An- Najah National University

Faculty of Graduate Studies

Determination of Some Proteins by the Interaction with Tris-Aminocarboxylate Metal Complexes

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Dedication

I dedicate this thesis to Allah first of all…..

To my great parents, who never stop giving of themselves in countless ways, who taught us the purpose of life…..

To my husbund, who leads me through the valley of darkness with light of hope and support…..

To my beloved brothers and sisters…..

To my beloved children Amr, and Jood whom I can't force myself to stop loving…..

To all my family, the symbol of love and giving…..

To my friends who encourage and support me…..

To all the people in my life who touch my heart, who supported my steps in any way and means….I dedicate this research.

Acknowledgement

First and foremost, I must acknowledge my limitless thanks to Allah, the Ever-Magnificent; the Ever-Thankful, for his help and bless. I am totally sure that this work would have never become truth, without his guidance…..

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I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis…..

Last but not least, deepest thanks go to all people who took part in making this thesis real.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان

Determination of Some Proteins by the Interaction with Tris-Aminocarboxylate Metal Complexes

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Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degrees or qualifications.

Student's Name: **اسم الطالب:مرفت فواز ابراهيم ابو زينة**

Signature **التوقيع:**

Date **التاريخ: 2016/9/21**

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List of Abbreviations

Abbreviation	Full Name
TAA	Tris amino ethyl amine
ATAA	acidified Tris amino ethyl amine
PVBC-PVPyridine	Poly vinyl benzyl chloride-poly vinyl pyridine
Mb	myoglobin
BHb	Bovine hemoglobin
ppm	Part per million
DMF	Dimethyl formamide
EDTA	Ethylenediaminetetraacetic acid

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Abstract

A sensing membrane made up of Tris- aminocarboxylate metal complex was prepared and characterized. This optical chemical sensor based on polymer swelling and shrinking was studied by optical transmission.

This sensor showed high response especially to copper ions. There was insignificant response to different pHs (5-12) . In addition, there was no detectable response towards alkali, alkaline earth metals and other metal ions such as Ni^{2+}, and Co^{2+}.

The aim of this research was to detect some of protein by the interaction with carboxylated tris-aminocarboxylate metal complexes, and to study the effect of various variables on the interaction of metal complexes with proteins.

it is found that the Cu^{2+} ions was the best cation to give a response, then this sensor was tested to different proteins such as Myoglobin from equine skeletal, Hemoglobin from bovine blood, and Albumin from human serum. Myoglobin was detected by the sensor with very high response. And also the effect of pH, and the relation between the concentration for both Cu^{2+} ions in the sensor and Myoglobin protein was studied. A linear relationship between the maximum absorbance and the concentration of myoglobin is

obtained, as the concentration of myoglobin increase, the absorbance increases.

This new detection method was easy, cheap, and fast way for analysis.

Chapter One

Introduction

1.1. Background:

The ever growing demand for ultrasensitive label free biosensors for rapid, early diagnostic applications is being catered to by the rapid advancements in micro- and nanotechnologies that the present decade is witnessing. Development of detection methodologies which are highly sensitive, selective, simple, rapid, robust, and cost effective is the current research trend in this area.

Nowadays, a great deal of interest is directed towards developing chemical sensors. A certain work has been done on the development of chemically selective sensors for analytical purposes.

A good example of this technology is the membrane which is prepared by suspending aminated polystyrene microspheres in a hydrogel. Thus, lightly cross-linked poly (vinylbenzyl chloride) microspheres with one micrometer in diameter was aminated with diethanolamine. Then, the microspheres were suspended in a poly (hydroxyethylmethacrylate) (poly HEMA) hydrogel membrance. The resulting membrane is turbid because the refractive index of the microspheres is greater than that of the hydrogel. In acidic media the protonation of the amino groups led to swelling by introducing a positive charge onto the polystyrene backbone. This increased the affinity of the polymer for aqueous media, and thus the turbidity decreased.[1,2]

In another previous study[3], dicarboxylate functionalized polymer microspheres were prepared and their sensing properties were tested. The

polymer exhibited good response to varying pH solutions and to solution of copper and calcium ions of different concentrations.

A sensor is a device that detects events or changes in quantities and provides a corresponding output, generally as an electrical or optical signal. Chemical and biochemical sensors are an important issue which have a great deal of interest toward developing it in our world today, especially for analyzing medical as well as biological samples.

1.2. Chemical sensor

A chemical sensor is a self-contained analytical device that can provide information about the chemical composition of its environment, that is, a liquid or a gas phase. The information is provided in the form of a measurable physical signal that is correlated with the concentration of a certain chemical species (analyte) as shown in figure 1. Two main steps are involved in the functioning of a chemical sensor, namely, recognition and transduction.

In the recognition step, analyte molecules interact selectively with receptor molecules or sites included in the structure of the recognition element of the sensor. Consequently, a characteristic physical parameter varies and this variation is reported by means of an integrated transducer that generates the output signal.

A chemical sensor based on recognition material of biological nature is a biosensor. However, as synthetic biomimetic materials are going to substitute to some extent recognition biomaterials, a sharp distinction

between a biosensor and a standard chemical sensor is superfluous. Typical biomimetic materials used in sensor development are molecularly imprinted polymers and aptamers. (4)

The primary motivations to develop chemical sensors are monitoring and controlling of environmental pollution, medical applications, reductions in measurement precision and accuracy, and process and quality control of industrial applications.

In biomedicine and biotechnology, sensors which detect analytes thanks to a biological component, such as cells, protein, nucleic acid or biomimetic polymers, are called biosensors. Whereas a non-biological sensor, even organic (carbon chemistry), for biological analytes is referred to as sensor. This terminology applies for both in vitro and in vivo applications. The encapsulation of the biological component in biosensors, presents a slightly different problem that ordinary sensors; this can either be done by means of a semipermeable barrier, such as a dialysis membrane or a hydrogel, or a 3D polymer matrix, which either physically constrains the sensing macromolecule or chemically constrains the macromolecule by bounding it to the scaffold.(4)

Sensor can be classified according to the property to be determined as; mass sensors, thermal sensors, catalytic sensors, electrochemical sensors, potentiometric sensors, amperometric sensors, conductimetric sensors, gas sensors, ionic sensors, and optical sensors.

1.3. Optical sensors

Optical sensors can be defined as devices that transform the change of optical phenomena. Optical sensors and spectroscopic measurements employ the same equipment, but the arrangement of the experiment itself is different. In spectroscopic measurements, the sample is generally placed in a well defined path of beam and the emerging radiation is captured by the detector. On the other hand, in optical sensors the beam is guided out of the spectrophotometer, allowed to interact with the sample, and then reintroduced into the spectrophotometer.[5]

The type of optical properties that should be available in chemical sensors include [6]:

1. Absorbance which is measured in a transparent and caused by absorptivity of the analyte itself or the reaction with suitable indicator.
2. Luminescence which is based on the measurement of the intensity of light emitted by chemical reaction in the receptor system.
3. Reflectance which is measured in non-transparent medium and usually using an immobilized indicator.
4. Fluorescence which is measured as the positive emission effect caused by irradiation.
5. Refractive index which is measured as result of change in solution composition.
6. Light scattering which is based on effects caused by particles of definite size present in the sample.

7. Optothermal effect which is based on a measurement of the thermal effect caused by light absorption.

1.3.1. Classification of optical sensors

1.3.1.1. Optical sensors based on immobilized indicator:

Recent years have seen an increasing interest in the development of fiber optic chemical sensors (FOCS) for ions or molecules in industry, environment, food processing…etc, as they have a number of advantages over conventional system. The development of FOCS during recent years is related to two of the most important scientific advances of the 1960s, the laser technology (1960) and modern low-cost optical fiber (1960). So, during the late 1960s and early 1970s some of these optical fibers were used in the development of the first chemical sensors, and then their applications have continued to spread to very different areas, particularly, environmental and industrial applications. [7,8]

These are optical sensors that employ indicator dye, which exist in two different color forms depending on whether or not the measured is bound to them. The interaction with the analyte is accompanied by a change in the absorbance or florescence. This means that the indicator acts as a transducer for the chemical species that cannot be determined directly by optical means.[7]

Cross-linked poly (vinyl alcohol) (PVOH) is used as a substrate for immobilizing indicators used in fiber optic chemical sensor. Since PVOH has hydrophilic properties which are suitable for ion sensing and due to its

ability to form very homogeneous film of high quality. It should be crosslinked in order to be useful for a wide range of applications.[9]

Some of the common cross-linking agents used for PVOH hydrogel preparation include glutaraldehyde, acetaldehyde and formaldehyde in the presence of acid like HCl which act as a catalyst.[9]

1.3.1.2. Optical sensor based on polymer swelling

There is an interest in developing fiber-optic chemical sensors based on optical changes accompanying polymer swelling. This is because they offer an improved robustness and calibration stability with low cost. In this sensing element a cross-linked polymer swells or shrinks as a function of analyte concentration. Since changes in analyte concentration cause changes in the polymer size, which affect the affinity of the polymer for external solvent. Thus, the intensity of light reflected into the optic fiber changes accordingly.[10]

Cross-linking prevents the polymer from dissolving in solvents. Instead, it absorbs solvent and swells to reach an equilibrium volume where the swelling forces due to the polymer solvation are balanced by the refractive force due to stretching of the chemical bounds in the polymer.

The degree of swelling is dependent on the affinity of the polymer for the solvent and the extent of cross-linking. If the polymer is ionic, the electrostatic forces can contribute to swelling. The driving force for swelling is the difference between the number of charges per unit volume in the polymer and the ionic strength of the external aqueous solution. So,

the solvent enters the polymer to equalize the change in densities inside the polymer.

As the ionic strength of the solution increases, the difference in charge density inside and outside the polymer decreases, so the driving force for the swelling will decrease.(11)

Polystyrene has been chosen as a polymer substrate because it is thermally stable, mechanically strong and amenable to a wide variety of derivatization chemistries. One common approach is to chloromethylate styrene, then the chloromethyl group can undergo a variety of reactions with primary and secondary amines, but poly (vinylbenzyl chloride) is directly prepared to avoid a separate chloromethylation step which might cause extra cross-linking that reduces dramatically the swelling process.

Recently, Shakhsher et.al developed an optical sensing element in which lightly cross-linked chemically derivatized polymer microspheres with dimensions of a few micrometers that are designed to swell and shrink as a function of analyte concentration (such as metal ions). Then these microspheres are suspended in a hydrogel membrane which change its volume reversibly in response to change in environment conditions.(3)

This chemical sensing is based on the changes in the optical properties of the membrane that accompany swelling and shrinking. In swelling, the refractive index of the microspheres become closer to that of the hydrogel, resulting in a decrease in the membrane turbidity.(12)

1.4. Methods for protein analysis

Many experimental methods are used for studying proteins e.g., for detecting proteins, for isolating and purifying proteins, and for characterizing the structure and function of protein. Often, requiring that the protein first be purified.

Myoglobin (Mb) is mainly from muscle tissues of vertebrates and mammals, and its main function is to bind oxygen reversibly for transport and storage as well as cytoprotection against reactive species and NO scavenging.[13]

Both qualitative and quantitative detections of Mb are of significant importance for early disease diagnosis, because it is a sensitive marker of muscle injury and a potential code for heart attack in patients with chest pain. When muscle tissues are damaged, Mb will be released and filtered by the kidneys. But it is toxic to the renal tubular epithelium, which may cause acute renal harm. [13,14]

Mb is absent or present in very low concentrations (below 0.01μg/mL) in the urine of healthy people. But, appears in the urine for patient with severe muscle damage sometimes at extremely high levels to reach 750 μg/mL. Mb in serum of healthy people is 0.008-0.098μg/mL and is increased to 79.9μg/mL when damaged. At present, the main method for detection of Mb in urine or serum is immunoassay; however, antibodies used in this are very expensive.[13,14]

In one of the studies, A novel gold nanoparticle (AuNP)-based optical sensing system has been developed for the detection of myoglobin (Mb),

which is of significant importance for early disease diagnosis. Two thiol molecules containing an iminodiacetic acid moiety (IDA) were synthesized. This detection is based on the Mb-induced aggregation of IDA-functionalized AuNPs resulting from the structures of Mb sandwiched between the functionalized AuNPs via Cu^{+2} bridges in the coordination interactions of IDA-Cu^{+2}-histidine residues available on the Mb surface, which was confirmed by UV-vis spectroscopy, transmission electron microscopy, dynamic light scattering, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The induction aggregation resulted in a red shift in plasmon resonance band of the AuNPs concomitant with a change in solution color from red to purple. The qualitative and quantitative detections of Mb can be achieved by colorimetric observations and UV-vis spectral measurements, respectively. The selectivity of protein assay with the functionalized AuNPs was further investigated, and it is found that the optical sensing of histidine-rich proteins is closely related to number and distribution of surface histidine residues as well as size of proteins.[13]

In this study, it was expected that a sensing membrane of tris-aminocarboxylate-metal complexes will be constructed and used for the specific detection of certain proteins which leads to a lot of future biochemical and biological applications.

1.5. Objectives

In this study we plan to construct a sensing membrane made up of Tris-aminocarboxylate polymer microspheres entrapped in a cross-linked Polyvinylalcohol (PVA) hydrogel. The microspheres will be allowed to form complexes with various metal ions, and then will be tested as a potential sensor for different proteins.s

The sensing membranes optical properties will be studied according to the following methodology:

- Testing the response with different metal ion complexes.
- Studying the effect of pH on response.
- Investigating the response time and the effect of temperature.
- Studying the reproducibility of the sensing membrane.

1.6. Hypothesis

this study involves some important aspects:

- Tris(2-aminoethyl) amine (TAA) is expected to form stable complexes with Cu^{2+} ions. This constitutes a basis for the determination of this metal by spectrophotometry.
- TAA undergoes a nucleophilic substitution reaction with PVBC-PVPyridine and followed by acidification with bromoacetic acid to form the sensing polymer then exchanging the ammonia to form Cu^{2+}-carboxylated TAA complex.
- Carboxylated TAA complex and its metal complexes are hypothesized to be stable for a long period of time.

- A new method for protein detection can be used to resolve the problem that arise from the fact of very cost and time spending for many immunoassay.

1.7. Novelty:

A membrane made up of Tris-aminocarboxylate polymer microspheres entrapped in a cross-linked Polyvinylalcohol (PVA) hydrogel was allowed to form complexes with various metal ions. The resulting membrane will be tested as a potential optical sensor for the detection of myoglobin protein.

The effect of various variables on the optical properties of the proposed sensor was studied. These variables include: type of metal ion, type and concentration of protein, pH, response time.

Finally, the reproducibility and stability of the sensing membrane was also investigated.

Chapter Two

Experimental

2.1 Reagents

Polyvinyl benzyl chloride-polyvinyl Pyridine was supplied from professor Seitz group (USA). N,N-Dimethylformamide (DMF), Tris(2-aminoethyl) amine (TAA), methylene chloride, polyvinyl alcohol, and glutaraldehyde (Sigma-aldrich) .

NaOH, KOH, NaCl, $MgSO_4$, and $CuSO_4.5H_2O$ (Frutarom). HCl (Merck). $CoSO_4.7H_2O$, $NiSO_4.6H_2O$, $HgCl_2$ (Riedel). EDTA disodium salt dehydrate (Alfa Aesar).

Myoglobin from equine skeletal, hemoglobin from bovine blood, albumin from human serum (Sigma-aldrich).

All chemicals were analytical grade.

All solutions were prepared with distilled water.

2.2 Apparatus

Shimadzu (UV-3101DC) UV-VIS-NIR scanning spectrometer was used for absorption measurements. Plastic cuvettes (1-cm bath length) were used. JENWAY (3510) pH Meter was used to measure pH. Fourier transform infrared spectrophotometer (Necolet Is5-Id3) was used to obtain IR-Spectra. TCC-CONTROLLER was used to control temperature. A transsomic 310 Sonicator was used to make the mixture uniform by dispersing the microspheres polymer.

2.3 Procedure

2.3.1 Solutions

The following aqueous solutions were prepared (using distilled water), and used:

1. Ammonia buffer solution (pH 5-12)
2. Aqueous solutions of Cu^{2+} with various concentrations (1.0×10^{-6} M up to 0.001M).
3. Different solutions of 0.001M Cu^{2+} in different pHs (5-12).
4. Aqueous solutions of different metal ions Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Co^{2+}, Ni^{2+}, Hg^{+} of various concentrations (5.0×10^{-6} up to 0.001 M).
5. Different solutions of different proteins stored in refrigerator
6. Different concentrations of myoglobin solution (50, 130, 200, 230, 300 ppm) dissolved in distilled water, phosphate buffer of pH 7.3, stored in refrigerator.
7. Saturated EDTA in buffer solution, pH 8.

2.3.2 Synthesis of the carboxylated Tris(2-aminoethyl) amine polymer

A 0.1g of the PVBC-PV Pyridine co-polymer was immersed in 0.3 g of tris(2-aminoethyl) amine and 10 mL of methylene chloride. The reaction mixture was stirred for two days at room temperature. The produced mixture was filtered, and placed in an aqueous solution containing 10mL of 0.1M NaOH and 0.12 g of bromoacetic acid (the amount of NaOH the same mmols of bromoacetic acid, in which both have the same amount of

Br group attached to the copolymer, related to the amount of tris-polymer), and left for one week at room temperature.

Finally, the mixture was washed several times with distilled water, and dried at room temperature for one week.

2.3.3 Preparation of the sensing element

In order to prepare the sensing element, the polymer microspheres were spread in the hydrogel as follows: 2.0 mg of the derivatized polymer microspheres were soaked in 3 drops of DMF for few minutes, then 1.0 mL of (5.0%) aqueous polyvinyl alcohol solution in water was added and the mixture was sonicated until the polymer microspheres were dispersed and the mixture was homogeneous.

Then, 50 microliters of 8% glutaraldehyde solution in water were added to the mixture and stirred for few seconds, followed by 50 microliters of 3M $HCl_{(aq)}$ as a catalyst.

The resulting mixture was mixed for few seconds. Then few drops were spread on the inner side of the measuring cell. The membrane was allowed to formulate and held in position. The membrane was washed and then stored in distilled water.

2.3.4 Optical measurements

The measuring cell containing the membrane was placed in the spectrophotometer, so that the sensing membrane was centered in the path of the light beam. Figure 2.1.

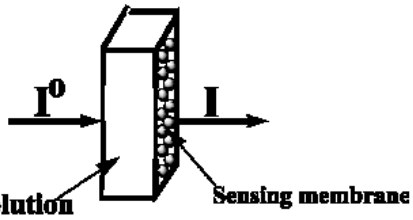

Figure (2. 1): optical sensing system

A disposable pipette was used to change the solution in the cell. The change in turbidity of the sensing element as a function of analyte concentration was measured as absorbance. All readings were taken at room temperature.

2.3.5 Testing the sensor response to pH

Solutions of different pHs; 5 – 12 were prepared, using ammonia buffer solutions. The solution in the cell was changed by using a disposable pipette, with an interval of 10.0 minutes between each spectrum run. The response of the sensor towards these buffer solutions was examined by measuring the UV-Vis spectrum in the range 340-800 nm.

2.3.6 Testing the sensor response to copper ions Cu^{2+}

The response to copper ions $Cu^{2+}$of various concentrations was tested as follow. Different concentrations of aqueous metal cation solutions, 5.0x 10^{-6} – 0.001M were prepared.

Solutions with different concentrations of Cu^{2+}metal ions were placed in the cell, one at a time starting with the lower concentration. A time interval of 5.0 minutes, was between runs. The signal of the sensing element was obtained by monitoring the change in absorbance spectrum in the range; 340 – 800 nm, (pH~7.3).

2.3.7 Testing the sensor response to other metal ions

The response to heavy metal cations of various concentrations was tested as follow. Different concentrations of aqueous metal cation solutions, 5.0x 10^{-6} – 0.001 M, of Co^{2+}, Ni^{2+}, Hg^{2+}, Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} were prepared.

Solutions with different concentrations of metal ions were placed in the cell, one at a time starting with the lower concentration. A time interval of 5.0 minutes, was between runs. The signal of the sensing element was obtained by monitoring the change in absorbance spectrum in the range; 340 – 800 nm, (pH~7.3).

2.3.8 Testing the reproducibility of the sensor

The reproducibility of the sensor response was tested by cycling between blank and 0.001M Cu^{2+} ions solution several times. Readings as turbidity absorbance at wavelength 800 nm were taken after 5 minutes of introducing the solution into the cell. After each reading, Cu^{2+} ions were eluted by a saturated solution of EDTA (pH~ 8), followed by a buffer solution (pH~ 7.3). Then, washing with distilled water until the blank reading is obtained.

2.3.9 Determination of the response time of the sensor

The absorbance of a 0.001M of Cu^{2+} solution was measured as a function of time until an equilibrium state is reached, (pH 7.3).

2.3.10 Regeneration of the sensor

After responding to a divalent metal ion, the sensing membrane is regenerated by adding a saturated solution of EDTA, followed by a buffer solution, and finally washing with distilled water (pH~7.3).

2.3.11 Testing the sensor response to different proteins

The sensor was tested to different proteins; myoglobin (from equine skeletal), hemoglobin (from bovine blood), and albumin (from human serum).

Each solution of different protein concentrations were placed in the sensing cell, one at a time starting with the lower concentration and going high (with 5 minutes intervals between runs). The response of the sensing element was obtained by measuring the absorption spectrum in the range 340 – 800 nm, at pH = 7.3.

2.3.12 Testing the sensor response to different concentrations of myoglobin

Different concentrations of myoglobin solutions in distilled water were prepared and tested toward the sensor without presence of copper ion. Started with lower concentration to the high one (50, 130, 200, 230, and 300 ppm) at pH = 7.3.

2.3.13 Testing the sensor response to different concentration of myoglobin in presense of Cu^{2+}

After the sensor response was tested toward different concentrations of myoglobin solutions, the response also was tested by fixing the concentration of myoglobin (50 ppm) with different concentrations of Cu^{2+} (1.0×10^{-6} up to 0.001M), then by fixing the copper ion concentration (0.001M) and changing the concentration of myoglobin solution (50, 130, 200, 230, 300 ppm) at pH~7.3.

Chapter Three

Results and Discussion

3.1 Identification of the acidified Tris-amine polymer:

The sensing polymer was formed from poly(vinylbenzylchloride)-Poly vinyl pyridine (PVBC-PVP), and Tris(2-aminoethyl)amine by a nucleophilic substitution reaction followed by acidification with bromoacitic acid as shown in figure 3.1.

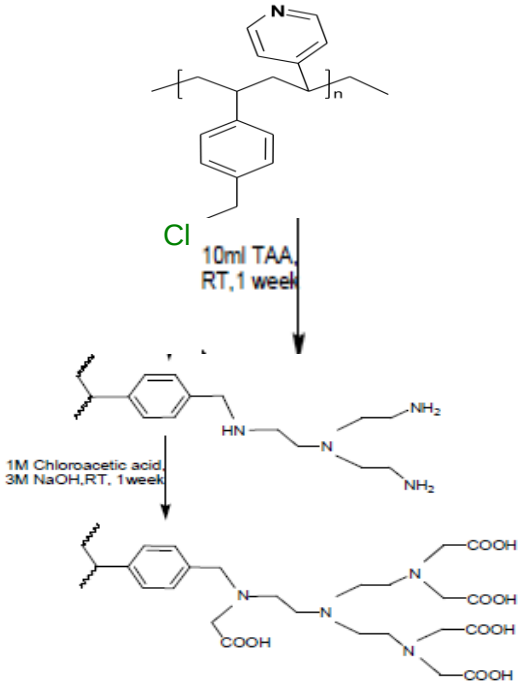

Figure (3. 1): Synthesis of acidified Tris-amine polymer[ATAA]

3.2 Formation of the sensing element:

The attachment of the Tris-amine functionality on the co-polymer was examined by IR. Comparing the IR-spectrum of (PVBC-PVP) with that of the tris-amine polymer, revealed that the intensity of the C-Br band peak at ~ 700 cm^{-1} has decreased after substitution with the amine, and the carboxyl groups peak and –C=O peak appeared at about 1600 &1700 cm^{-1} respectively. Figures (3.2,3.3, and 3.4).

The sensing element was formed by dispersing the acidified Tris(2-aminoethyl)amine-polymer beads and entrapping them as microspheres in a hydrogel membrane formed by cross linking of poly (vinylalcohol) with glutaraldehyde.

Acidified Tris-amine (ATAA) was chosen as a ligand because of its similarity to EDTA (common chelating agent for metal ion), insensitive to changes in pH particularly above 4, it is consider as a highly selective ligand, high stability in water, and it has stable complex formation with metal ion (this functional group is similar to EDTA, which has formation constant(K_f) with Cu^{+2} =6.3 x10^{18})[(5)].

The response is based on the interaction between the metal cations and the deprotonated carboxylated Tris(2-aminoethyl)amine functional group, whereby the swellable polymer undergoes shrinking as a result of neutralization of the negative charges of this functional group by metal ion as shown in figure 3.5. This complex formation of a metal cation with carboxylated Tris(2- aminoethyl)amine functionality causes significant changes in the optical properties of the sensing element. Shrinking of the polymer microspheres resulted in a decrease in the optical transmission through the sensing membrane. This is due to the increasing difference in the refractive indices between the microspheres and the dispersing hydrogel membrane.

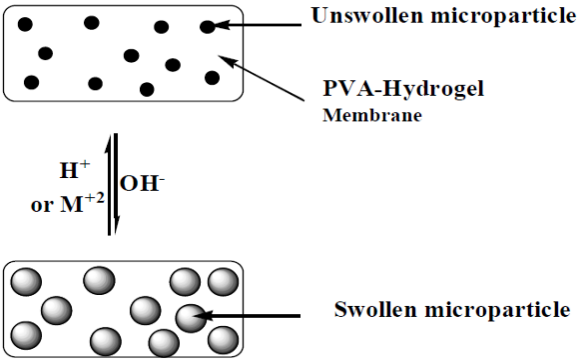

Figure (3. 2): Schematic diagram of sensing response to pH and metal ions

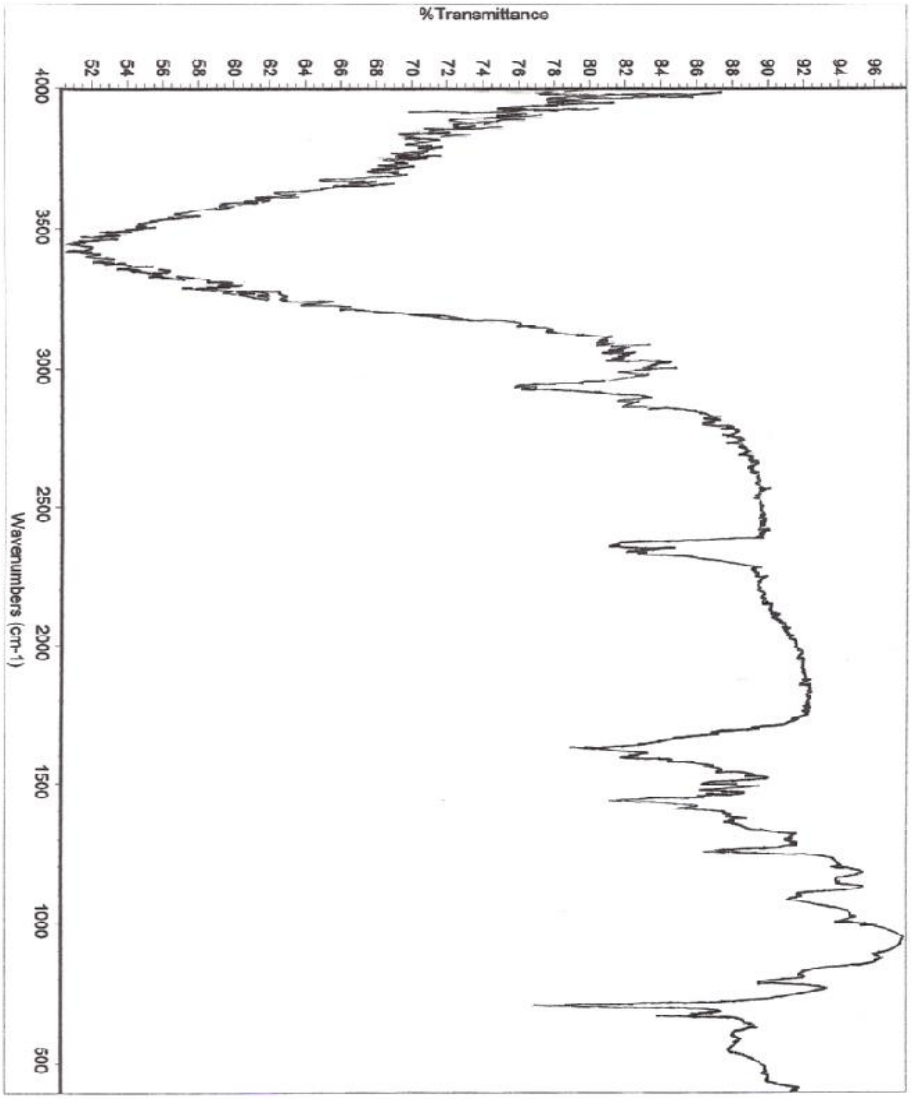

Figure (3. 3): IR spectrum for the copolymer of PVBC_PVP

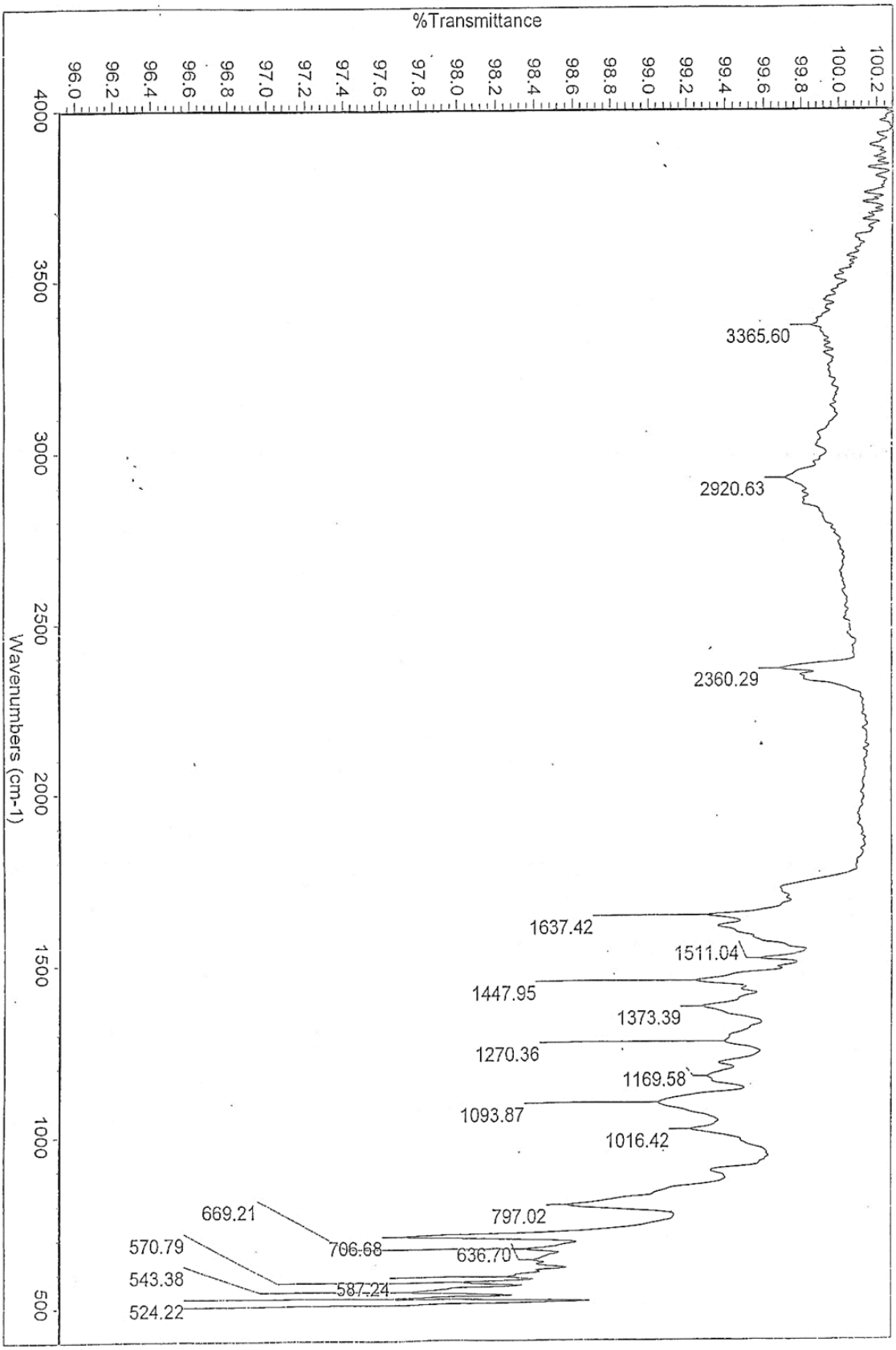

Figure (3. 4): IR spectrum of Tris-amine [TAA] co-polymer .

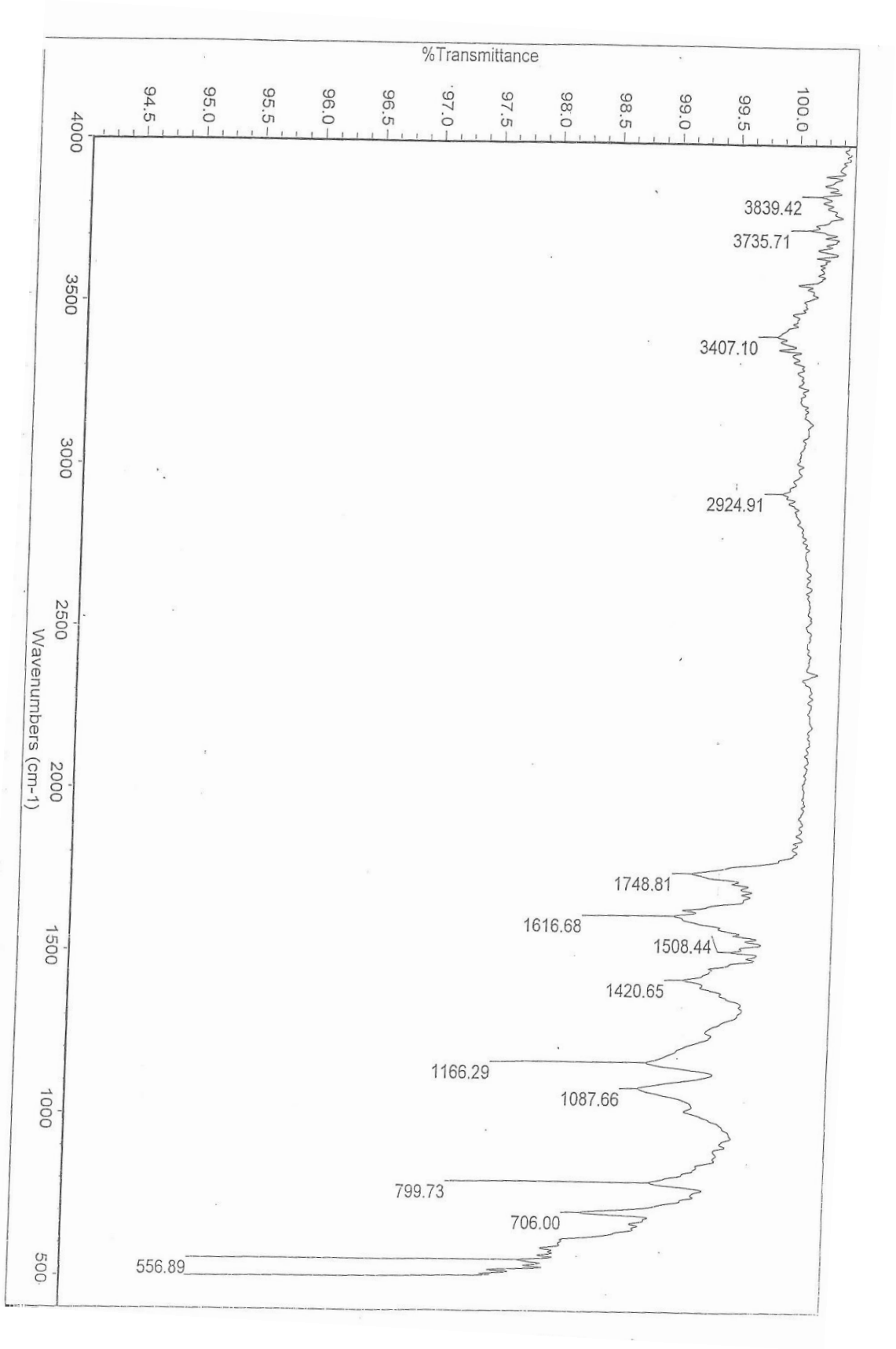

Figure (3. 5): IR spectrum of carboxylated Tris-amine [TAA] co-polymer.

3.3 The response of the sensor to pH

The sensing element was examined with different buffer solutions (pH5-12). It showed insignificant changes in absorbance as shown in figure 3.6. This may be attributed to the fact that the acidified tris-amine polymer contains both basic (**amine groups**) and acidic groups (**COOH**). Both the nitrogen and the oxygen are protonated, where positive charges of nitrogen repel each other causing the polymer to be in a swollen state. The oxygen on the carboxylic tris-amine group becomes deprotonated as the pH is raised; the repulsion between similar negative charges forces the polymer to stay in a swollen state. Thus no observable shrinking process can occur, and so no detectable change in absorbance will be noticed.

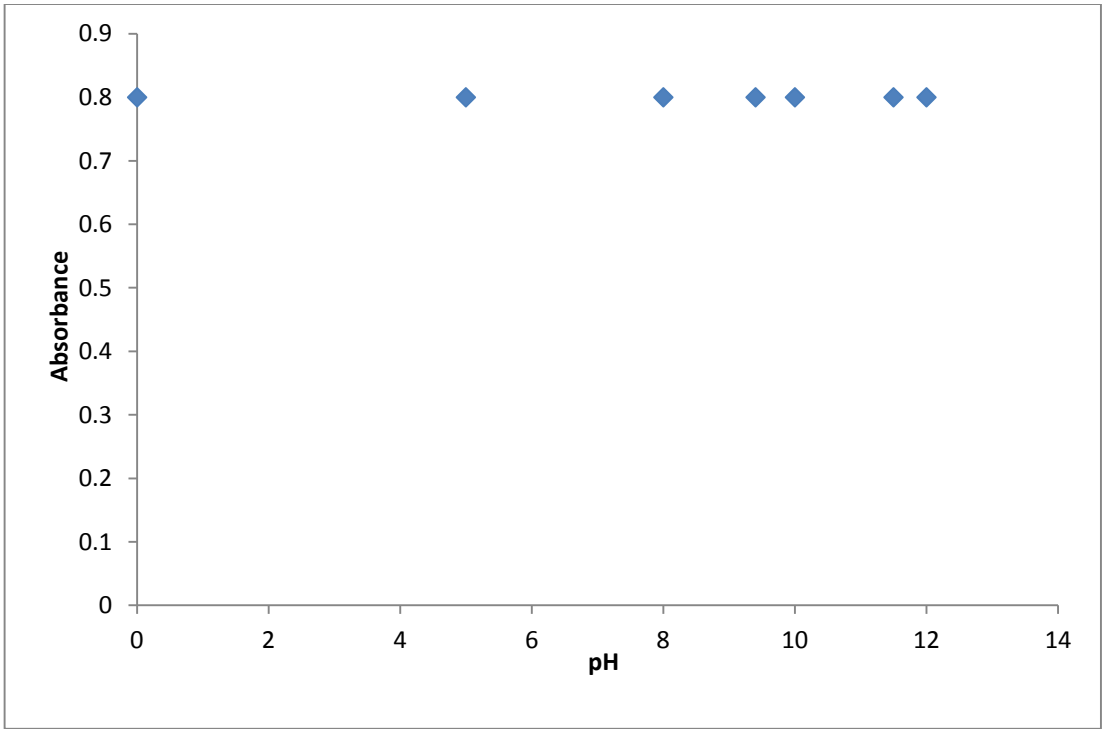

Figure (3. 6): Turbidity absorbance of the sensing element without metal at different pHs.

3.4 The response of the sensor to heavy metal ions:

The deprotonated carboxylated tris-amine polymer didn't show any optical response with respect to the tested metal ions such as Co^{2+}, Hg^{2+}, and Ni^{2+} at all concentration. On the other hand, the carboxylated Tris-amine ligand showed high affinity toward the cation Cu^{2+} as shown in figures (3.7, 3.8, 3.9, 3.10). This is due to the precipitation of $Cu(OH)_2$ with ksp = 1.6×10^{-19} which increase the turbidity of the solution. The response of the sensing membrane to Cu^{2+} ions is based on the interaction between these ions and the aminodicarboxylate functional group situated on the co-polymer microspheres. As a result, an increase in the turbidity of the sensing element occurred due to a change in the refractive index of the derivatized polymer microspheres relative to that of the hydrogel. Thus, as the concentration of the Cu^{2+} solution was varied an increase in absorbance was observed. The sensing element demonstrated good reproducibility in response towards Cu^{2+} ions. The formation constant (Kf) of binding of Cu^{2+} ions with the tris-aminodicarboxylate-polymer was obtained from the plot of figure 3.7. As the amounts of the free and the bonded ligand will be equal at the point half-way to the maximum response, Kf is equal to $1/[Cu^{2+}]$, where the concentration of Cu^{2+} ion is 1×10^{-5} M. Thus, the formation constant (Kf) is calculated to be 1×10^{5} M^{-1}. This result ensures the high selectivity of the prepared sensing element toward Cu^{2+}ion.

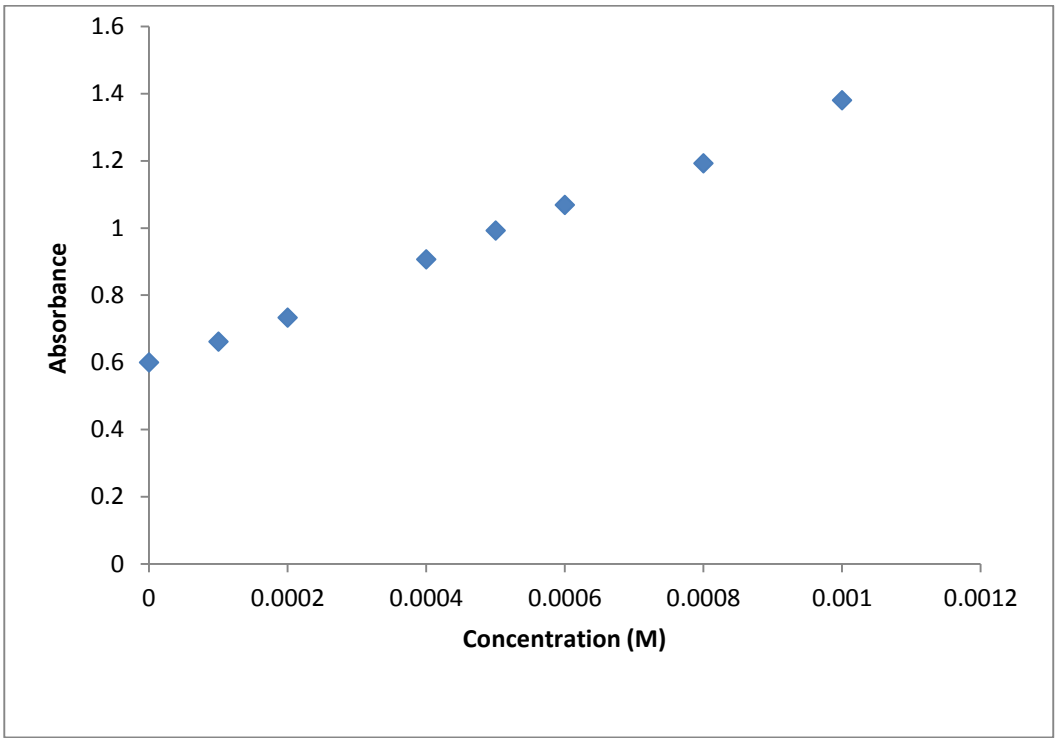

Figure (3. 7): Turbidity absorbance of sensing element vs concentration of Cu2+ in distilled water at 400 nm, 25 ºC, pH~7.3.

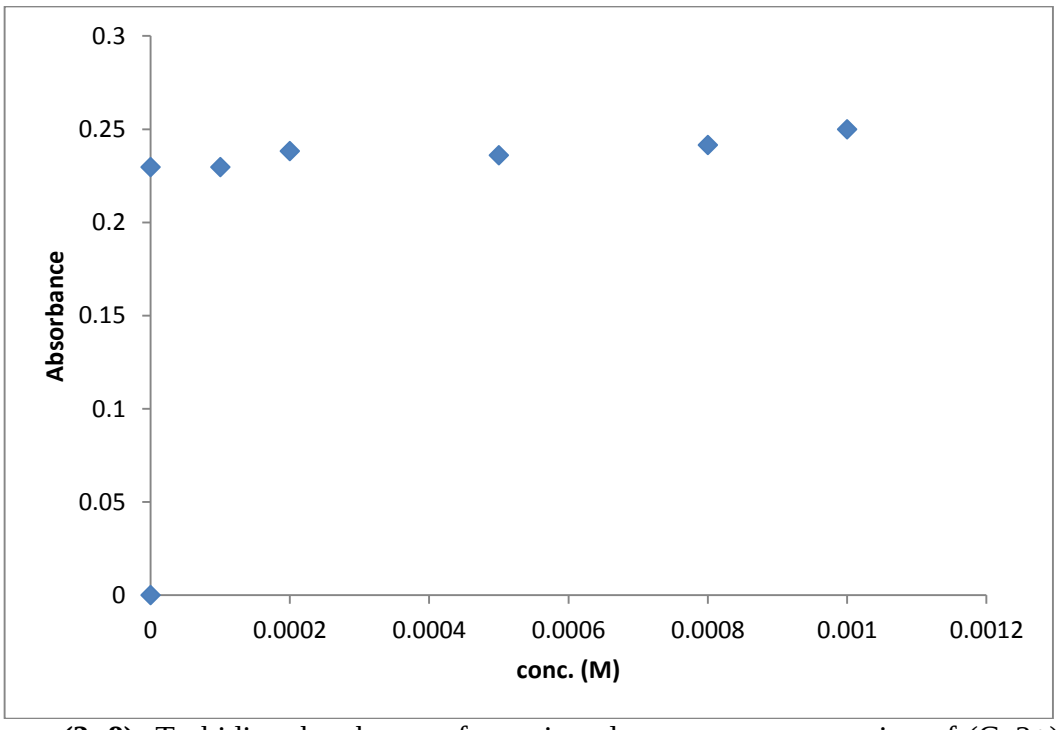

Figure (3. 8): Turbidity absorbance of sensing element vs concentration of (Co2+) in distilled water at 400 nm, 25 ºC, pH~7.3.

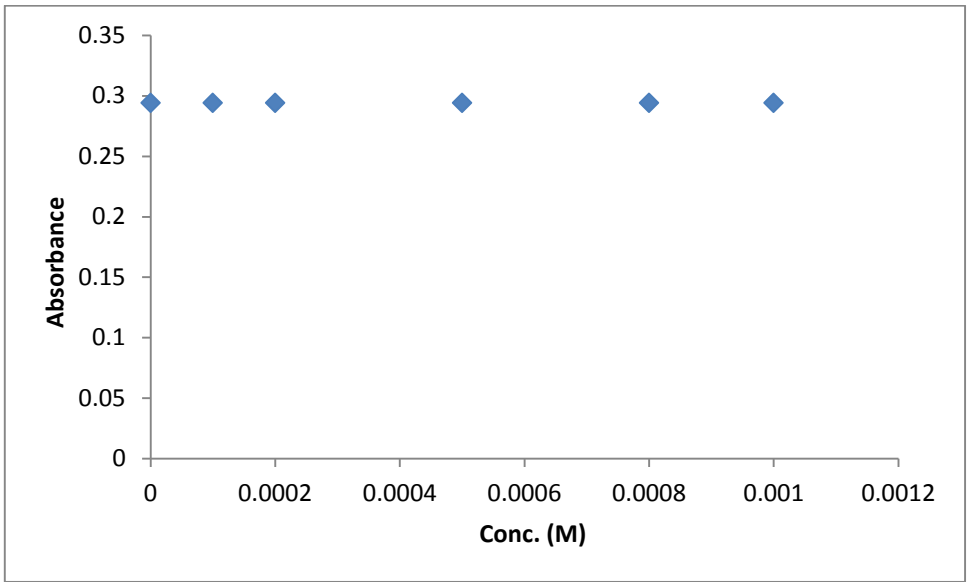

Figure (3. 9): Turbidity absorbance of sensing element vs concentration of (Ni2+) in distilled water at 400 nm, 25 ºC, pH~7.3.

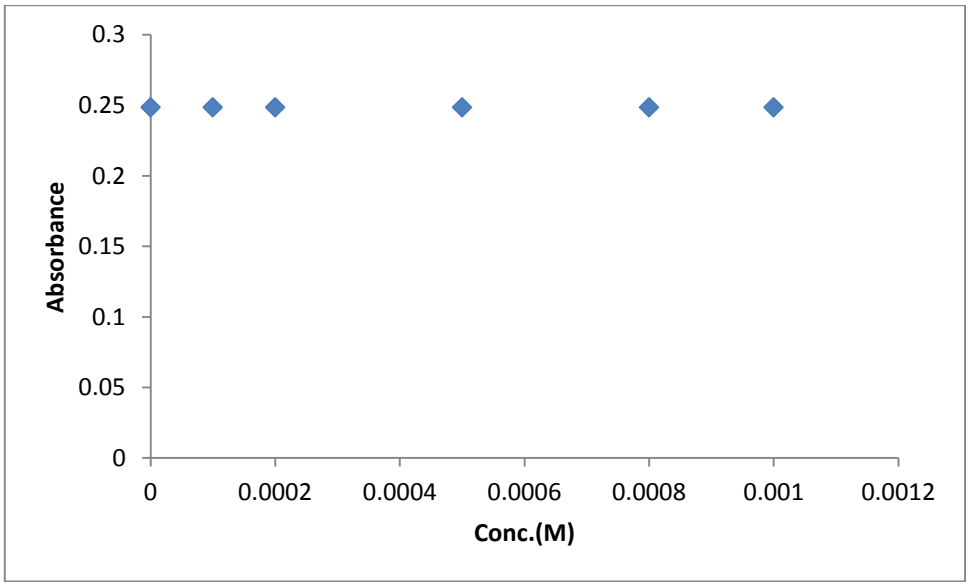

Figure (3. 10): Turbidity absorbance of sensing element vs concentration of (Hg2+) in distilled water at 400 nm, 25 ºC, pH~7.3.

A significant response to Cu^{2+} was observed. This indicates that the acidified tris-amine functional group didn't bind with the other tested transition metal ions regardless of their concentrations. The complex formation between Cu^{2+} and the acidified tris-amine functional group

caused the polymer microspheres to shrink. This shrinking causes an increase in the turbidity and the absorbance.

Since the sensor showed a significant response to Cu^{2+}, with an optimum pH of 8.0. This is probably due to at pH of about 8; the acidified tris-amine functional groups are completely deprotonated and are free to bind the Cu^{2+} ions. The response of the sensor to various concentrations ofCu^{2+} aqueous solutions ($1x10^{-6}$ up to 0.001 M) was tested.

As the concentration of Cu^{2+} was increased, the corresponding turbidity and absorbance in the range 3400-800 nm also increased.

3.5 The response time of the sensor:

The response time of the sensing membrane toward Cu^{2+} ions was obtained by measuring the change in absorbance of 0.001 M Cu^{2+} with time.

The absorbance increased sharply up to about 5 minutes as the time increased, with a response of ~ 90%. A slight increase in the absorbance was noticed up to ~ 10 minutes, then it leveled off as shown in Figure 3.11. This is faster than the response of sensor made by PVBC alone. This is related to the existence of hydrophilic group of pyridine.

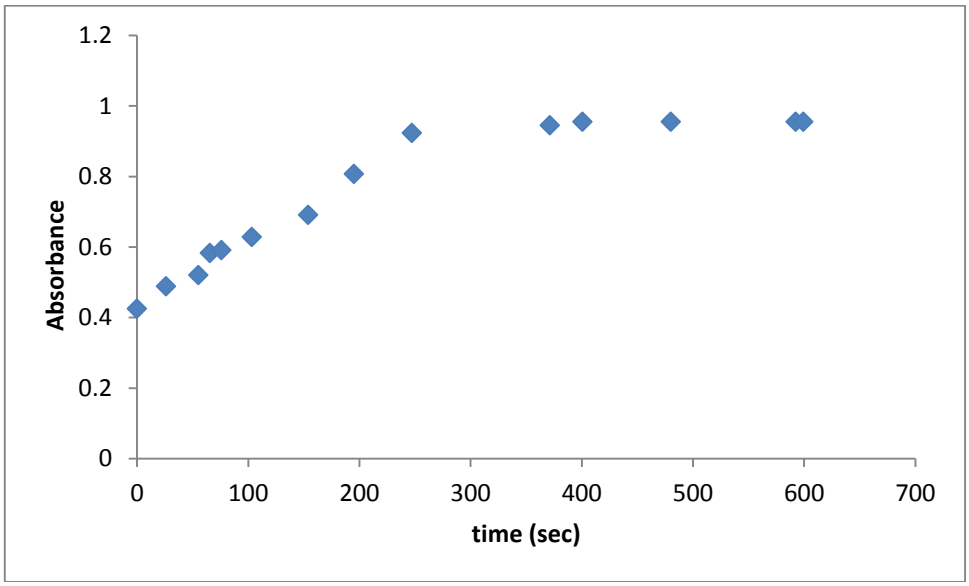

Figure (3. 11): The response time of the sensing membrane of Cu2+ in distilled water (pH~7.3).

After 5 minutes, there may be an increase in the thickness of the shrinking outer layer of the microsphere surface that results in a significant reduction in the rate of diffusion of ions through it. As it becomes less hydrophilic as a result of chelation between the metal ion and deprotonated functional groups on the polymer microspheres. Also, saturation of the complex take place.

3.6 Reproducibility of the sensor response:

The reproducibility of the sensor was tested by measuring the absorbance of 0.001 M Cu^{2+} aqueous solution at 800 nm. The results are shown in Table (1). In distilled water, the sensor gave an absorbance of 0.8062. An absorbance of about 1.2356 was observed when the sensing membrane was placed in a 0.001M Cu^{2+} solution (pH~7.3).

After each run, the sensor was washed with a saturated solution of EDTA and then with buffer solution, then washed successively with distilled water. So this sensor response was reproducible.

Table (3. 1): Reproducibility of the sensor

Conc.(Cu^{2+})	1st run	2nd run	3rd run	4th run
0.000 M	0.8062	0.8062	0.8062	0.8075
0.001M	1.2356	1.2356	1.2336	1.2336

3.7 Effect of pH on the sensor response

The effect of pH on the binding of Cu^{2+} with the acidified tris-amine co-polymer was examined by using buffer solutions of pH 2 - 8. Figure 3.12 shows the dependence of the complex formation between Cu^{+2} and acidified tris-amine co-polymer on pH. Potassium phosphate buffer was used to adjust the buffer solution.(15)

The values of pKa of amino and carboxyl groups are about 10 and 3 respectively, because of that, spectral variations of the sensing phase at high and low pH values would be observed.(16) It also due to the precipitation of $Cu(OH)_2$ at high pH.

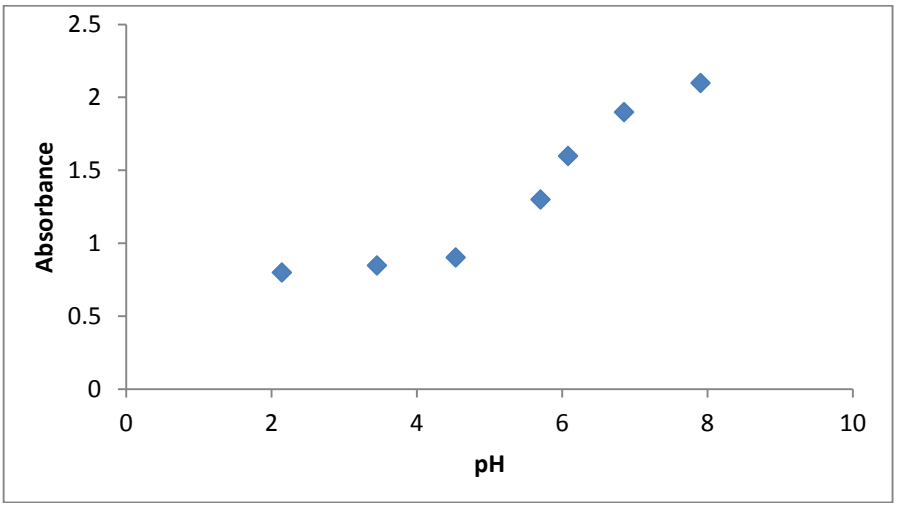

Figure (3. 12): Turbidity absorbance of the sensing element for (0.001M) Cu2+ at different pHs.

We detected a small change in absorbance up to about pH 7. But, above that, significant increases in absorbance were observed. This is probably due to an increase in the fraction of the deprotonated functional groups that resulted in an enhancement of the complex formation between ligand and Cu^{2+} ions.

3.8 Sensor response to other alkali and alkaline earth metal ions:

Figures (3.13, 3.14, 3.15, 3.16), show that the sensing element has no response toward alkali and alkaline earth metal cations. This can be explained by the fact that oxygen atoms of the tris-amine group are classified as soft ligands that can't bind hard metal ions of alkali and alkaline earth metals [17]. So the existence of these metal cations will have no effect on the optical properties of the sensing membrane. This result allows the application of this sensor on real samples regardless to any concentration of these ions; Na^{+},K^{+} ,Mg^{2+}, and Ca^{2+}.

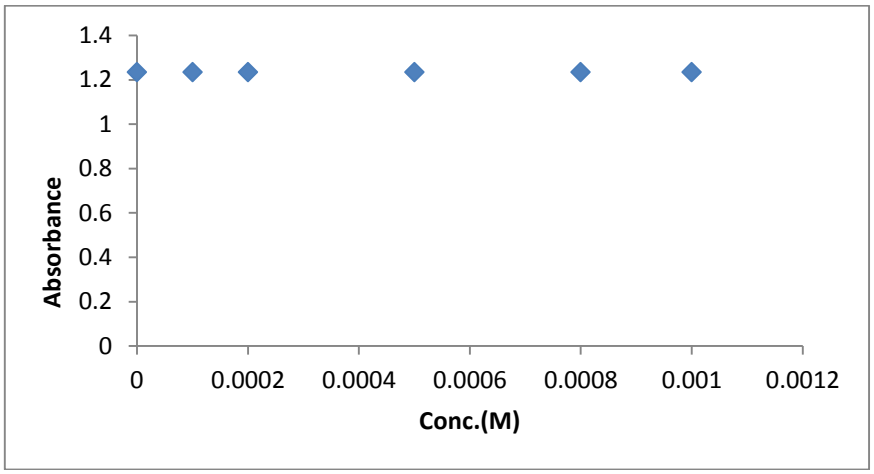

Figure (3. 13): Sensor response to Na+ at pH=7.3, at RT, and 400nm.

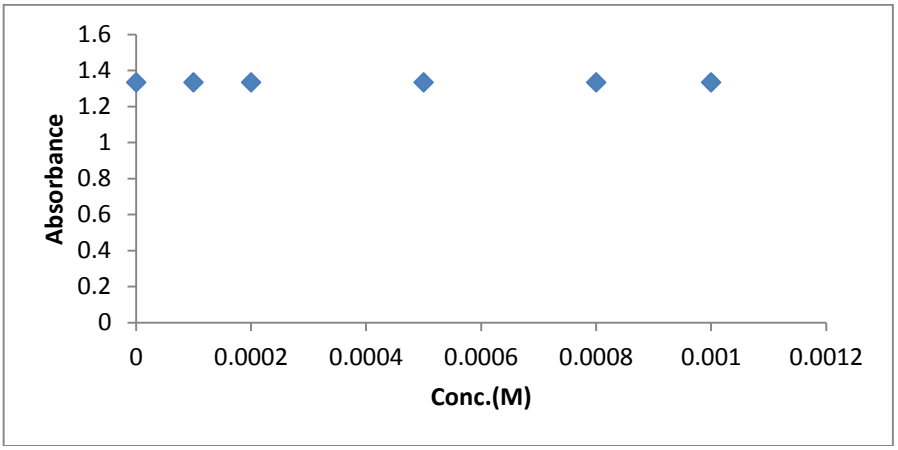

Figure (3. 14): Sensor response to K+ at pH=7.3, at RT, and 400nm.

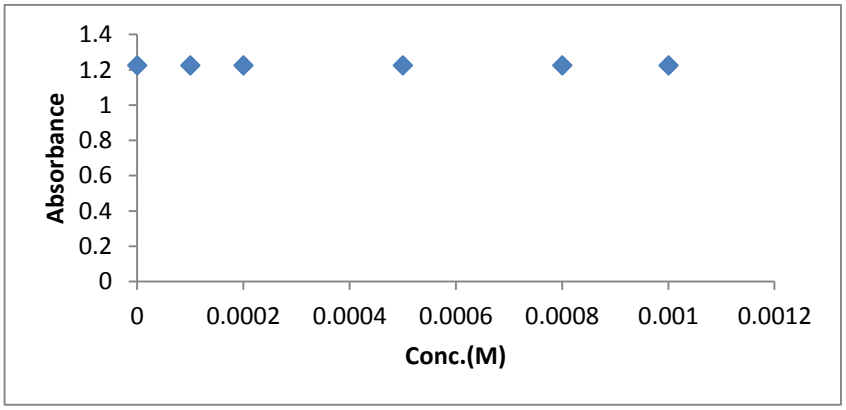

Figure (3. 15): Sensor response to Mg2+ at pH=7.3, at RT, and 400nm.

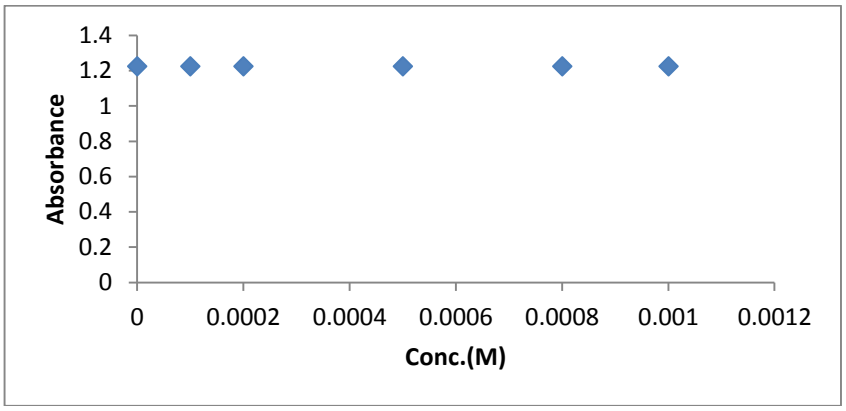

Figure (3. 16): Sensor response to Ca2+ at pH=7.3, at RT, and 400nm.

3.9 Sensor response towards myoglobin protein

The sensor response was tested to different proteins; myoglobin (from equine skeletal), hemoglobin (from bovine blood), and albumin (from human serum). It has good response for myoglobin and acceptable one for hemoglobin, and has no response for albumin.

The sensing membrane response was studied especially for myoglobin protein which was the target in this study.

Absorbance for only myoglobin was measured as shown in figure (3.17). As the concentration increase, the absorbance increase linearly.

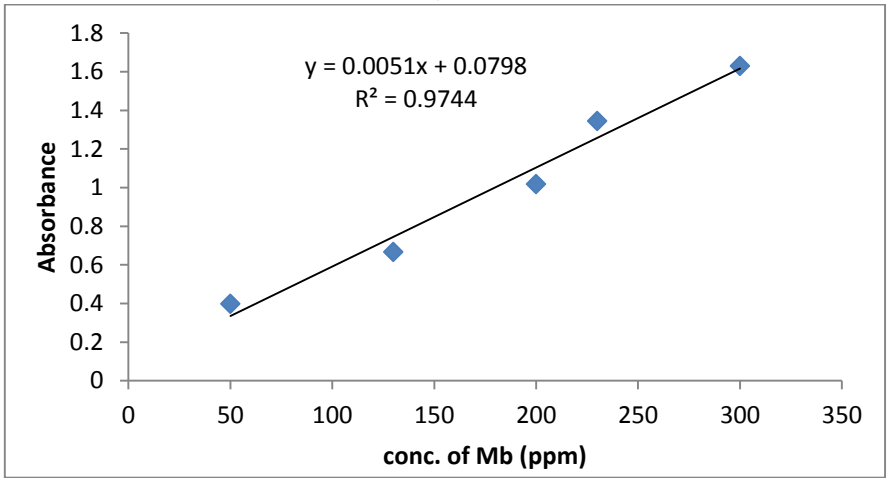

Figure (3. 17): Sensor response of different concentrations of myoglobin (50, 130, 200, 230,and 300ppm) at λ=400, in distilled water (pH= 7.3)without membrane.

The response of the sensing membrane to myoglobin (50 ppm) upon the addition of various concentrations of Cu^{2+} was observed as shown in figure (3.18, and 3.19). The results were different from those in the absence of myoglobin. As the concentration of Cu^{2+} ions attached to the membrane increased, high absorbance was appeared. In the presence of myoglobin, the absorbance was increased with increasing Cu^{2+} concentration. This may be attributed to formation of aggregates of myoglobin with the complex TAA- Cu^{2+} – His[13].

No change in the spectra was observed when Cu^{2+} ions or myoglobin was individually added to the membrane complex. The peak at 408 nm is due to the Soret absorption band of myoglobin associated with a six-coordinated high-spin ferric heme with covalently bound water and a highly polar distal pocket.[18]

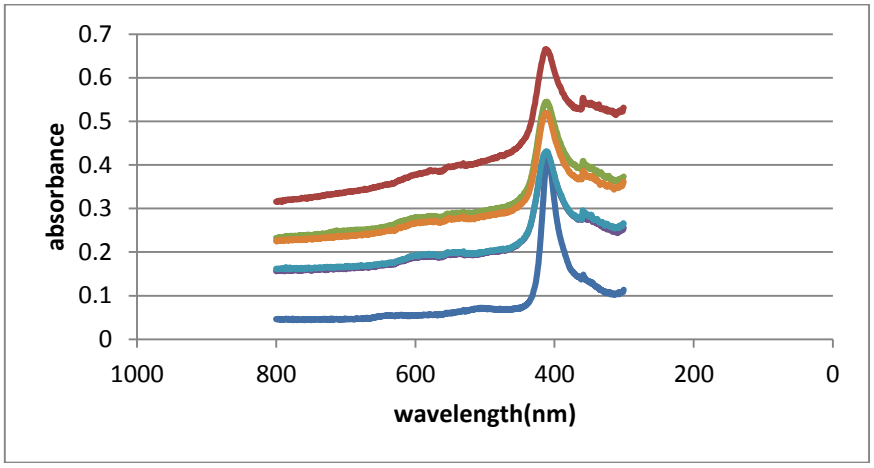

Figure (3. 18): Absorbance vs. wavelength(nm) of Mb. (Different conc. of Cu2+ (0.0002, 0.0004, 0.0005, 0.0006, 0.0008, and 0.001M) with 50 ppm myoglobin in presence of membrane.

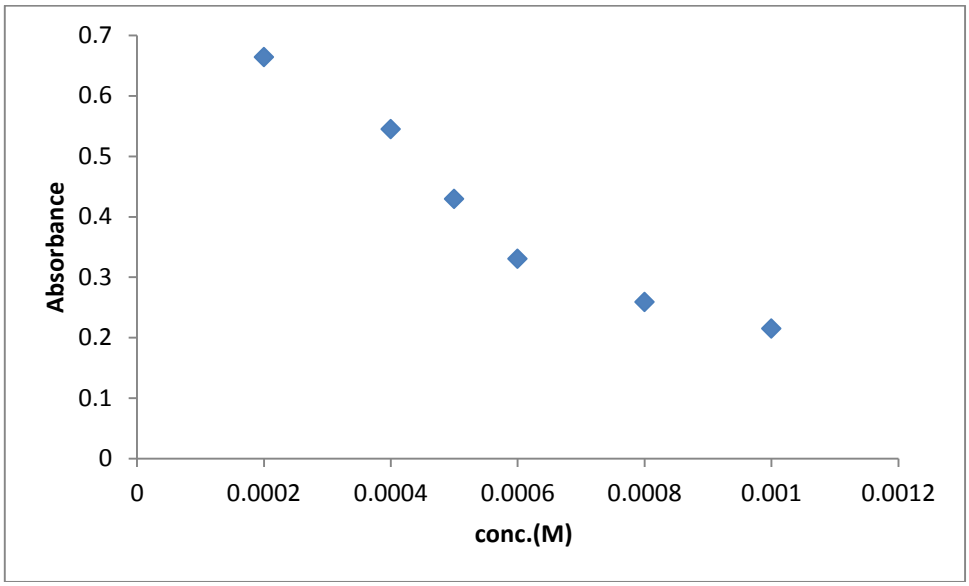

Figure (3. 19): Turbidity absorbance of the sensing element for Mb (50ppm) of different concentrations of Cu2+ (0.0002, 0.0004, 0.0005, 0.0006, 0.0008, and 0.001ppm) in distilled water (pH=7.3),λ=400, and at room temperature.

Myoglobin has eleven His residues, four of which are exposed on the surface[19]. The imidazole moieties of the surface His residues can bind with Cu^{2+} [20]. Therefore, the aggregates of myoglobin are expected to be formed through the coordination interaction of TAA complex-Cu^{2+}-His.

3.10 Determination of Myoglobin

Quantitative determination of myoglobin was achieved by addition of various concentrations of myoglobin to the carboxylated TAA-Cu^{2+} membrane (in the presence of Cu^{2+} ions). As shown in figure 3.20.

A linear relationship between the maximum absorbance and the concentration of myoglobin is obtained, as the concentration of myoglobin increase, the absorbance increases. The sensitivity with the sensing system is greater than that can be obtained by the absorption band of the protein itself.

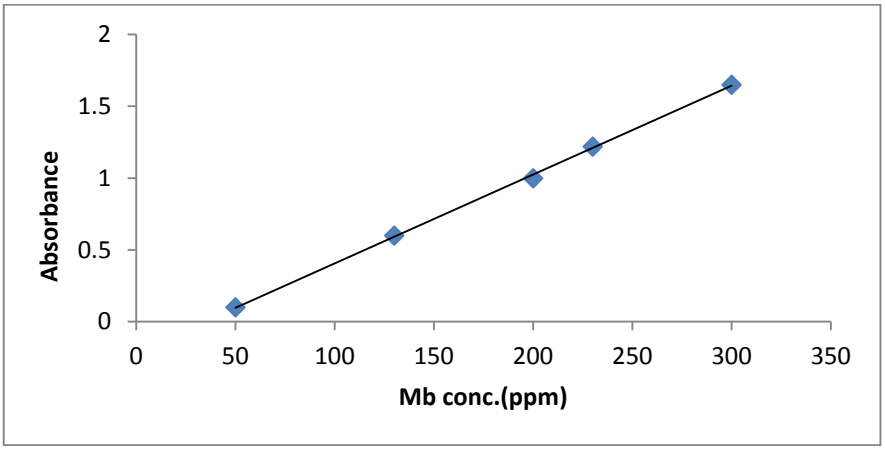

Figure (3. 20): Response of sensing membrane to different conc. of myoglobin(50, 130, 200, 230, 300ppm), (0.001M Cu2+) .

3.11 Calibration Curve

A calibration curve was constructed by plotting the absorbance that measured at 400 nm, vs Mb- Cu^{2+}-complex concentration**.** The curve is shown in figure 3.21. A linear relationship was obtained over the studied concentration range.

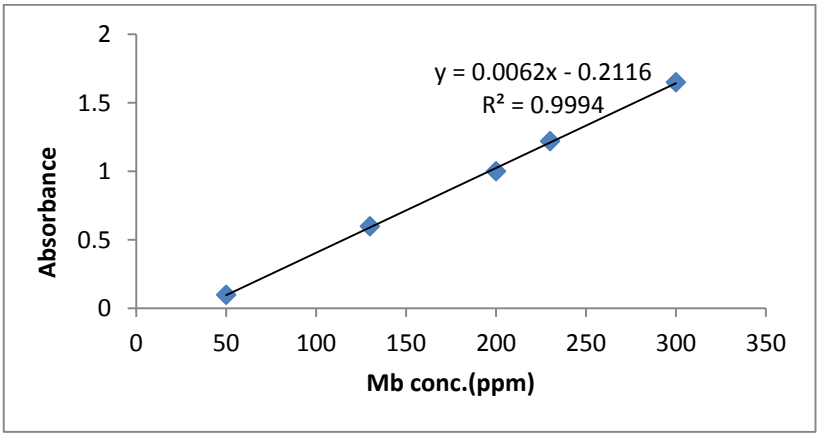

Figure (3. 21): calibration curve for Mb- Cu2+-complex at pH 7.3, 400 nm.

3.12 Performance characteristics

3.12.1 Limit of Detection (LOD)

For analyst, it is important to know when analyte signal is acceptable or not. Instrumental error applies also to blank measurement, which is the instrument response when no analyte is found in the sample. In analytical chemistry, it must be at least three times greater than the blank signal[22,23].

The analyte's signal ($S_{analyte)}$ at the detection limit is given by equation (3.1):

$$S_{analyte} = S_{blank} + z\sigma_{blank} \quad (3.1)$$

Where S_{blank} is the average blank signal, σ_{blank} is the standard deviation of the blank signal, z is the factor of the desired confidence level, is set to 3 at 95% confidence level. [22, 23]

The analyte's concentration ($C_{analyte}$) at the detection limit is given by the equation(3.2).[24]

$$C_{analyte} = (S_{analyte} - S_{blank})/slope \quad (3.2)$$

The standard deviation of the blank was determined by measuring the absorbance of the blank (at 400nm) ten times.

The detection limit was calculated to be 6.174×10^{-6} M.

3.12.2 Limit of Quantitation (LOQ)

LOQ defined as the lowest analyte concentration in a sample that can be determined with acceptable value of accuracy and precision[23].

The analyte's signal ($S_{analyte}$) at the quantitation limit is given by the equation (3.3).[22,23]

$S_{analyte} = S_{blank} + 10\sigma_{blank}$ (3.3)

Where S_{blank} is the average blank signal, σ_{blank} is the standard deviation of the blank signal [22,23].

The analyte's concentration ($C_{analyte}$) at the quantitation limit is given by equation (3.4): [23]

$C_{analyte} = 10\sigma_{blank} / slope$ (3.4)

The quantitation limit of the method was calculated to be 1.317×10^{-5} M.

3.13 Protein assay

The response of the TAA-Cu^{2+} membrane to other proteins, including hemoglobin (from bovine blood), and albumin (from human serum) was also examined. When albumin protein was added, no changes in the absorbance were observed; whereas there was clear change upon the addition of Mb and bovine hemoglobin BHb. However, the change in the absorbance is larger for Mb compared to that of BHb.

These observations may be attributed basically to insufficient His residue on the surface of albumin and the larger volume of BHb compared to that of Mb. The magnitude of the band shifts depends on strength of the PR coupling which decays with the inter-particle gap[21].

It is clear that the optical sensing of His-rich proteins with TAA complex depends on the number and distribution of surface His residues as well as the size of proteins.

This method has the advantages of being easy to perform, inexpensive, and time saving.

Conclusion

In this work, a modified sensing membrane with a selective functional group made up of Tris-aminocarboxylate as optical sensor based on swellable polymer microspheres is developed. Carboxylated tris-amine functional group covalently bonded to the co-polymer was prepared. These derivatized microspheres are entrapped in a hydrogel membrane. The acidified tris-amine group showed a selective response to Cu^{+2} ion. It did not show respone to other metal ions, especially alkali and alkaline earth metal and other heavy metal ions regardless of their concentrations.

Chemical sensors based on polymer swelling and shrinking have several advantages: They are very simple to make and they are of low cost which makes them good candidates for remote sensing upon using a LED light source and a photodiode detector. In addition to the possibility of introducing a variety of functionality in place of the carboxylated tris-amine making them capable of targeting other different metal ions.

The optical sensing of Mb with tris-aminocarboxylate metal complexes has been demonstrated. The membrane was showed a high response when tested to myoglobin which gave a very good result which lead to a big change in future.

Determination of myoglobin is of a significant importance for early diagnosis of diseases. The main method for detection of Mb is immunoassay which is usually very expensive. The proposed method in this work offer the advantage of being easy to perform, with low cost, and time saving.

Suggestions for further studies

The following recommendations are suggested for future work:

1. Testing the membrane with other bioorganic samples.
2. Testing the membrane behavior and response in real samples(urine, and blood), and food samples.
3. Try flourometers as another detection method.

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جامعة النجاح الوطنية
كلية الدراسات العليا

تحديد بعض البروتينات من خلال تفاعلها مع معقدات ثلاثي امينوكاربوكسيليت

اعداد
ميرفت فواز ابراهيم ابوزينة

اشراف
د.ابراهيم ابوشقير
د.زياد الشخشير

قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في الكيمياء بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس- فلسطين

2016

تحديد بعض البروتينات من خلال تفاعلها مع معقدات ثلاثي امينوكاربوكسيليت

اعداد

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اشراف

د.ابراهيم ابوشقير

د.زياد الشخشير

الملخص

في هذه الدراسة و عن طريق تقنية الامتصاص البصري تم تحضير ودراسة مجس كيميائي معتمدا على الانكماش والانتفاخ للبوليمر. حيث تم تحضير بوليمر مرتبط بمجموعة ثلاثي-2-أمينو ايثل أمين وتشخيصة, البوليمر المتكون ينتج من تفاعل الاستبدال الحاصل بين بوليمر بولي(متعدد) فينيل بنزيل برومايد-بولي فينيل بيريدين و ثلاثي-2-أمينو ايثيل أمين مرتبط بمجموعة كاربوكسليت.

بعد تشخيص المجس ودراسة خصائصه البصرية, وجد ان ايون النحاس اعطى استجابة عالية ولم يعط اي استجابة لأيونات العناصر القلوية والقلويات الترابية والانتقالية مثل الايونات الثنائية كالكوبالت, النيكل, والزئبق. ولم يعط اي استجابة لدرجات حموضة مختلفة.

تم تحديد بروتين الميوجلوبين من خلال تفاعله مع هذا المعقد المرتبط بايونات النحاس , وتمت دراسة العديد من الخصائص وتأثرها بالتركيز ودرجة الحموضة التي اظهرت نتائج جيدة نتوقع من خلالها ايجاد طرق تحليل سهلة, غيرمكلفة, وسريعة.