results were encountered. We expected to get all three peaks (i.e. ATP, ADP and AMP) with the decreasing amount per time, but instead of getting three different peaks sometimes two peaks resulted with increasing or decreasing amount of analyte. It was also difficult to judge the actual analyte because the retention times of the peaks were different than expected. The reason behind this result may be due to the catalytic property of Zirconia.
- ii. When Zirconia 500 was treated with ATP, ADP and AMP at pH 10 (Solution C), it was found that this is the only condition where all three peaks could be

detected, but the amount of AMP was increasing and ADP and ATP was decreasing.

Solid Phase Extraction:

After solid phase extraction it is confirmed that at basic pH all 3 Adenosine Phosphates species get absorbed on the Zirconia 500 surface and as compared with other condition their degradation is less. Further, after washing with 0.1 M of NH_4OH , all three Adenosine Phosphates species could be recovered.

Purification of FGF Protein:

- i. Silica cannot be used for the purification of FGF protein
- ii. Zirconia is very promising for purification of FGF protein. More work needs to be done to optimize the conditions for the purification of FGF protein.
- iii. Zirconia 500 proved to be very efficient for the purification of FGF.
- iv. Tris and Phosphate buffers can provide better condition than HEPES buffer for the purification of FGF protein.

SEM study:

- i. Blank Sample: Each monolith has rounder or circular shape and number of pores in Zirconia 700 is more than Zirconia 500.
- ii. Adenosine Phosphate species changed the size of Zirconia monolith. It can also be seen that the Zirconia became more porous.
- iii. It can be seen that after washing, Zirconia regained its original size and shape.

As stated in the introduction section, one of the aims of our experiments was to find out the perfect conditions for the absorption of Adenosine phosphate species on the surface of Zirconia. Dr. Luis Colon's work indicates that hafnia can enrich mono- and

tetraphosphorylated peptides while titania and zirconia showed a bias towards the tetraphosphorylated peptides. After our experiments, it can be concluded that ATP, ADP and AMP get absorbed on the Zr500 surface at basic pH.

Dr. Colon's work tells about the biasness of Zirconia with tetraphosphorylated peptides. Our work also shows the biasness of Zirconia with various Adenosine Phosphate species, but we found one condition which is very less bias as compared to the other conditions. When ATP, ADP, AMP together treated with Zr500 at basic pH, then after HPLC analysis all three peaks could be seen. As per kinetics study, amount of AMP increased on the other hand amount of ADP and ATP decreased with respect to time.

V. FUTURE WORK

A procedure for the synthesis of high purity Zirconia must be developed to avoid complication from the catalytic properties. Most of the impurity is created by Aluminum and Silicon. Special attention should be given to elimination of these two elements.

Actual phosphorylated protein should be used in the separation studies to examine the possible practical uses of Zirconia in the analysis of phosphorylated proteins

Other materials such as Hafnium oxide and Titanium oxide should be studied to optimize conditions for the analysis of phosphorylated protein.

In FGF purification more work needs to be done to optimize the condition for the purification. Variables such as the types and concentration of salt and buffers should be examined. KCl can be effective to get rid of unwanted protein.

VII. REFERENCES

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