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Synthesis And Characterization Of Molecularly Imprinted Cross-Linked Poly (4-Vinylpyridine) For The Recognition And Concentrationdetermination Of Bilirubin In Solution

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Synthesis and Characterization of Molecularly Imprinted Cross-Linked Poly (4-vinylpyridine) for the Recognition and Concentration Determination of Bilirubin in Solution

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Dedication

To Hannah Daniels (Grandmother) and Perpetua Christiana Oppong (Mother) whom I lost during the pursuit of this degree but are here in spirit. I love you both.

Biographical Sketch

Alex Aboagye was born on August 4, 1982, in Takoradi, Ghana, West Africa. He received his Bachelor of Science degree in Chemical Engineering from Kwame Nkrumah University of Science and Technology, Kumasi, Ghana in 2006. He is a candidate for the Master of Science degree in Chemical Engineering.

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Abstract

Molecular imprinting is a procedure for the preparation of artificial receptors for specific binding with target molecule. In this work molecularly imprinted polymer (MIP) film for the recognition of bilirubin was synthesized and studied using the Quartz Crystal Microbalance (QCM). The MIP was coated on the QCM crystals and analyzed using QCM. 4-vinylpyridine was used as functional monomer, divinylbenzene as cross-linking agent and benzophenone as initiator. As a control, another polymer was prepared in the same way as the MIP but without the template monomer. The control polymer or the non-MIP exhibited very little affinity for the target molecule compared to the MIP. A calibration curve for bilirubin concentration was obtained from the frequency shifts given by different concentrations of bilirubin solution using the QCM. The effects of pH and ionic strength on recognition were also discussed. To ensure that the MIP synthesized was selective, biliverdin recognition was studied and compared to that of bilirubin. The MIP showed a higher affinity for bilirubin than biliverdin.

CHAPTER 1

Introduction

1.1 Background

Biological recognition is highly specific and selective. This ability of one molecule to selectively recognize a specific molecule in a pool of similar molecules is crucial to biological and chemical processes. Molecular recognition occurs when a molecule is geometrically complementary to another molecule such that both can fit together spatially or bind to each other usually utilizing non-covalent forces (Chen, Piletsky, & Turner, 2002). An example of this process is the binding of starch and amylase (enzyme) to form complexes that catalyze breakdown of the starch. Other examples include antigen/antibody recognition in the immune system(Jimenez, Salazar, Baldridge, & Romesberg, 2003) and RNA and DNA molecules as receptors for a variety of substrates(Bunka & Stockley, 2006).

Following nature's cue, the biosensor, an analytical device for the detection of an analyte has been developed. The biosensor's sensitive element interacts (binds or recognizes) the analyte under study. This sensitive element is usually a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.), a biologically derived material or bio-mimic component. The blood glucose biosensor, which uses the enzyme glucose oxidase to break blood glucose down, is a common example of a commercial biosensor. The biosensor is very useful and has many potential applications. Some of these potential applications include clinical diagnosis and biomedicine, environmental monitoring, and pharmaceutical product analysis. Other applications include fermentation control analysis of food and drinks. They may also be used for explosives, chemical agents and biological toxins detection, drug detection and testing, and military applications. Although the biosensor is very

useful and has a wide array of applications, its bio sensitive component makes it unsuitable for certain applications. These bio sensitive elements of the biosensor do not thrive in extreme temperatures and pressures(Yan & Ramström, 2005). They possess little or no tolerance towards external factors such as exposure to acidic, basic or organic solvents(Yan & Ramström, 2005). They are expensive and expensive to handle. Storage by refrigeration and other specialized means will increase the cost of operation of these biosensors in remote parts of the world such as some African villages with no source of electrical power and means of refrigeration. This will eventually defeat the purpose of using the biosensor as a cheap and reliable alternative of diagnosis of diseases in very remote parts of the world where quality healthcare services is not easily accessible. Also, it is difficult to engineer these biomaterials to be able to detect a wide range of chemical and biochemical analytes and as such the number of analytes that can be detected by these bio sensitive elements are relatively limited.

1.2 Significance of Work

To overcome the shortcomings of the bio sensitive element of the biosensor and with increased knowledge in molecular recognition, scientists are seeking to fabricate completely polymeric materials that can specifically bind analyte (target molecules) to mimic their biological counterparts. These mimics can be applied in fields such as food industry, environmental monitoring, health care for diagnosis of disease and controlled drug delivery(Burkoth & Anseth, 2000). The polymeric materials are made in the presence of the target molecule. In this way, they can ‘memorize’ the shape, size and chemical functionalities of the target molecule to mimic biological recognition. The synthetic polymers have a strong tolerance towards external factors such as exposure to acidic, basic or organic solvents. They possess higher storage and operational stability than their biological counterparts. Also, a wide

range of chemical functionalities can be put into the synthetic system, and the mechanical properties can be readily tailored(Bergmann, 2006).

An entirely synthetic scheme that can recognize specific target molecules could be a great recognition element in a wide range of applications. For example, such a system could target undesirable substances in the blood such as carcinogens or viruses such as the human immunodeficiency virus (HIV). The binding of these substances would effectively remove them from the bloodstream and, as such, act as an alternative route for disease treatment.

Sensing taste by human panel is often employed in the food and beverages industries to achieve taste consistence in their products. However, the variability between taste panelists can sometimes give rise to more than 50% variation in terms of flavor units(Leonte, Sehra, Cole, Hesketh, & Gardner, 2006). In addition, the tasting process is expensive, time consuming, not possible for 24 hours continuous taste monitoring, and the results are often subjected to the influence of a highly opinionated experienced worker. This leads to the development of an alternative and more consistent means through artificial sensors for quality control of taste in food products. MIPs responsible for specifically and selectively picking taste causing compounds in food products will be a good alternative.

Following the 2001 anthrax attacks in the United States, also known as Amerithrax from its Federal Bureau of Investigation (FBI) case name, a lot of research has been done in synthesizing MIPs that can specifically bind biological toxins such as anthrax. In 2005 the Johns Hopkins University Applied Physics Laboratory (JHU/APL) signed an agreement with Link Plus Corporation granting worldwide exclusive rights to its Molecularly Imprinted Polymer (MIP) detection technology for explosives. In 2006, additional license agreements expanded the grant to include MIPs for toxins, chemical agents, biological agents, pesticides, poisons, elements,

chemical compounds and an apparatus for detecting mines and improvised explosive devices (IEDs) buried in the ground.

In the development of a reliable MIP sensor two significant tasks that must be accomplished:

- The development of a specific chemical recognition element that allows a molecule, or a class of molecules, to be identified.
- A means of signal transduction in which the presence of the molecule causes a measurable change in the physical property of the material.

The MIP together with a suitable transducer could be an alternative route in the diagnosis of diseases. This would be a quick, reliable and less expensive way of detecting marker molecules responsible for diseases in test samples. This will be very useful in very remote parts of the world where quality health facilities are not easily accessible. The setup will be simple and will not require very skilled personnel to man. This will enhance diagnosis of diseases and in turn help in saving lives.

1.3 Objectives

In this project, molecularly imprinted polymer was synthesized using bilirubin as a template. Bilirubin is a brownish yellow substance found in bile. It is produced when the liver breaks down old red blood cells. Bilirubin can be regarded as an important index for judging liver functions and is used in this context to identify a variety of liver diseases. Disorders in the metabolism of bilirubin may cause a yellow discoloration of the skin and other tissues, called hyperbilirubinemia. High bilirubin concentration may even cause hepatic or biliary duct dysfunction, and also permanent brain damage or death in the most severe cases (Cotler et al., 2000). Quartz crystal microbalance (QCM) is the transducer used for this project. The QCM is

portable and highly sensitive. The mass sensing technique of the QCM eliminates the need for any specific labeling step to be part of the signal transduction mechanism. Also, the technique is relatively easy to use, and the basic equipment is inexpensive to purchase. Combining the advantages of the QCM and MIP makes the biosensor developed in this project cost effective.

The objectives of this project are to:

1. Synthesize and characterize bilirubin imprinted polymer.
2. Obtain a bilirubin concentration calibration curve for the bilirubin imprinted polymer.

CHAPTER 2

Literature Review

2.1 Molecular Imprinting

Molecular imprinting is a noted path to the synthesis of molecular recognition materials. It involves the copolymerization of functional and cross-linking monomers in the presence of a target molecule (imprint molecule) which acts as the molecular template (Haupt & Mosbach, 2000). First, the template molecule is dissolved in a solvent and mixed with a functional monomer. The functional monomer interacts with the molecular template through covalent interaction, non-covalent interaction or both, (Hwang & Lee, 2002; Sellergren, 1997; Whitcombe, Rodriguez, Villar, & Vulfson, 1995). Second, the pre-polymer complex is usually copolymerized by free radical initiation in the presence of excess cross-linking monomer. Finally, after polymerization, the template molecule is extracted from the formed polymer, leaving behind a 3-D cavity in the polymer matrix. The cavity, also known as the binding site, is similar in size, and shape as the template molecule. Due to this, a molecular memory is created in the polymer which is able to selectively rebind the template molecule(Haupt & Mosbach, 2000).

The origin of the molecular imprinting technology can be traced back to the early theories on the functions of enzymes and antibodies (D. A. Spivak & Shea, 2001). Emil Fischer (D. A. Spivak & Shea, 2001) postulated the idea of a “lock and key” fit to describe the specificity of enzymes in the 1890s as shown in Figure 2.1. Frank Dickey (D. A. Spivak & Shea, 2001) conducted initial experiments toward the development of “synthetic antibodies”. He prepared silica gel through the polymerization of sodium silicate in the presence of a dye. When the dye compound was removed, he observed that the silica gels preferentially rebound the organic dye in which it was prepared. Takagashi and Koltz (Takagishi & Klotz, 1972) and Wulff and Sarhan

(Wulff & Sarhan, 1972) in 1972 separately reported the first examples of molecular imprinting in synthetic polymers. Since then, a lot of research has been done in this area. Wulff initially suggested a 'covalent approach' for the synthesis of molecularly imprinted polymers (MIPs). Later on, Mosbach (Mosbach & Ramström, 1996; Sellergren, Lepistoe, & Mosbach, 1988) and his co-workers came up with a non-covalent interaction approach to synthesize MIPs.

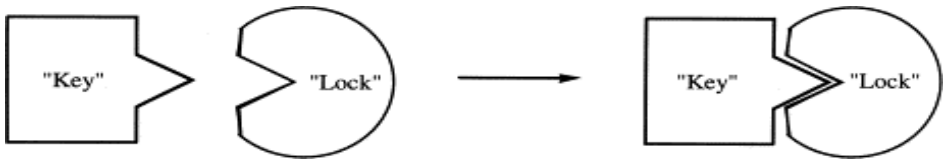

Figure 2.1. Emil Fischer's 'lock and key' principle

2.1.1 Covalent Molecular Imprinting. Reversible covalent bonds between the template molecule and the functional monomer are employed to create a stable monomer-template complex. After polymerization reaction, the covalent bond is chemically split permitting the template to be extracted from the polymer matrix. On reintroduction of the template into the polymer matrix, the covalent bond reforms in the binding sites (Wulff, 1995). The principal advantage of this approach is that, the synthesized polymer will have a homogenous distribution of binding sites. This approach attains a uniform distribution of imprinting sites than can be attained by the non-covalent method. Nevertheless, it is limited to templates and monomers that form covalent bonds whose formation kinetics are adequately fast in order to allow for use in many common separation techniques (Andersson, 2000). The success of covalent imprinting depends on the chemical association that forms the template-monomer complex. This association needs to be stable under the reaction conditions and at the same time the bond must be easily broken for template removal. In order not to disrupt the imprinted polymer network, the breaking of bond must be done under mild cleavage conditions. Also, the rebinding of the template molecule in covalent imprinting is slower than the recognition attained in non-covalent

imprinting (Komiyama, 2003). As such, covalent imprinting is not as widely studied as its non covalent counterpart.

2.1.2 Non-Covalent Molecular Imprinting. Non-covalent molecular imprinting is a commonly applied procedure to create MIPs. In this approach, non-covalent interactions occur between the target molecule and functional monomers. Hydrogen bonding, electrostatic interaction, π-π complexation, hydrophobic interactions, and metal – ligand complexation (Andersson & Mosbach, 1990; Dunkin, Lenfeld, & Sherrington, 1993; Fu, Sanbe, Kagawa, Kunimoto, & Haginaka, 2003; Hart & Shea, 2002; Sellergren, Ekberg, & Mosbach, 1985) are typical types of interactions utilized. These interactions are weaker compared to covalent bonding allowing a rapid and reversible binding as needed. Once polymerized, a non-evasive wash cycle is employed to fully remove the template. The advantage is that, the intermolecular interactions utilized in the imprinting process have very fast kinetics, thus many different types of templates can be imprinted. Figure 2.2 is a schematic representation of covalent and non-covalent imprinting procedures.

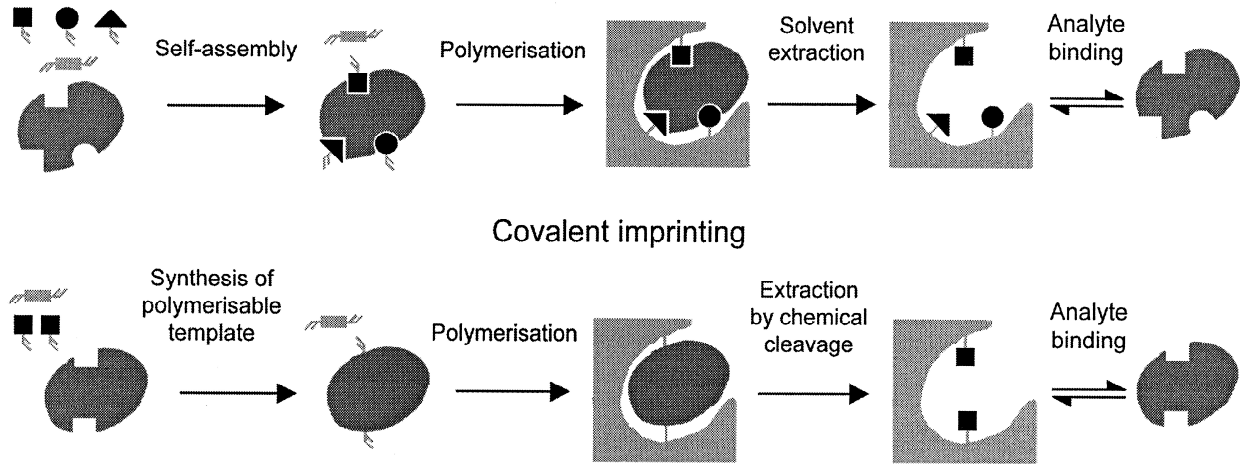

Figure 2.2. Schematic representation of covalent and non-covalent molecular imprinting procedures

2.2 Polymer Components

2.2.1 Template. The templates direct the organization of the functional groups pendant to the functional monomers. Templates offering several interaction sites for the functional monomers are likely to yield binding cavities with high affinity and specificity for the template. Templates possessing conformational rigidity can fit well in the cavity of the polymer matrix with minimal change in conformation and will increase the affinity and selectivity in the recognition (Shea, Spivak, & Sellergren, 1993; D. Spivak, Gilmore, & Shea, 1997). The ratio of template to functional monomer is known to play a vital role in the selectivity and sensitivity in imprinted polymers and has to be determined for each template (Yu & Mosbach, 1997). Not all templates are directly amenable to templating for a variety of reasons. In terms of compatibility with free radical polymerization, templates should be chemically inert under the polymerization conditions. Thus, different imprinting strategies may have to be sought if the template can take part in radical reactions or is for any other reason, it is unstable under the polymerization conditions. The following are justifiable questions to ask of a template:(1) Does the template have any polymerizable groups, (2) Does the template have functionality that could inhibit or retard a free radical polymerization, e.g. a thiol group or a hydroquinone moiety, and (3) Will the template be stable at moderately elevated temperatures (e.g. at or around 60 °C if azobisisobutyronitrile (AIBN) is being used as the chemical initiator) or upon exposure to UV irradiation (Cormack & Elorza, 2004).

2.2.2 Functional Monomers. The ability of the polymer structure to have a high affinity for a template molecule defines the success of an imprinting system, which depends on the template-monomer interaction (Bures, Huang, Oral, & Peppas, 2001). Functional monomers are responsible for the binding interactions in the imprinted binding sites. It is obviously essential to

match the functionality of the template with that of the functional monomer in a complementary manner (e.g. H-bond donor with H-bond acceptor). In this way, the complex formation as well as the imprinting effect is maximized. In general, templates containing basic functional groups are best imprinted with monomers containing acidic functional groups and vice versa. Functional monomers are usually used in an excess mole ratio to the template, usually starting at 4:1 and increasing (Cormack & Elorza, 2004). Some common functional monomers used are shown in Figure 2.3. Methacrylic acid is one of the most widely used monomers. It interacts ionically with amine functional group and via hydrogen bonding with a variety of polar functional groups such as alcohols, carboxylic acids, carbamates and carboxylic esters.

2.2.3 Cross-linkers. The rigidity of the imprint cavities in MIPs enhances their selectivity. Using a high degree of cross-linking, high selectivity and specificity in MIPs is achieved. Cross-linkers fulfill the following three major functions in MIPs:

- Controls the morphology of the polymer matrix;
- Stabilizes the imprinted binding sites; and
- Imparts mechanical stability to the polymer matrix.

High cross-link ratios are generally preferred in order to access permanently porous (macroporous) materials and in order to be able to generate materials with adequate mechanical stability. Polymers with cross-link ratios in excess of 80% are often the norm (Cormack & Elorza, 2004). The chemical structures of some well-known cross-linkers are shown in Figure 2.4. Ethylene glycol dimethacrylate (EGDMA) is one of the widely used cross-linker in non-covalent molecular imprinting.

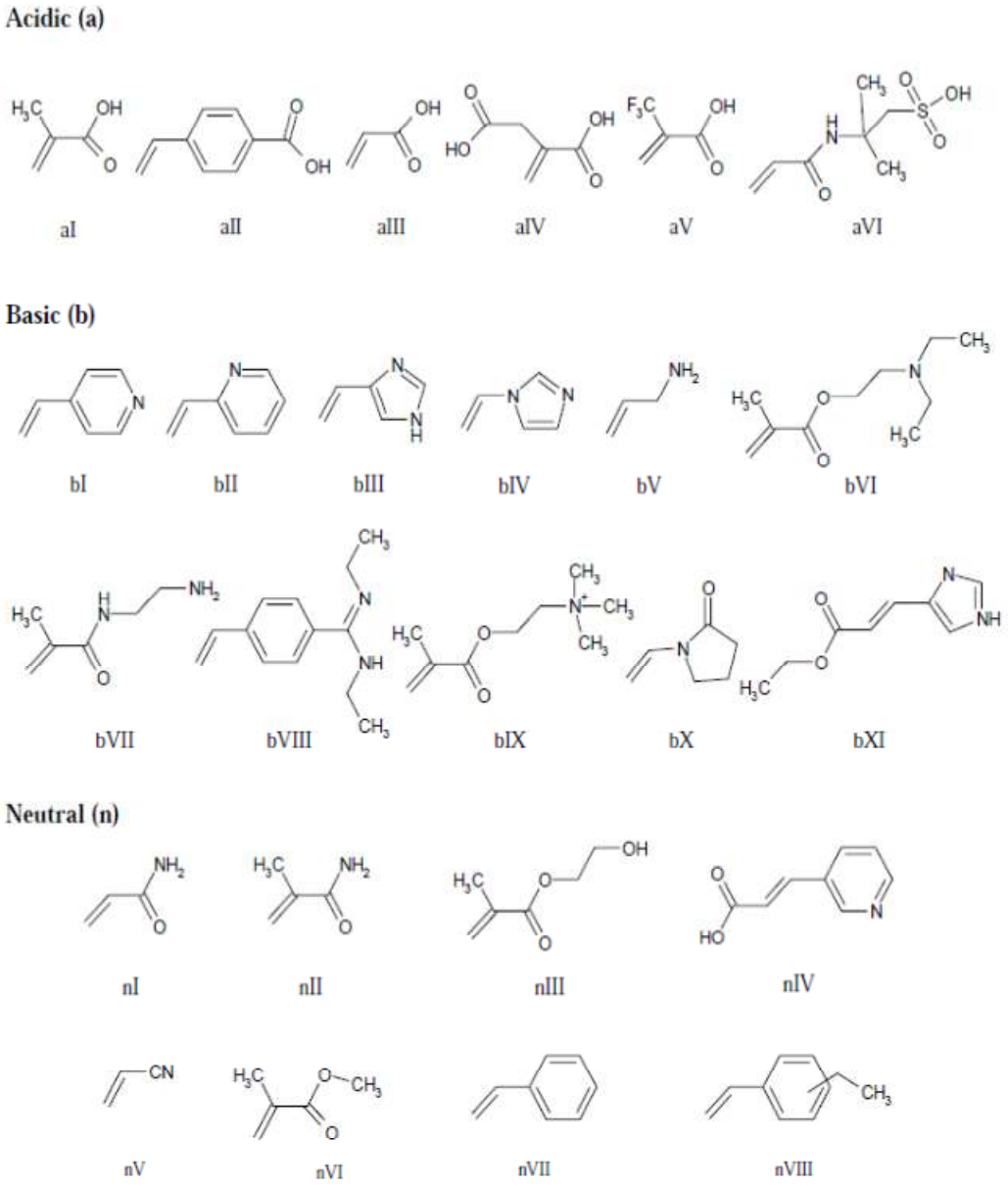

Figure 2.3. Selection of monomers used in the non-covalent approach. Acidic; aI: methacrylic acid (MAA); aII: p-vinylbenzoic acid; aIII: acrylic acid (AA); aIV: itaconic acid; aV: 2-(trifluoromethyl)-acrylic acid (TFMAA); aVI: acrylamido-(2-methyl)-propane sulfonic acid (AMPSA). Basic; bI: 4-vinylpyridine (4-VP); bII: 2-vinylpyridine (2-VP); bIII: 4-(5)-vinylimidazole; bIV: 1-vinylimidazole; bV: allylamine; bVI: N,N-diethyl aminoethyl methacrylamide (DEAEM), bVII: N-(2-aminethyl)-methacrylamide; bVIII: N,N-diethyl-4-styrylamidine; bIX: N,N,N,-trimethyl aminoethylmethacrylate; bX: N-vinylpyrrolidone (NVP); bXI: urocanic ethyl ester. Neutral; nI: acrylamide; nII: methacrylamide; nIII: 2-hydroxyethyl methacrylate (2-HEMA); nIV: trans-3-(3-pyridyl)-acrylic acid; nV: acrylonitrile (AN); nVI: methyl methacrylate (MMA); nVII: styrene; nVIII: ethylstyrene.

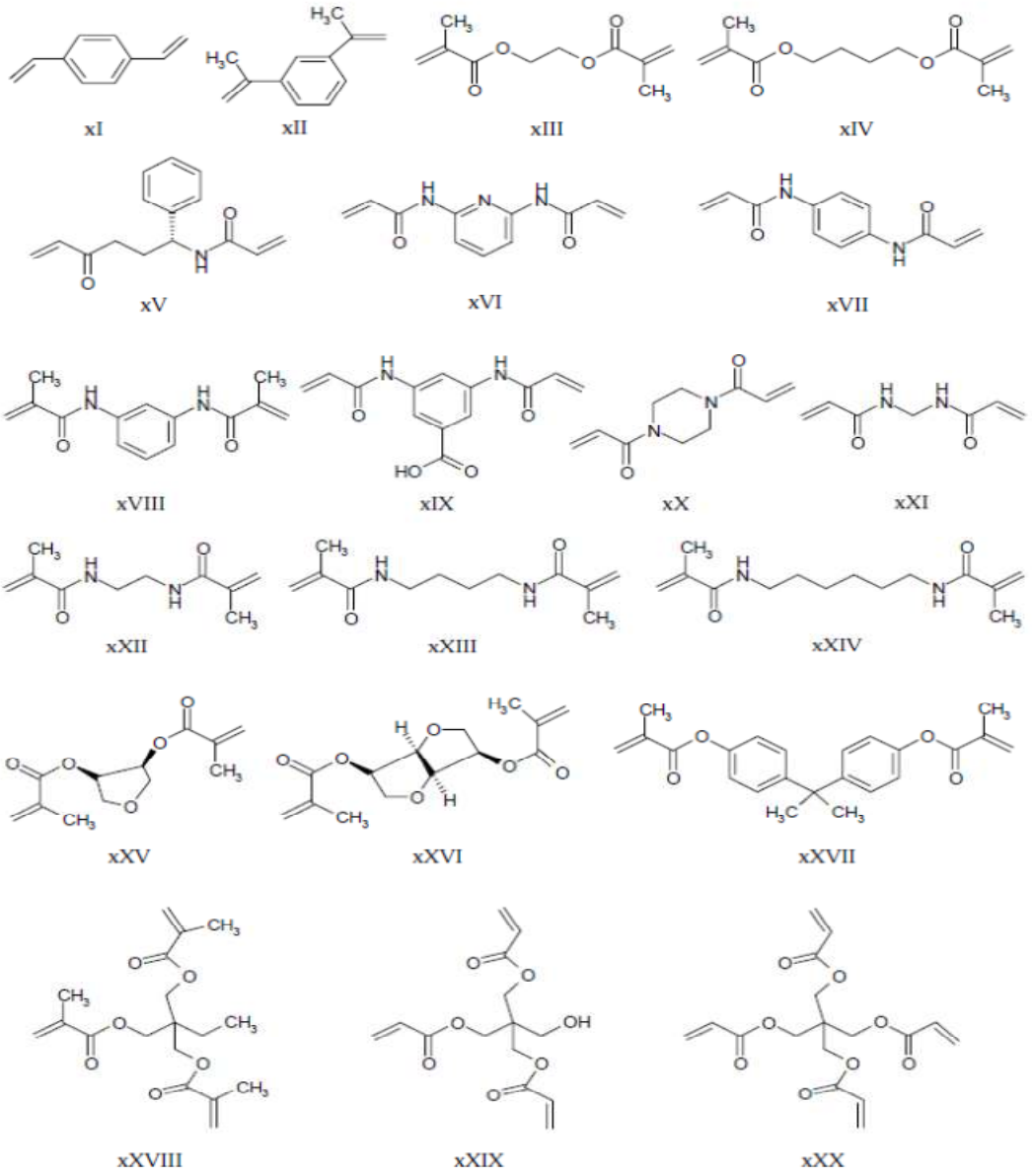

Figure 2.4. Selection of cross-linkers used for molecular imprinting. xI: p-divinylbenzene (DVB); xII: 1,3-diisopropenyl benzene (DIP); xIII: ethylene glycoldimethacrylate (EGDMA); xIV: tetramethylene dimethacrylate (TDMA); xV: N,O-bisacryloyl-l-phenylalaninol; xVI: 2,6-bisacryloylamidopyridine; xVII: 1,4-phenylene diacrylamide; xVIII: N,N-1,3-phenylenebis(2-methyl-2-propenamide) (PDBMP); xIX: 3,5-bisacrylamido benzoic acid; xX: 1,4-diacryloyl piperazine (DAP); xXI: N,N-methylene bisacrylamide (MDAA); xXII: N,N-ethylene bismethacrylamide; xXIII: N,N-tetramethylene bismethacrylamide; xXIV: N,N-hexamethylene bismethacrylamide; xXV: anhydroerythritol dimethacrylate; xXVI: 1,4;3,6-dianhydro-d-sorbitol-2,5-dimethacrylate; xXVII: isopropylenebis(1,4-phenylene) dimethacrylate; xXVIII: trimethylpropane trimethacrylate (TRIM); xXIX: pentaerythritol triacrylate (PETRA); xXX: pentaerythritol tetraacrylate (PETEA).

2.2.4 Solvents (Porogens). The solvent serves two important functions. First, it brings all the components in the polymerization into one phase. Second, it is responsible for creating the pores in macroporous polymers and thus usually referred to as 'porogen'. Pores are necessary to permit the diffusion of the template out of and subsequently in the polymer network during recognition. Generally, solvent are chosen with the idea that it will not interfere with the template-monomer complex. The interaction between solvent and template-monomer complex could hinder the formation of imprinted sites. Due to this, many imprinting systems avoid polar solvents and instead utilize nonpolar solvents in order to maximize attraction of the template by the functional monomers (Cormack & Elorza, 2004; Komiyama, 2003; Oral & Peppas, 2000).

Sellergren and Shea (Sellergren & Shea, 1993) studied a variety of different solvents used in the preparation of L-phenylamine anilide imprinted polymer. They showed convincingly that the largest influence on polymer selectivity could be attributed directly to the porogens ability to form hydrogen bonds. They observed that polymers made in poor hydrogen bonding solvents such as chloroform, hexane, and acetonitrile, showed a greater chromatographic selectivity than those prepared in strong hydrogen bonding solvents such as isopropanol or acetic acid. They concluded that, generally, imprinted polymer selectivity would be remarkably better if performed in low-hydrogen bonding capacity solvents, based on their results.

In general, the best recognition properties of MIPs occur when the same solvent is used during the synthesis and the rebinding process (Sharma & Borovik, 2000; D. Spivak, et al., 1997; Umpleby II et al., 2001; Zhu, Haupt, Knopp, & Niessner, 2002). Furthermore, the solvating ability of the solvent for the polymer impacts the shape of the imprint cavities and may reorganize the functional groups inside the binding sites. Thus, selective rebinding of template molecules is typically achieved when the difference in the solvating ability is minimized between

the solvent used in the synthesis of MIP and the solvent used for rebinding (D. Spivak, et al., 1997).

2.2.5 Initiators. Many initiation methods can be used to initiate the polymerization in the presence of templates. There are however drivers for selecting one method over the other after a careful study of the system If the template were thermally or photochemically unstable, then initiators that can be triggered thermally or photochemically respectively, would not be useful. When a non-covalent interaction such as hydrogen bonding is utilized, lower polymerization temperatures are preferred. In such situations, photochemically active initiators are used since they can operate efficiently at low temperatures (Cormack & Elorza, 2004). The chemical structures of some initiators are shown in Figure .2.5.

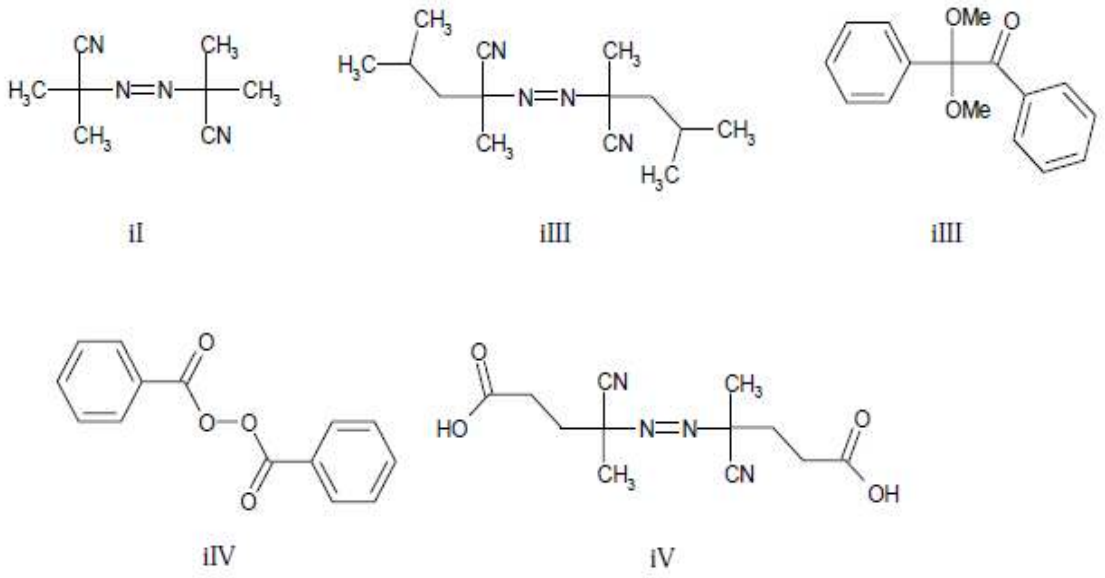

Figure 2.5. Chemical structures of selected chemical initiators: iI: azobisisobutyronitrile (AIBN); iII: azobisdimethylvaleronitrile (ABDV); iIII: dimethylacetal of benzil; iIV: benzoylperoxide (BPO); iV: 4,4azo(4-cyanovaleric acid).

2.3 Applications of Molecularly Imprinted Polymers

Three unique features have made MIPs very attractive synthetic materials (Andersson, 2000). These are:

1. Their high affinity and selectivity, which are similar to those of natural receptors;
2. Their unique stability which is superior to that demonstrated by natural biomolecules; and
3. The simplicity of their preparation and the ease of adaptation to different practical applications.

Since the development of MIPs for sugar and amino acid derivatives by Wulff's group in the early 1970s, MIPs for many classes of compounds have been reported for a variety of applications in many fields.

2.3.1 Chromatography. Much of the initial work on molecularly imprinted polymers was devoted to the field of chromatography. They were used as the stationary phase. The most studied application of imprinting technology is the use of MIPs as stationary phase. Shea and his group (D. Spivak, et al., 1997) developed imprinted polymers for the nucleic acid derivative, 9-ethyl adenine. These imprinted polymers showed high affinity over other nucleic acid derivatives. The particularity of molecularly imprinted polymers compared with conventional chiral stationary phases is that they are tailored for a specific target molecule, hence their selectivity is predetermined.

Most work has concentrated on the resolution of chiral compounds (Vallano & Remcho, 2000). Application of imprinted polymer in enantio-separation of cinchonidine and cinchonine was demonstrated by Takeuchi's group (Matsui, Nicholls, & Takeuchi, 1996), with chiral separation as high as 31. Also, Mosbach and his coworkers (Kempe & Mosbach, 1994) successfully developed an imprinted polymers for (S)-naproxen. EGDMA and 4- vinylpyridine were copolymerized in the presence of the template, (S) - naproxen. (R, S)-Naproxen was well resolved on this chiral separation phase. A separation factor of 1.65 and a resolution factor of 0.83 were obtained when 2 μg of the racemate was loaded on the column. In addition, this

imprinted polymer was able to separate naproxen from the structurally related ibuprofen and ketoprofen.

2.3.2 Antibody Binding Mimics. Mimic of antigen-antibody interaction is one of the driving forces for the research in molecular imprinting. MIPs have several advantages over natural antibodies. The chemical, physical and thermal resistance of imprinted polymers makes them suitable to be under harsh conditions where natural antibodies would not survive, and hence MIPs are sometimes referred to as 'plastic antibodies' (Mosbach & Ramström, 1996). Important features of antibodies like the ability to specifically bind a target molecule are shared by MIPs. Because of this, MIPs have been employed in immunoassay-type binding assays in place of antibodies. This was first demonstrated by Mosbach's group (Schweitz, Andersson, & Nilsson, 1998).

2.3.3 Sensors. The use of MIPs as recognition elements in chemical sensor and biosensor devices is one of their most promising applications. Chemical sensors and biosensors are able to selectively recognize a target molecule in a complex sample. The recognition element, which is in close contact with an interrogative transducer, is the central part of chemical sensors and biosensors. It is responsible for specifically recognizing and binding the target molecule in a complex matrix (Haupt & Mosbach, 2000). Some of the widely used mass sensitive acoustic transducers are the surface acoustic wave (SAW) and the love wave oscillator or quartz crystal microbalance (QCM).There is a change in one or more physical and or chemical parameters whenever there is an interaction between the analyte and the recognition element. The transducer converts these changes such as production of heat, electrons, photons, etc. into an electrical signal that can be amplified and displayed in a suitable form (Risti 1994). The binding sites that are produced during the imprinting process often have affinities and selectivity nearing those of

antibody-antigen systems. Therefore, molecularly imprinted materials have been dubbed 'antibody mimics' (Haupt & Mosbach, 2000). The 'antibody mimics' exhibit some obvious advantages over real antibodies for sensor technology. Due to their highly cross-linked polymeric nature, they are intrinsically stable and robust. This facilitates their application in severe environments such as in the presence of acids or bases, in organic solvents, or at high temperatures and pressures. Moreover, these materials are cheap to produce and can be stored in the dry state at room temperature for long periods of time (Haupt & Mosbach, 2000).

Kriz and coworkers (Kriz, Ramström, Svensson, & Mosbach, 1995) constructed the optical-fiber-like device, a biomimetic sensor based on MIPs. Dansyl-L-phenylalanine was used as template to produce MIPs. These MIPs upon excitation generated fluorescent emission signals that varied as a function of the concentration of the target molecule in solution.

2.4 Methods for Preparing MIPs

Conventionally, MIPs have been synthesized by bulk free radical copolymerization. By this method, functional monomers are bound either covalently or non-covalently to a template molecule in the presence of cross-linking agent and solvent. This method yields polymer monoliths that must be ground and sieved before use. The major setbacks of this method are loss of material and disruption of imprint sites during grinding and sieving, large size distribution of particles, and irregular shapes of particles.

Free radical polymerization is utilized because it can be performed under mild conditions e.g. ambient temperatures and atmospheric pressures, in bulk or in solution. Also, many of the vinyl monomers are available commercially at low cost. Again, the method is tolerant of functional groups in the monomers and impurities in the system (e.g. water) (Cormack & Elorza, 2004). Free radical polymerization has three principal steps (Fried, 2003):

- Initiation of the active monomer;
- Propagation or growth of the active (free radical) chain; and
- Termination of the active chain to give the final polymer product

Below is a simple schematic of free polymerization mechanism.

$I\sim I \longrightarrow 2\ I\bullet$		Initiation (dissociation)
$I\bullet + M \longrightarrow IM\bullet$		Initiation (association)
$IM\bullet + M \longrightarrow IMM\bullet$		Propagation
$IM_{x-1}M\bullet + \bullet MM_{y-1}I \longrightarrow$	$IM_{x-1}M - MM_{y-1}I$	Termination (combination)
$IM_{x-1}M\bullet + \bullet MM_{y-1}I \longrightarrow$	$IM_x + IM_y$	Termination (disproportionation)

Several new techniques have been used recently to synthesize MIPs. In one of such approaches, the pre-polymerization mixture (template, monomers, initiator, solvent) were filled into silica gel pores followed by polymerization that is initiated by either UV radiation or heating. The silica gel was dissolved after polymerization and separated to produce 100% MIP spherical beads (Yilmaz, Ramström, Möller, Sanchez, & Mosbach, 2002). This study concluded that, the spherical MIP beads showed higher mass transfer rates and lower back pressures than traditionally prepared MIPs, when used as chromatographic stationary phase materials.

2.5 Transducers

The transducer or the detector element is the component of the biosensor that transforms the signal resulting from the interaction of the analyte with the sensitive element into another signal that can be more simply measured and quantified. Transducers can be subdivided into the following four main types(Eggins, 2002).

2.5.1 Electrochemical Transducers. The following are some of the types of electrochemical transducers:

i. *Potentiometric*. These involve the measurement of the EMF (potential) of a cell at zero current. The EMF is proportional to the logarithm of the concentration of the substance being determined.

ii. *Voltametric*. An increasing (or decreasing) potential is applied to the cell until oxidation (or reduction) of the substance to be analysed occurs and there is a rise (or fall) in the current to give a peak current. The height of the peak current is directly proportional to the concentration of the electroactive material. If the appropriate oxidation or reduction potential is known, one may step the potential directly to that value and observe the current. This mode is known as amperometric. A procedure was suggested for determination of the 2,4-dichlorophenoxyacetic acid content in milk using immobilized molecular imprinted polymers and an amperometric cholinesterase biosensor(Medyantseva et al., 2010).

iii. *Conductometric.* Most reactions involve a change in the composition of the solution. This will normally result in a change in the electrical conductivity of the solution which can be measured electrically.

iv. *Field Effect Transistor (FET)-based sensors*. Miniaturization can sometimes be achieved by constructing one of the above types of electrochemical transducers on a silicon chip-based field effect transistor. This method has mainly been used with potentiometic sensors, but could also be used voltammetric and conductometric sensors.

2.5.2 Optical Transducers. These have taken a new lease of life with the development of fibre optics, thus allowing greater flexibility and miniaturization. The techniques used include absorption spectroscopy, fluorescence spectroscopy, luminescence spectroscopy, internal reflection spectroscopy, surface Plasmon spectroscopy and light scattering. Wu and Feng (Wu, Feng, Tan, & Hu, 2009) demonstrated for the first time that optical reflection technique combined with the molecular imprinting polymer can selectively detect lower concentrations of formaldehyde molecules.

2.5.3 Piezoelectric Transducers. These devices involve the generation of electric currents from a vibrating crystal. The frequency of vibration is affected by the mass of material adsorbed on its surface, which could be related to changes in a reaction. Surface acoustic wave (SAW) and quartz crystal microbalance (QCM) are related systems.

2.5.4 Thermal Sensors. All chemical and biochemical processes involve the production or absorption of heat. This heat can be measured by sensitive thermistors and hence be related to the amount of substance to be analyzed.

The QCM is portable and highly sensitive. The mass sensing technique of the QCM eliminates the need for any specific labeling step to be part of the signal transduction mechanism. Also, the technique is relatively easy to use, and the basic equipment is inexpensive to purchase. With these advantages, the QCM was the preferred transducer for our study.

CHAPTER 3

Experiments

3.1 Reagents

The bilirubin imprinted polymers were prepared using 4-vinylpyridine (Figure 3.1), as functional monomer (formula: C_7H_7N, MW=105.10g/mol, colorless liquid). Cross linking agent used was divinylbenzene (Figure 3.2), (formula: $C_{10}H_{10}$, MW=130.19g/mol, colorless liquid). Benzophenone (Figure 3.3) was used as initiator for the free radical polymerization reaction, (formula: $C_{13}H_{10}O$, MW=182.22g/mol, crystalline).Template used was bilirubin (Figure 3.4) (formula: $C_{33}H_{36}N_4O_6$, MW=584.66g/mol, orange powder) The solvent used was chloroform, (formula: $CHCl_3$, MW= 119.38g/mol, colorless liquid.). All chemicals were supplied by Sigma Aldrich and were used as supplied.

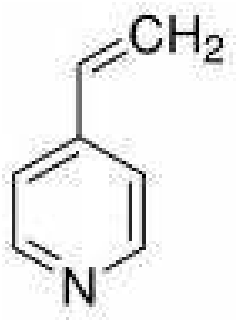

Figure 3.1. 4-vinylpyridine

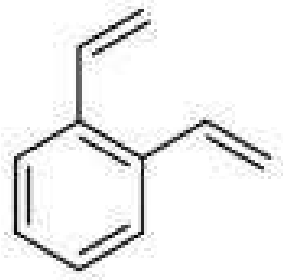

Figure 3.2. Divinylbenzene

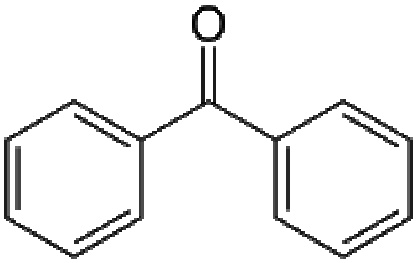

Figure 3.3. Benzophenone

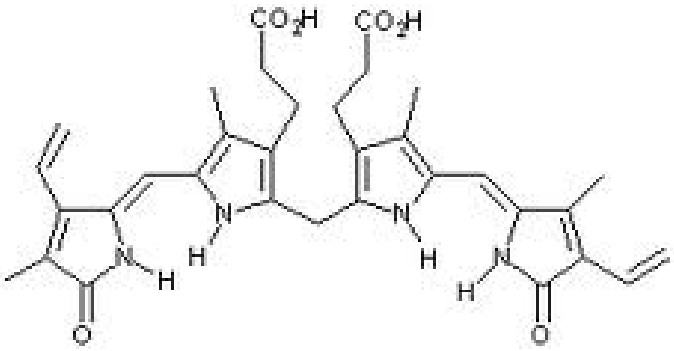

Figure 3.4. Bilirubin

3.2 Instrumentation

The QCM system consisted of a QCM 200 Quartz Crystal Microbalance controller, QCM 25 crystal oscillator, and a crystal holder with LABVIEW software obtained from Stanford Research Systems (Sunnyvale, California). QCM crystals purchased from Stanford Research Systems were AT-cut spherical quartz crystals (1 inch diameter) with a basic resonant frequency of 5 MHz and two gold electrodes on opposite sides (Figure 3.5). The spincoat G3P was obtained from Specialty Coating Systems (Indianapolis, Indiana). The UV light source used for the polymerization reaction was Blak-Ray®, Model B 100SP obtained from Fisher Scientific.

3.3 Polymer Synthesis

3.3.1 Preparation of QCM Crystals. The QCM crystals were cleaned by repeated and sequential immersion in 1N HCl, deionized water, 1N NaOH and deionized water. The gold

surface of the cleaned QCM crystals were later treated with a solution 2-propene-1-thiol (allyl mercaptan) and methanol for 24 hours. The crystals were washed with methanol, dried and stored prior to use.

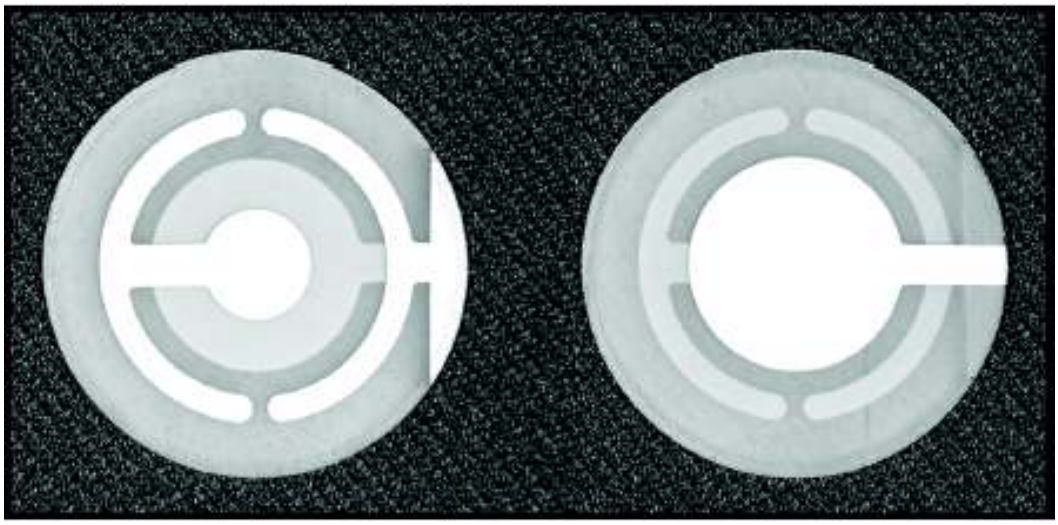

Figure 3.5. Standard 1 inch QCM sensor crystal. The crystal on the left shows the contact surface, while the one on the right shows the liquid surface.

3.3.2 Pre-polymerization Solution. Bilirubin (0.25 mmol) was dissolved in 3 ml chloroform in a borosilicate glass vial. The initiator, benzophenone, functional monomer, 4-vinylpyridine (2 mmol), and the cross linking agent, divinylbenzene (10 mmol) were then added to the solution and mixed thoroughly. Nitrogen gas was bubbled through pre-polymerization solution for about 15 minutes to remove dissolved oxygen which slows down free radical polymerization. The pre-polymerization solution (Figure 3.6) was placed under UV light to initiate polymerization. The change from solution to gel form is very slow. It takes about 12-24 hours. The gel form of the polymer was coated onto the QCM crystal at 2000 rpm for 2 minutes. The QCM crystal was placed under UV light with N_2 gas flow in a glass chamber (Figure 3.7) for further curing for about 2 hours. The non imprinted polymer (blank) was prepared in the same but without bilirubin (template molecule)

Figure 3.6. Prepolymerization solutions showing various template to monomer ratios

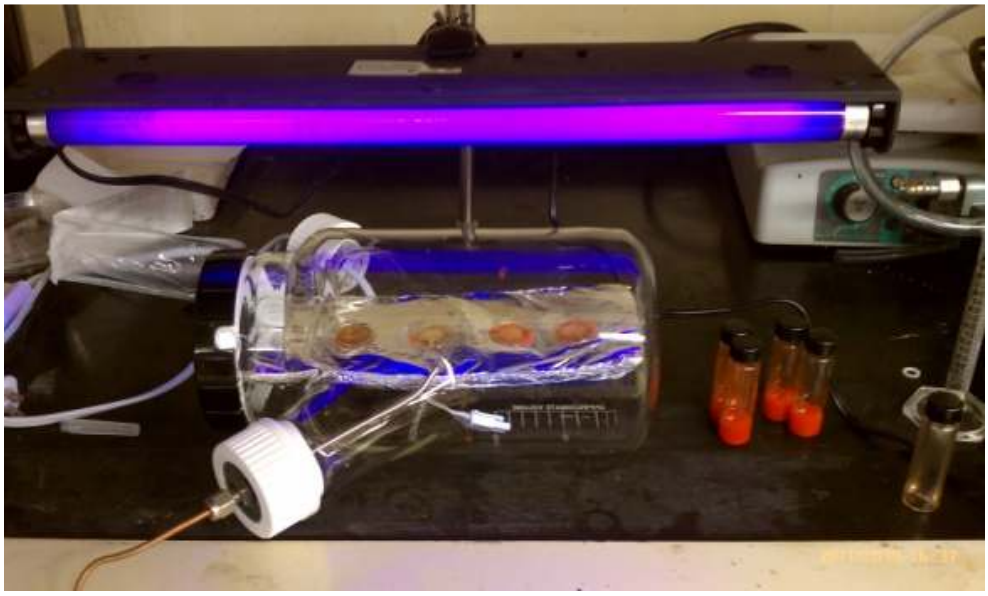

Figure 3.7. Polymerization of thin MIP film on QCM crystals inside photoreactor with nitrogen gas flow.

3.3.3 Coating of QCM Crystals. Spincoat G3P-B supplied by Specialty Coating Systems was used to apply a thin film of prepolymerization solution onto the QCM crystals. The QCM crystal was mounted horizontally on the top of a motor shaft. The pre-polymerization solution was added dropwise on to the surface of the QCM crystal until the surface was covered entirely. The Spincoat G3P-B was turned on and the excess solution was spun off. A thin film of solution was left on the surface of the QCM crystal. Coating was done in the presence of N_2 gas.

3.4 Template Extraction

The removal of the target molecules from the imprinted polymer creates cavities that are able to specifically rebind target analyte upon reintroduction. During template extraction, other unwanted chemical residues are also removed. After the polymerization reaction, the bilirubin was washed out of MIP. 10 mM was used as solvent to wash the template molecule, bilirubin, from the synthesized MIP. The washing was achieved by repeatedly immersing the QCM crystal coated with bilirubin MIP in the 10mM NaOH solution. This was done till there was no significant change in mass of the QCM crystals from consecutive washes. To further validate the washing procedure, the washing solvent from the initial and final washes were analyzed for remaining template and monomer via UV Vis spectroscopy. Frequencies of the QCM crystal were measured before surface modification and after washing of the template molecule from the polymer film. The mass of the MIP film was determined from the difference between these two states.

3.5 Binding Experiments

The detection of bilirubin concentration from the oscillation frequency change of the QCM crystal coated with MIP thin film was carried out by the QCM200 instrument (Stanford Research Systems, California). The QCM crystal coated with thin MIP film was placed in the

crystal holder of the QCM. The flow cell was fixed on the crystal holder to create a chamber where the analyte solution will be in contact with the MIP modified QCM crystal. The experimental setup is illustrated in Figure 3.8. Known concentrations of analyte solutions were prepared. Hundred and fifty microlitres of the analyte solution was injected into the flow cell. The frequency change was monitored and recorded on the computer attached to the QCM until a stable frequency was reached. After each detection cycle, the QCM crystals were washed with 10mM NaOH to remove the absorbed target molecule.

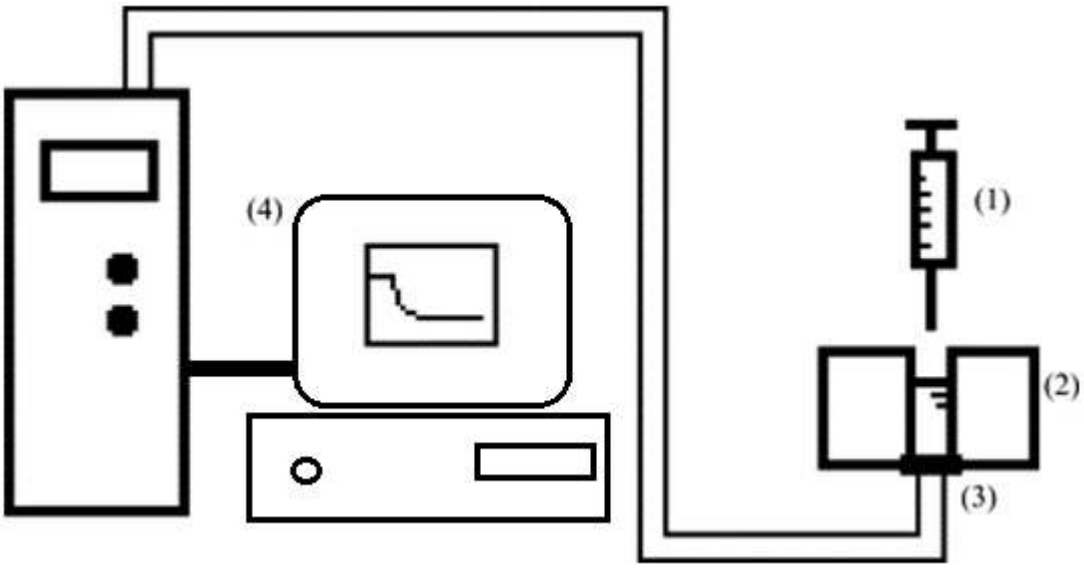

Figure 3.8. The schematic diagram of MIP-QCM: (1) syringe; (2) QCM flow cell; (3) QCM electrode; (4) computer.

3.6 Effects of pH on Recognition Properties

The effect of the change in pH of analyte solution was studied. A pH change can alter the overall charge of either the polymer, target molecule or both. Bilirubin solution was analyzed in the pH range from 2.62 to 12.05. Different concentrations of bilirubin solution were prepared. The pH of the solutions were altered to the desired pH by adding 1M HCl drop wise. This was achieved whiles the solution was being stirred with a pH probe present to ensure accurate pH

measurements as shown in Figure 3.9. The interaction of MIP with bilirubin solution of different concentrations was studied using the QCM system. Frequency shift data were recorded for further processing.

Figure 3.9. Setup for altering pH of bilirubin solution

3.7 Effect of Ionic Strength on Recognition Properties

Different concentrations of salt were investigated to find out their effects on recognition. The presence of ions can compete with the target molecule for recognition. Sodium chloride was dissolved in deionized water at differing concentrations. The salt solution was then added to bilirubin to make bilirubin solution (1.23mg/dl). The QCM system was used to follow the recognition and how the presence of ions affects interaction between bilirubin and the MIP.

3.8 Specific Recognition Studies (Selectivity of Imprinted Polymers)

To ensure that the imprinted polymer is able to specifically bind the target molecule, another compound; biliverdin which is similar in size and structure to bilirubin was studied.

Different concentrations of biliverdin solutions were prepared. The frequency shift given by bilivervin on interacting with the MIP was studied with the QCM system in the same way as it was done for bilirubin. The data obtained was recorded, analyzed and compared to data obtained from bilirubin rebinding.

CHAPTER 4

Results and Discussion

4.1 Polymer Preparation

In order to make highly selective polymers, factors that could likely affect the selectivity of the imprinted polymer towards the template were examined. Bilirubin dissolves easily in basic solutions and insoluble in solvents that are acidic. 4-Vinyl pyridine was chosen as the functional monomer because of its hydrophobic and weak basic nature. It was thought to have a better chance to form a complex with the bilirubin template. Divinyl benzene is chosen as cross linking agent because it is one of the well known cross linkers for non-covalent imprinting.

Polymerizations were carried out in a number of commonly used solvents for molecular imprinting; toluene, chloroform, acetonitrile and acetone. Chloroform, acetone and acetonitrile have good capabilities to dissolve the template bilirubin, whereas toluene does not. Chloroform is finally chosen as the preferred solvent from preliminary screening because polymers formed in this solvent are durable. For the non-covalent protocol of molecular imprinting, the effect of polymerization temperature is not of much concern. Lower polymerization temperatures are preferred, when a non-covalent interaction such as hydrogen bonding is utilized. Polymerization reactions are initiated by UV irradiation. Polymerization was performed at 25^{o}C. Nitrogen gas was bubbled through pre-polymerization solution for about 15 minutes to remove dissolved oxygen which slows down free radical polymerization. The pre-polymerization solution was placed under UV light to initiate polymerization. The change from solution to gel form is very slow. It takes about 12-24 hours. The gel form of the polymer was coated onto the QCM crystal at 2000 rpm for 2 minutes. The QCM crystal was placed under UV light with N_2 gas flow for further curing for about 2 hours.

4.2 Template Extraction

The extraction of the template (imprint molecule) from the imprinted polymers creates cavities that are capable of specific binding of the template and may also remove residual monomers and initiator fragments. The MIP/QCM crystal was immersed in solvent for 1 hour periods. After each batch, the frequency of the crystal (mass) was measured. This was repeated till almost all of the bilirubin was washed out. The frequency and mass profile is shown in Figures 4.1 and 4.2 respectively. It can be seen from Figure 4.1 that, the frequency after first two washes increased significantly. The signal almost leveled off after the fourth wash.

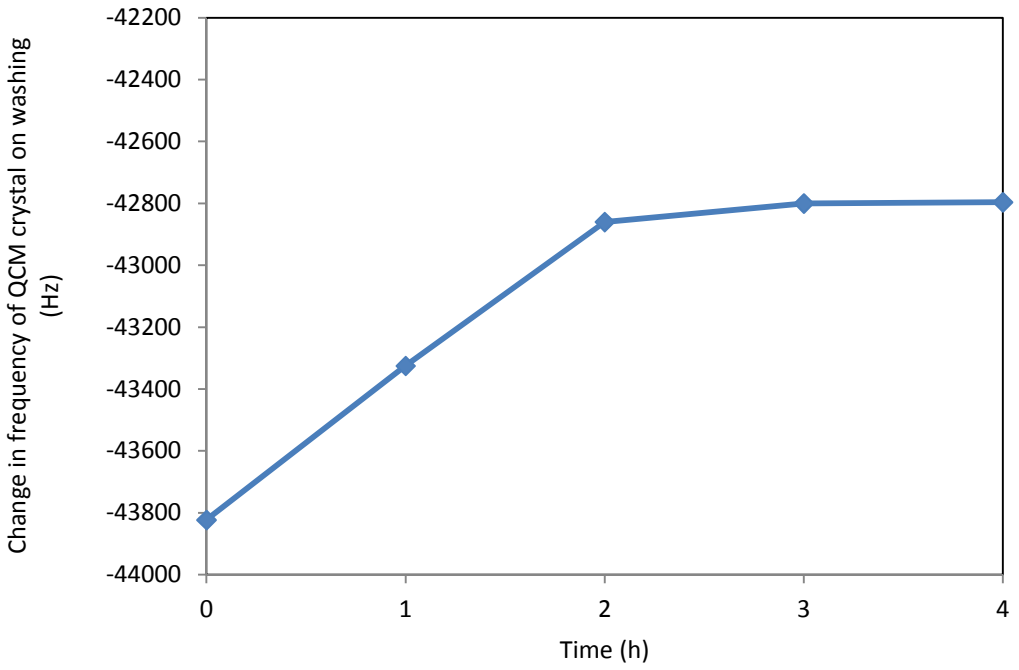

Figure 4.1. Response frequency and mass profile detected from MIP/QCM crystal operated with different washing batches.

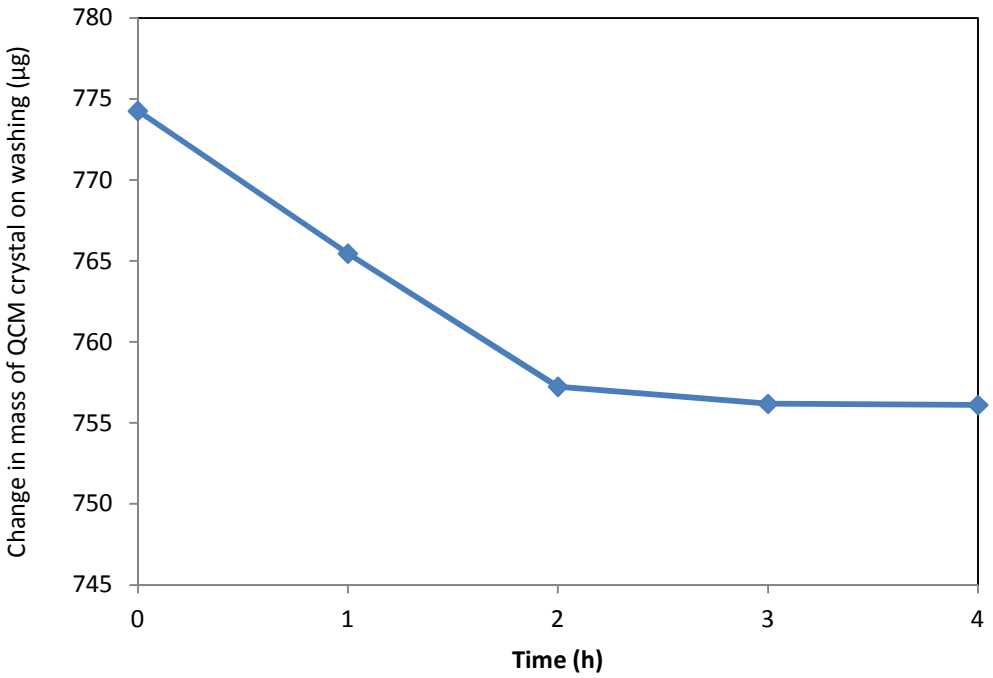

Figure 4.2 Response frequency and mass profile detected from MIP/QCM crystal operated with different washing batches.

To further ensure that template extraction was almost perfect, the washing solvent from the initial and final washes were analyzed for remaining template and monomer via UV Vis spectroscopy. Figure 4.3 shows UV spectrum of template (bilirubin) extraction solutions. After the last was there was no bilirubin in the washing solution to absorb UV light.

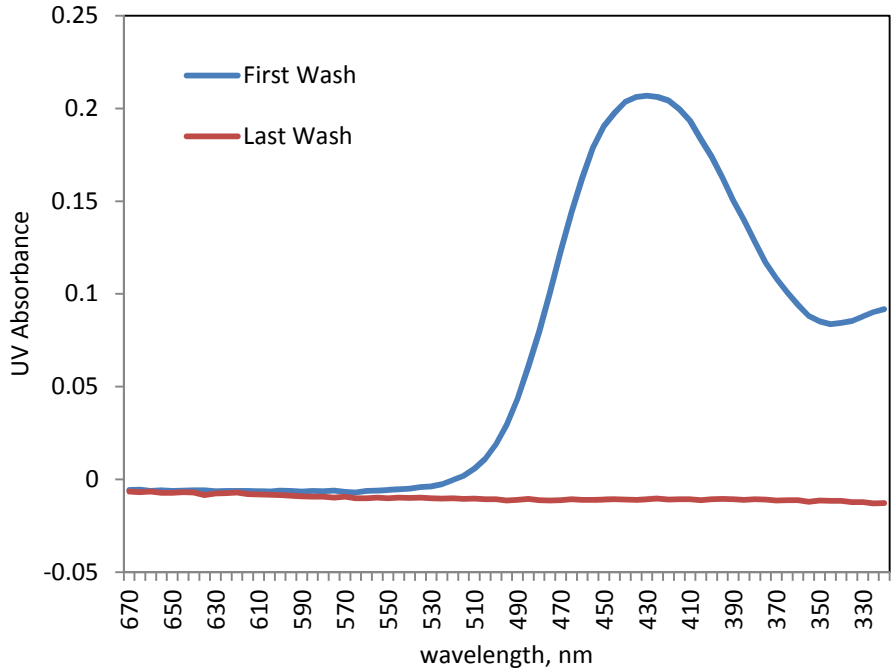

Figure 4.3. UV spectrum of template (bilirubin) extraction solution

4.3 Recognition Studies

The success of imprinting can be assessed by a number of factors. The quantity of the template molecule that can be absorbed at equilibrium is important in identifying the difference between imprinted and non-imprinted polymers in binding of the template molecule. To ensure imprinting was successful, recognition studies of both the MIP and non-MIP were performed using the QCM system to determine how much of the target molecule were picked up from the analyte solution. By comparing frequency shifts from both QCM crystals; the MIP coated crystal and non-MIP coated crystal, it was concluded that imprinting had been achieved. When both MIP and non-MIP coated crystals were tested using equal volumes of the same concentration of analyte solution, the MIP coated crystal showed a greater frequency shift than the non-MIP. This can be attributed to the strong interactions between the target molecules and the imprint sites. This interaction causes the frequency shift. When the analyte solution is injected into the flow

cell, the binding of the target molecule to the imprint sites causes frequency shift that is far more than that of the non-MIP coated crystal. The imprint sites are such that their size and shape are complementary to the target molecule. In this way, the target molecules in solution fit into these binding cavities on the QCM crystals causing the frequency shifts. On the other hand the non-MIP coated crystals have no imprint sites and therefore, there is no binding of target of molecule to imprinted site. Hence the relative lesser frequency shift observed.

4.4 Characterization of MIP

There are three major levels used to characterize MIPs: (1) chemical characterization, (2) morphological characterization, and (3) molecular recognition behavior characterization (Cormack & Elorza, 2004). Some of the convenient analytical methods for chemical characterization include elemental micro-analysis, Fourier-transform infra-red (FTIR) spectroscopy, and solid state nuclear magnetic resonance (NMR). Morphological characterization is achieved through the following; solvent uptake experiments, nitrogen sorption porosimetry, mercury intrusion porosimetry, inverse size exclusion chromatography, scanning electron microscopy (SEM), and atomic force microscopy (AFM). Molecular recognition characterization is achieved by rebinding (recognition) experiments. The most convenient way of characterizing the MIP was through the use of SEM and AFM techniques. Polymer film was synthesized directly on QCM crystal and makes SEM and AFM characterization desirable than other morphological characterization methods.

4.4.1 Characterization by QCM sensor. The QCM crystal surface is usually coated with a thin film of stationary phase that is capable of adsorbing the analytes or target molecules. Initially, the QCM crystal has a frequency of approximately 5 MHz. The QCM works such that there is a frequency shift when there is an increase in mass at the surface of the crystal. The

difference in frequency shift of the crystal before and after polymerization are recorded and compared. After the comparison of the two frequencies recorded, the difference was significant. This was clear evidence that there is a polymer film coating on the surface of the QCM crystal. This makes the QCM device a sensitive instrument for characterizing thin films and for analyzing the interaction between analyte (target molecules) and film coating. The mass of polymer film obtained for various templates to polymer ratio is given in Table 4.1.

Table 4.1

Template/monomer ratio and mass of MIP

Crystal	Template:Monomer Ratio	Mass of MIP (µg)
1	1:8	56.99
2	1:16	65.11
3	1:32	76.93

4.4.2 Surface Characterization by SEM and AFM. SEM images were taken to observe the surface morphology of the bare gold surface of crystal, MIP coated crystal and non-MIP coated crystal. These images are shown in Figures 4.4, 4.5, and 4.6 respectively. The bare gold surface (Figure 4.4) is very rough compared to the MIP coated surface (Figure 4.6) and non-MIP coated surface (Figure 4.5). This is an indication that a polymer film has been successfully synthesized on the gold surface of the QCM crystals in Figures 4.5, and 4.6. Comparing the MIP and non MIP surface images, it can be seen that the non-MIP film had a lot of continuous cracks than the MIP. This can be attributed to the fact that the MIP prepolymerization solution had more mass than that of the non-MIP. This made the MIP film relatively thicker than the non-MIP. Thus, the MIP coated surface showed a smoother finish than its non-MIP counterpart. Also from Figure 4.6 are some holes scattered which I believe to be the imprinted sites responsible for rebinding the template molecule; bilirubin.

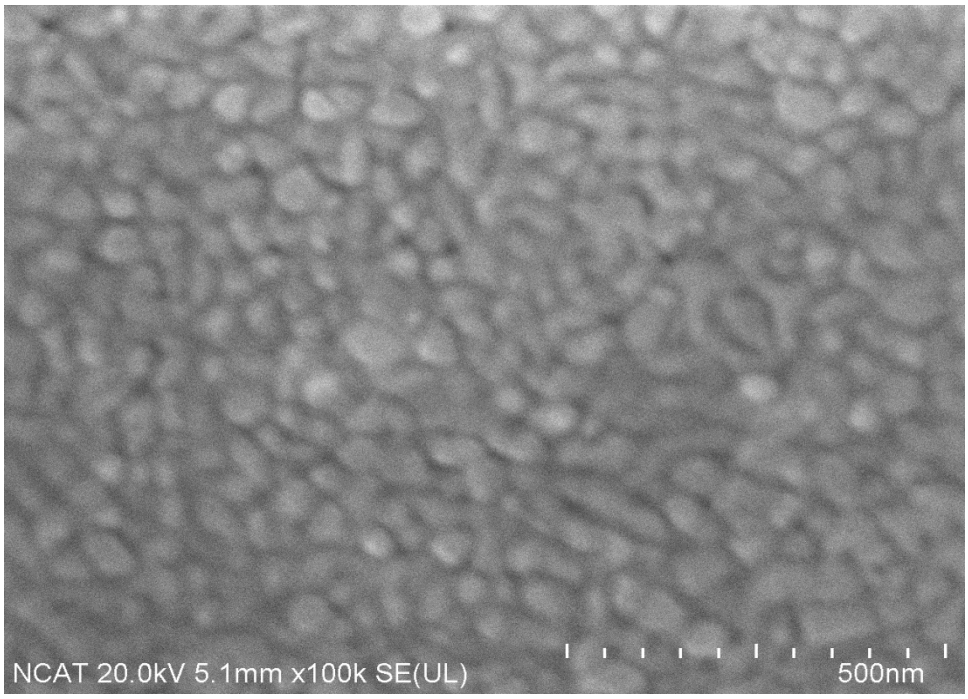

Figure 4.4. SEM image of bare gold surface

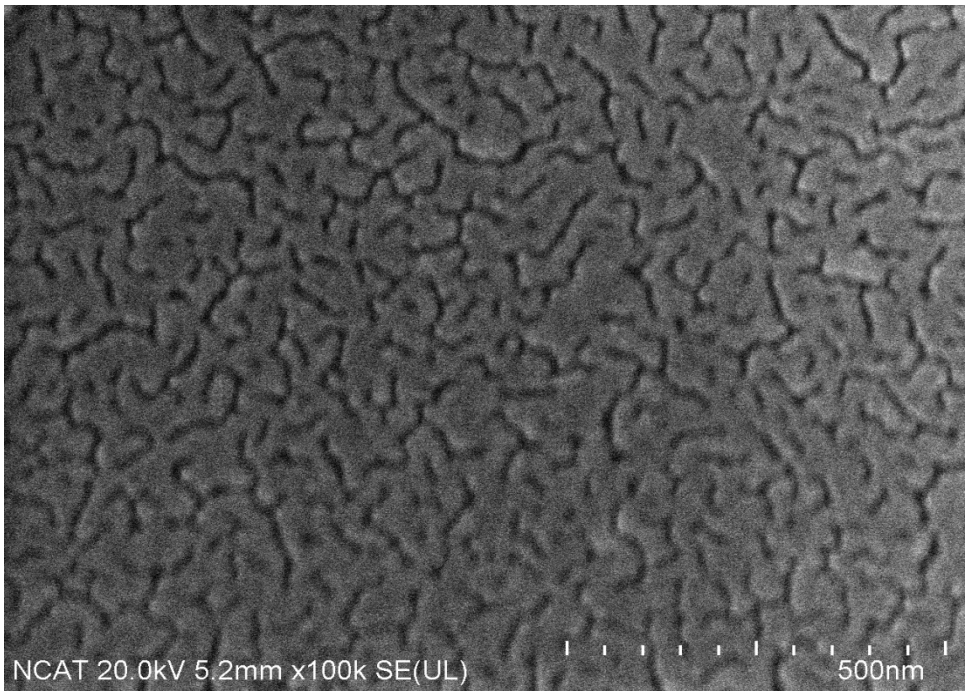

Figure 4.5. SEM image of non-MIP coated crystal surface

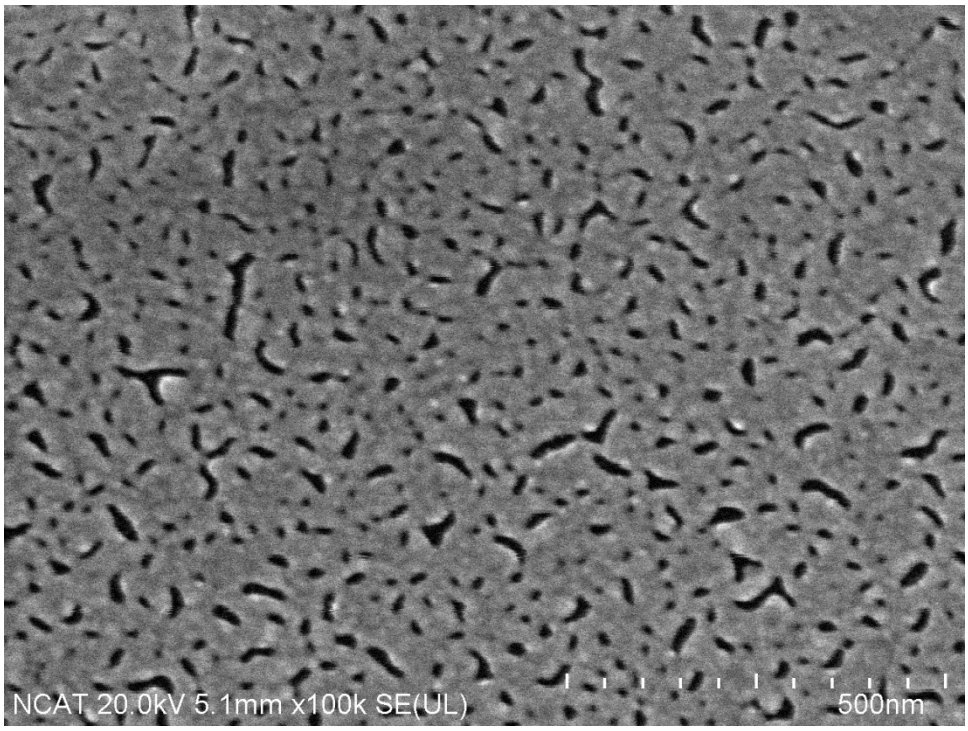

Figure 4.6. SEM image of MIP coated crystal surface

The surface of the coating of the various crystals was further studied with the AFM. The bare gold surface, the non MIP coated surface , and the MIP coated are shown in Figures 4.7, 4.8, and 4.9 respectively. Just as in the SEM imagery, the surface of the bare gold was rough. The application of the thin polymer film on the surface of the gold conealed the roughness. The MIP coated crystal image shows has some pores which I believe to be the imprinted sites responsible for recognizing the target molecule, bilirubin in solution.

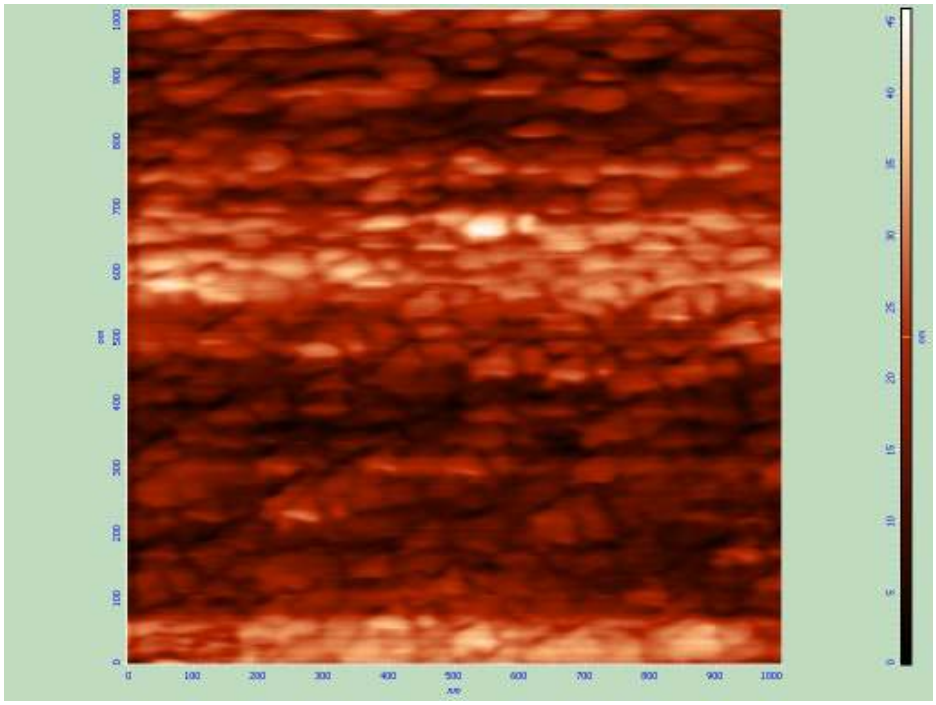

Figure 4.7. AFM image of bare gold surface of QCM crystal

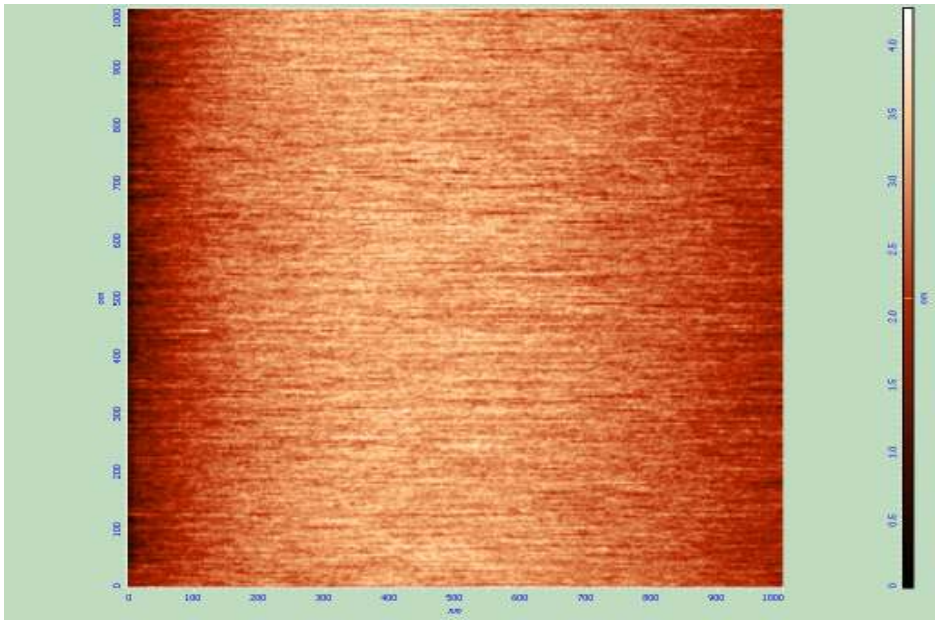

Figure 4.8. AFM image of non-MIP coated QCM crystal

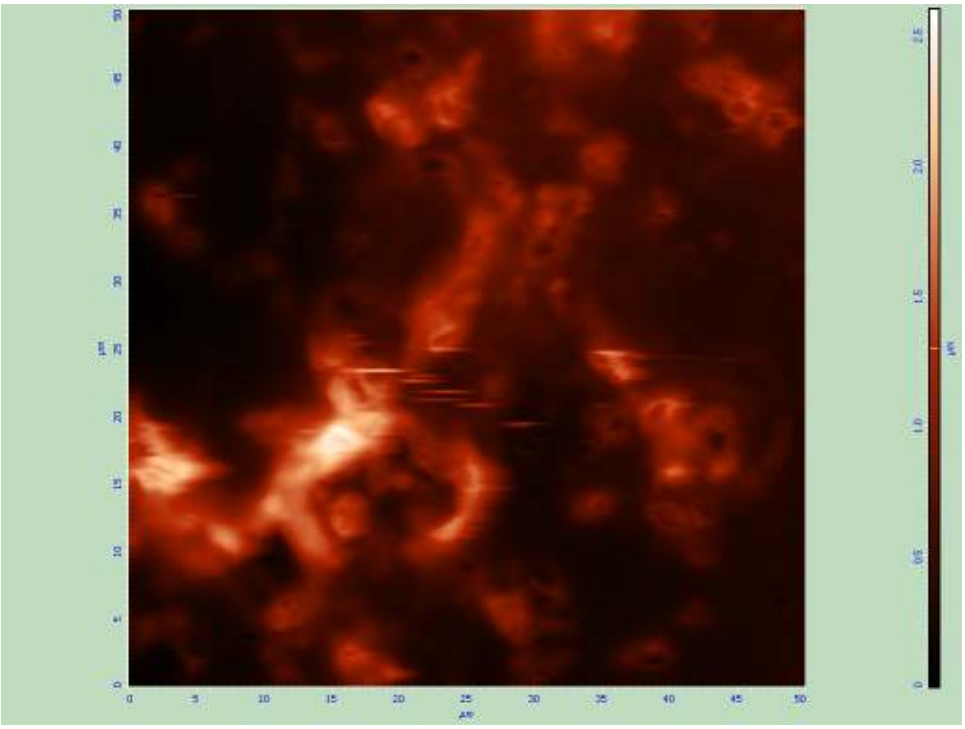

Figure 4.9. AFM image of MIP coated QCM crystal

4.5 Calibration Curve

A calibration curve is curve that permits us to calculate desired experimental results in terms of another. The calibration curve is a general method used in analytical chemistry in determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentrations (Harris, 2003). This curve is a plot of how the instrumental response (analytical signal) changes with the concentration of the analyte. A series of standards are prepared to cover the expected concentration range of the analyte. These standards are analyzed using a chosen technique to produce a series of measurements (analytical signals). Mostly, a plot of analytical response vs. analyte concentration yields a linear relationship. The analytical signal of the unknown analyte is measured and using the calibration curve, the concentration of the unknown analyte is determined by interpolation.

Obtaining a calibration curve was one of the principal objectives of this study. The analytical signal was the steady state frequency shift of the QCM observed upon the introduction of the analyte solution into the flow cell containing the MIP coated QCM crystal. A series of known concentrations of bilirubin solutions were prepared. The concentration of these solutions ranged from 0.07 to 1.5mg/dl. These standards were first calibrated with the UV Vis to ensure accuracy. The calibration curve obtained from UV visible spectroscopy at 438 nm is shown in Figure 4.10.

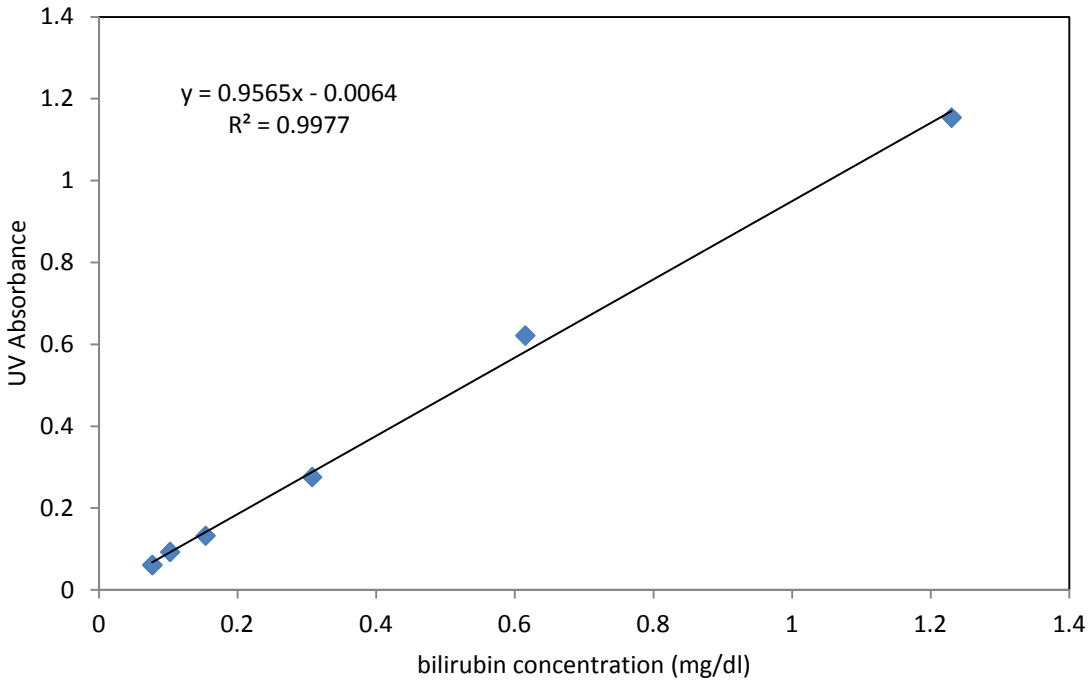

Figure 4.10. Calibration of bilirubin solution concentration using UV Vis spectroscopy at 438 nm.

Once the accuracy of the bilirubin solutions' concentrations was checked, a calibration curve for bilirubin concentration was obtained for the QCM crystal coated with bilirubin imprinted polymer. Frequency shift was plotted against bilirubin concentration. A typical calibration curve obtained from the bilirubin imprinted polymer is shown in Figure 4.11. The

curve shows a linear fitting with a slope of 248.58 Hz/mg/dl. The correlation coefficient was 0.92. From this curve, bilirubin solutions whose concentration is unknown can be determined. From the calibration curves in Figures 4.11, 4.12 and 4.13, increasing concentration resulted in increasing frequency shifts. This can be attributed to the fact that, there are a fixed number of imprinted sites capable of binding to the target molecule bilirubin. At low concentrations, there are not enough bilirubin molecules in solution to interact with the imprinted sites. This gives a relatively smaller frequency shift because not all the imprinted sites rebind with the bilirubin molecules. As the concentration is increased, there are more bilirubin molecules in solution to interact with the imprinted sites resulting in the increase of frequency shift.

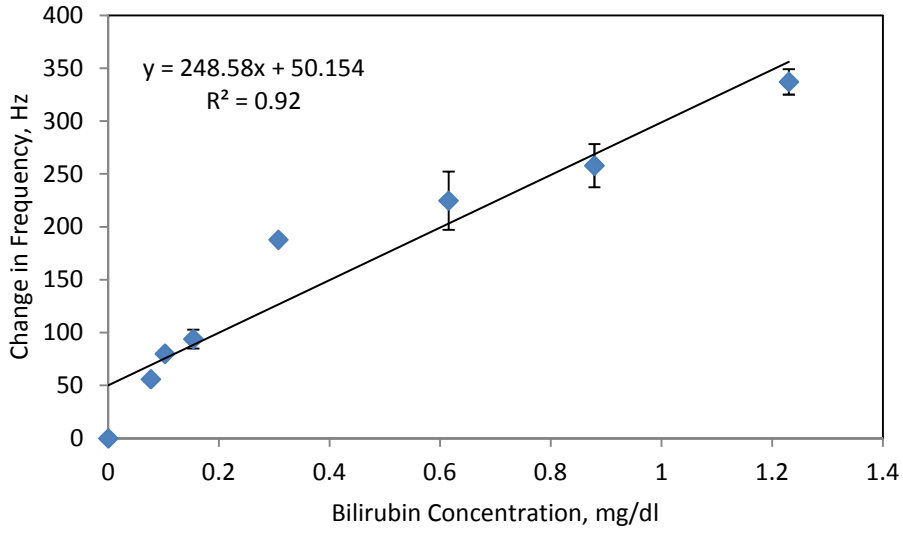

Figure 4.11. Calibration curve of bilirubin concentration from QCM/MIP crystal (1:8)

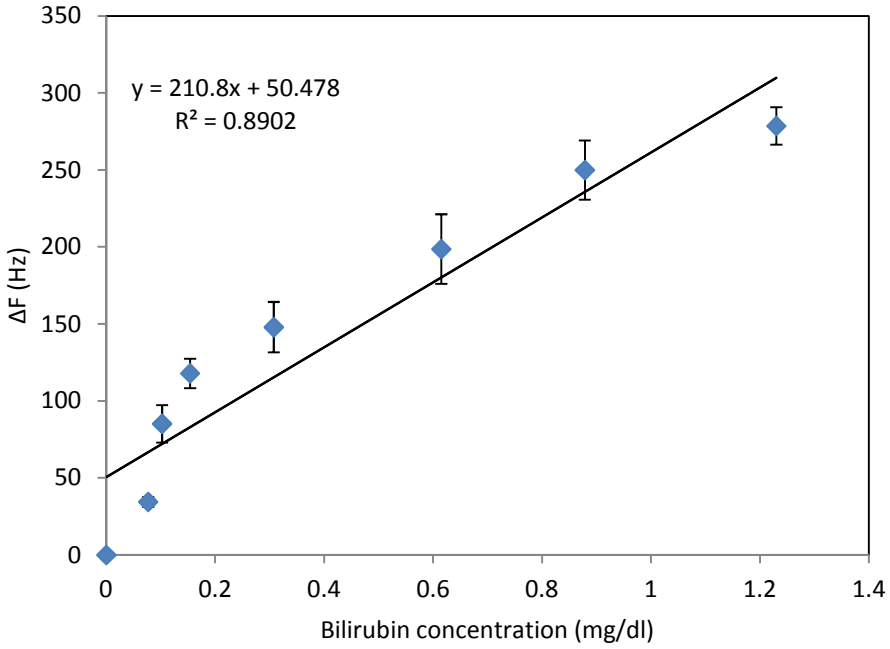

Figure 4.12. Calibration curve of bilirubin concentration from QCM/MIP crystal (1:16)

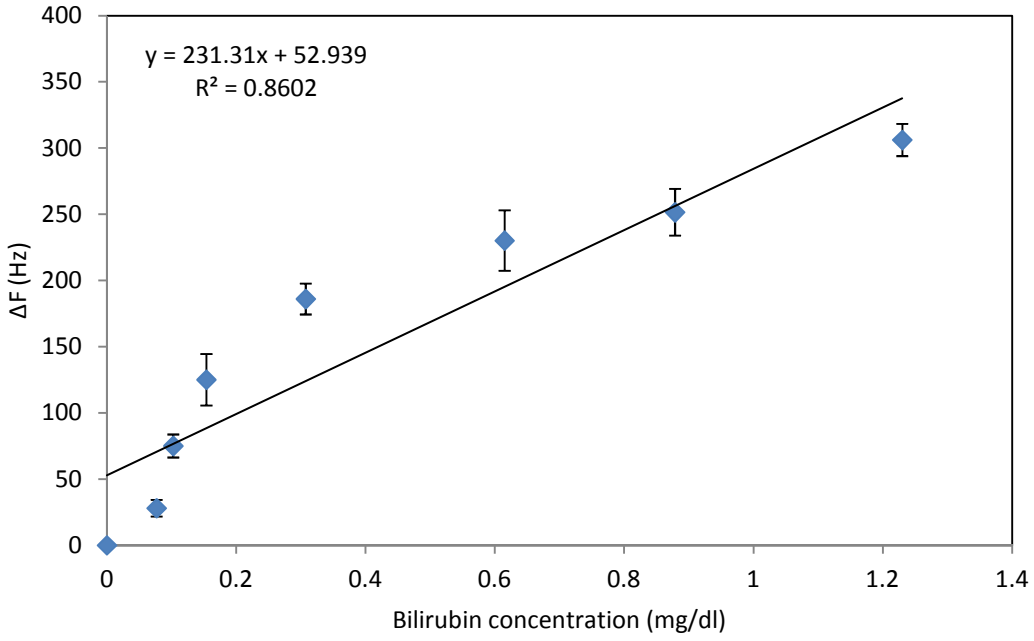

Figure 4.13. Calibration curve of bilirubin concentration from QCM/MIP crystal (1:32)

Once we know the equation of the regression line, the bilirubin concentration can be easily calculated from a given frequency shift. Because we are going from frequency (y-value) to bilirubin concentration (x-value) (instead of the other way around), we need to find the standard deviation in x(Stone & Ellis, 2006). With no replications and a measured signal within the calibrated range of the instrument (interpolation), the standard deviation in x is given by(Stone & Ellis, 2006):

$$s_{x0} = \frac{s_{y/x}}{b}\sqrt{1 + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum (x_i - \bar{x})^2}}$$

Where,

$s_{y/x}$ is the standard deviation in the residuals = $\sqrt{\frac{\sum (y_i - bx_i - c)^2}{n-2}}$

b is the slope of the line

n is the number of samples(standards)

x_i are the concentration of the standards

$\bar{x}$ is the average concentrations of the standards

y_0 is experimental signal

$\bar{y}$ is the average signal measurement of the standard

c is the y-intercept of the line

If replications are performed, the computation of the error in x must be modified to account for the extra degrees of freedom, as a result of the extra measurements. The formula for the standard deviation in x with m replications is (Stone & Ellis, 2006);

$$s_{x0}, R = \frac{s_{y/x}}{b}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum (x_i - \bar{x})^2}}$$

Where,

m is the number of replicates.

In cases, where the measurement value for the sample is outside the measured range of the calibration curve, the standard deviation for extrapolation is used. Despite the fact that this situation is undesirable, due to the possibility of nonlinear effects outside the measurement range, it is sometimes unavoidable, and the results can still be used. The standard deviation is calculated by(Stone & Ellis, 2006);

$$s_{xE} = \frac{s_{y/x}}{b}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{b^2 \sum(x_i - \bar{x})^2}}$$

4.6 Comparison Imprinted and non-imprinted QCM Crystals

Two QCM crystals coated with polymer were studied for their detection signal. The first crystal was synthesized with the template bilirubin present (MIP). The second crystal was prepared the same way as the first but without bilirubin (non-MIP or control polymer). The detection signal of bilirubin solution for the MIP and non-MIP crystals were studied and compared. From Figures 4.14 to 4.16, it can be seen that the MIP showed a greater frequency shift than the non-MIP. The reason for this is that, the MIP has imprinted sites that have the affinity to bind bilirubin molecules in solution resulting in the greater frequency shift. On the other hand, the relatively less frequency shift exhibited by the non-MIP coated crystal can be attributed to the fact the polymer film coating has (1) no cavities complementary to the shape and size of the target molecule, bilirubin, (2) no or very less affinity to bind bilirubin molecules in the analyte solution. From the comparison of these two crystals; imprinted and non-imprinted, it is clearly evident that the MIP coated crystal has much more interaction with the bilirubin

molecules in solution than the non-imprinted polymer. Figures 4.17 to 4.19 show the mass of bilirubin recognized by various test crystals.

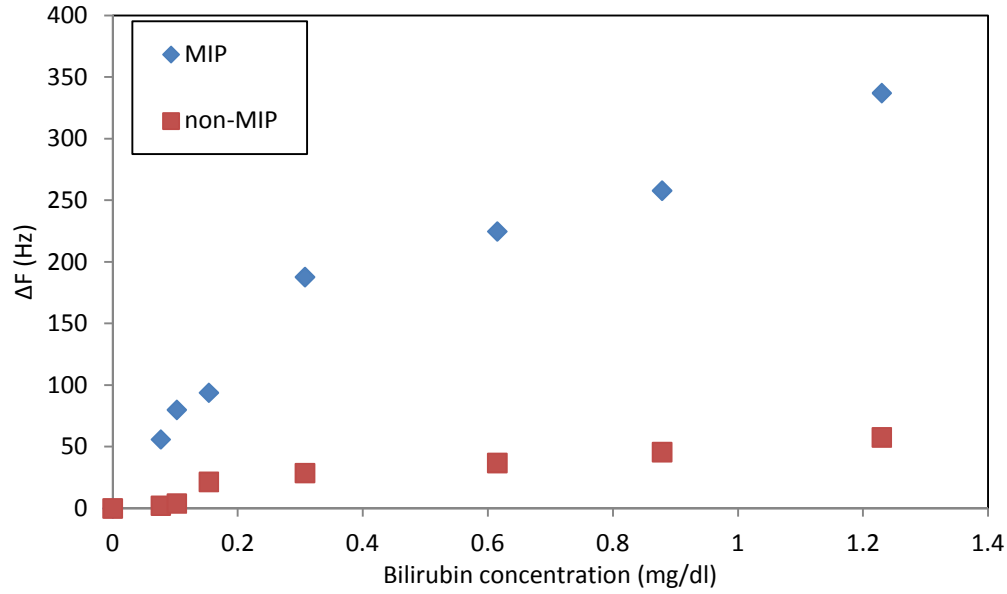

Figure 4.14. Comparison of detection frequency shift from crystals coated with MIP and non-MIP films (1:8)

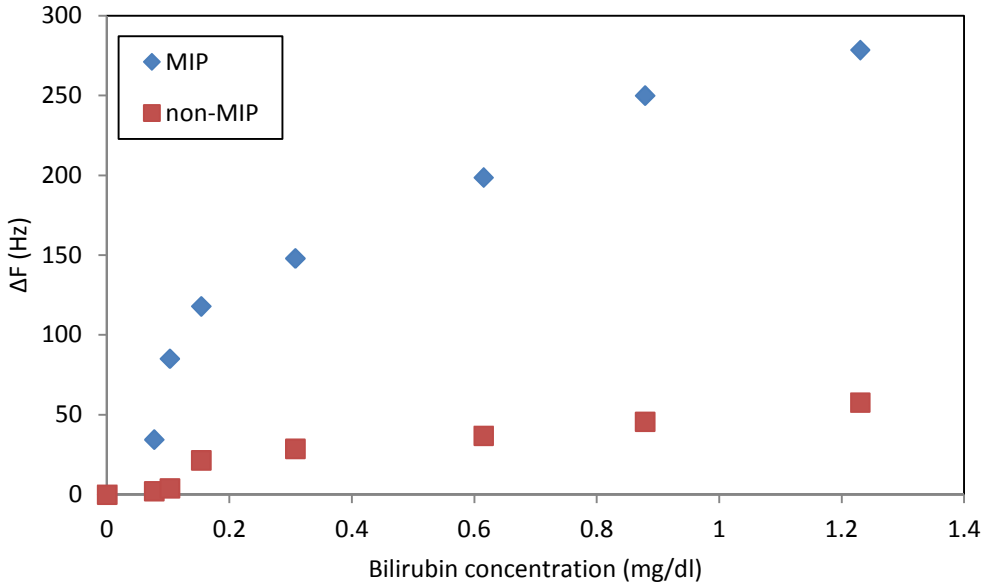

Figure 4.15. Comparison of detection frequency shift from crystals coated with MIP and non-MIP films (1:16)

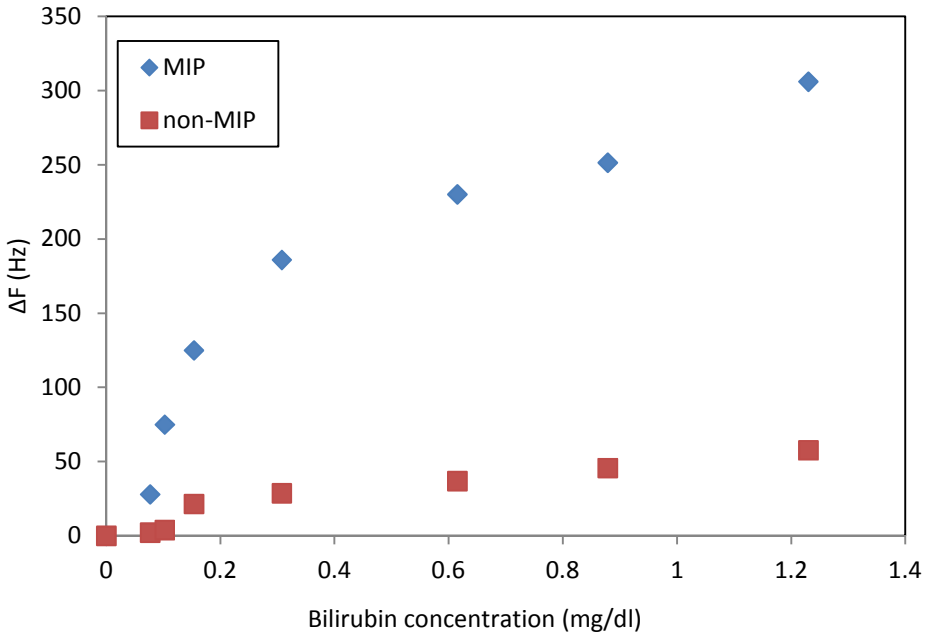

Figure 4.16. Comparison of detection frequency shift from crystals coated with MIP and non-MIP films (1:32)

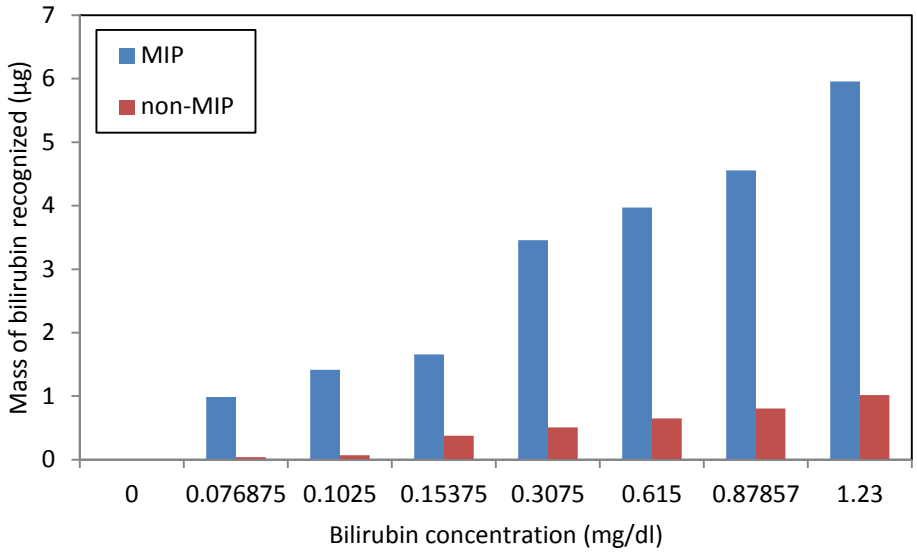

Figure 4.17. Comparison of mass of bilirubin recognized by crystals coated with MIP and non-MIP films (1:8)

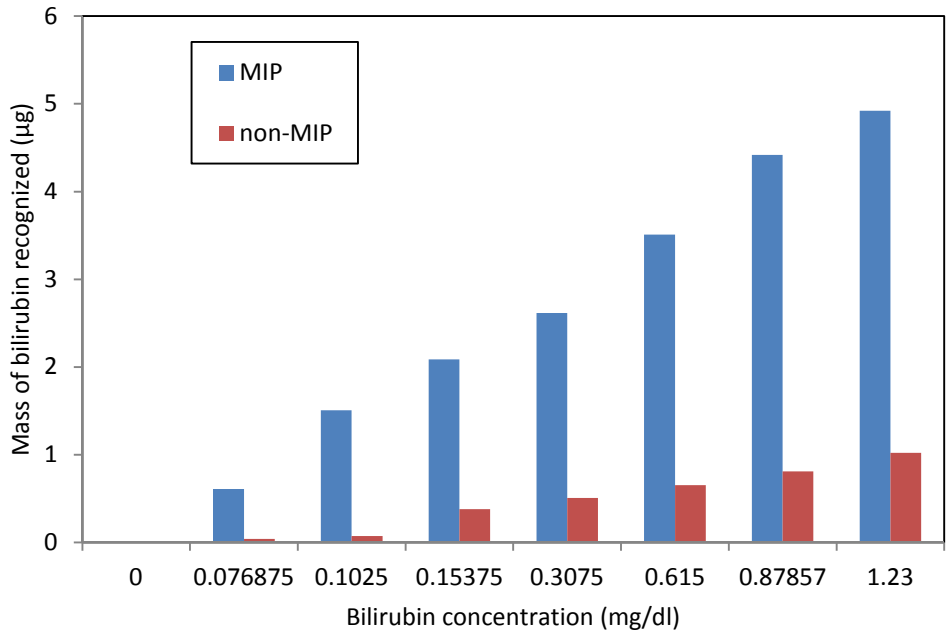

Figure 4.18. Comparison of mass of bilirubin recognized by crystals coated with MIP and non-MIP films (1:16)

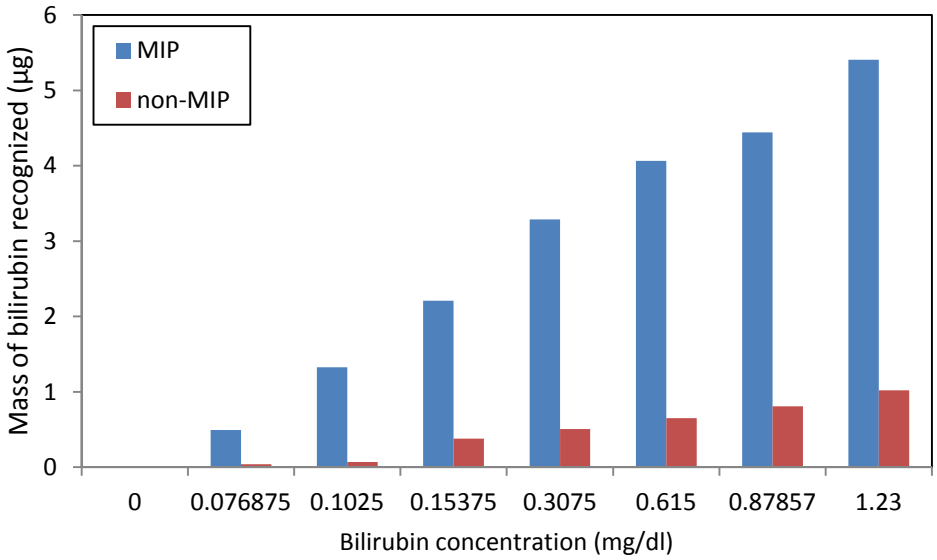

Figure 4.19. Comparison of mass of bilirubin recognized by crystals coated with MIP and non-MIP films (1:32)

4.7 Effect of pH on Recognition

The effect of pH on recognition was studied. Two bilirubin concentrations were chosen for the effect of pH study. The 0.615 mg/dl solution's recognition was studied within the pH range from 2.62 to 12.05. The 0.1025 mg/dl was studied from pH of 3.11 to 11.89. Figures 4.20, 4.21, 4.22, 4.23, 4.24, and 4.25 show the amount of bilirubin recognized by the test crystals from the solutions with varying concentrations and pH.

Generally, the lower pH (2.62 and 3.11) showed a relatively lower recognition than the higher pH values did. This trend was evident in the 0.615 mg/dl solution as depicted in Figures 4.20, 4.21, and 4.22. Figure 4.20 shows that the amount of bilirubin recognized increased with increasing pH. Similar trends are seen in Figures 4.21 and 4.22, except that in both pH 9.12 showed some exception. In Figure 4.21 more bilirubin was recognized from solution than that with pH 12.05 whereas in Figure 4.22, the amount of bilirubin recognized was less than solution with pH 4.36, 7.1, and 12.05. The effect of pH on recognition at lower concentration (0.1025 mg/dl) did not show any general trend at all.

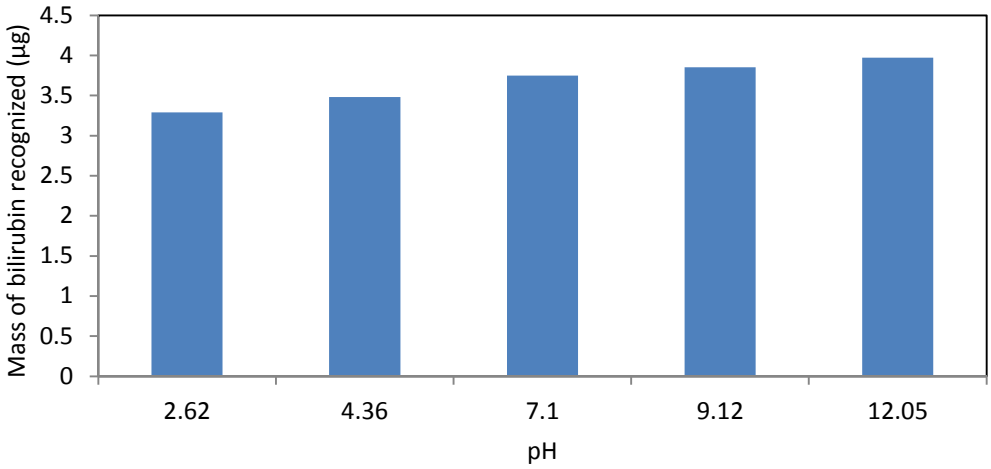

Figure 4.20. Effect of pH on the amount of bilirubin recognized 1:8 ratio MIP crystal (0.615 mg/dl)

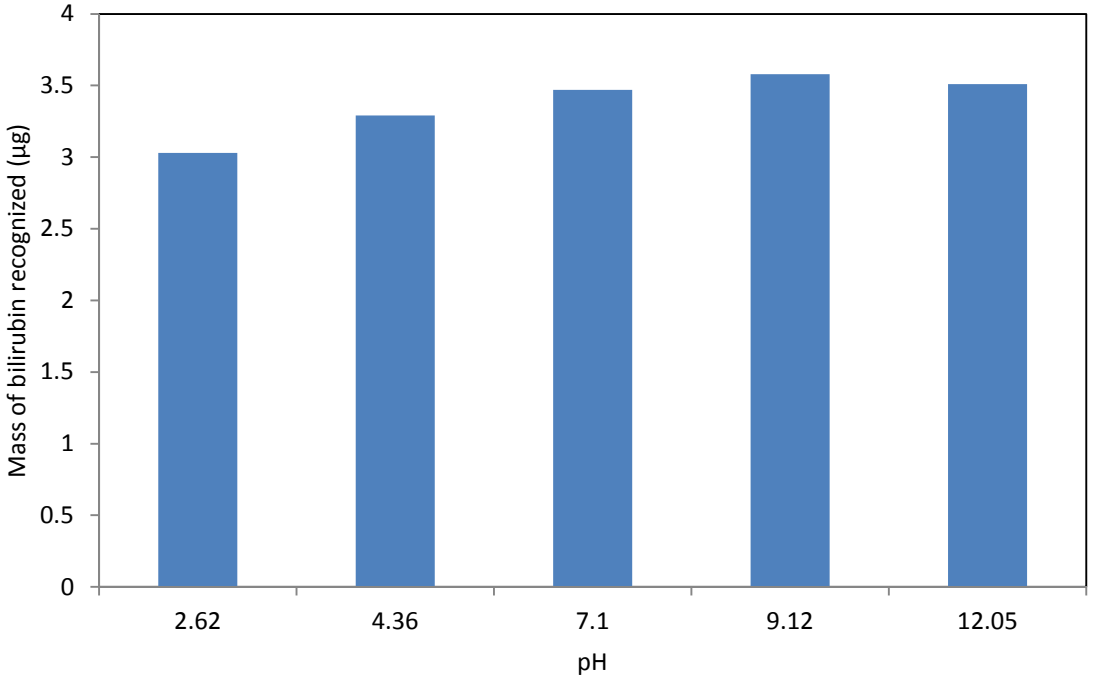

Figure 4.21. Effect of pH on the amount of bilirubin recognized by 1:16 ratio MIP crystal (0.615 mg/dl)

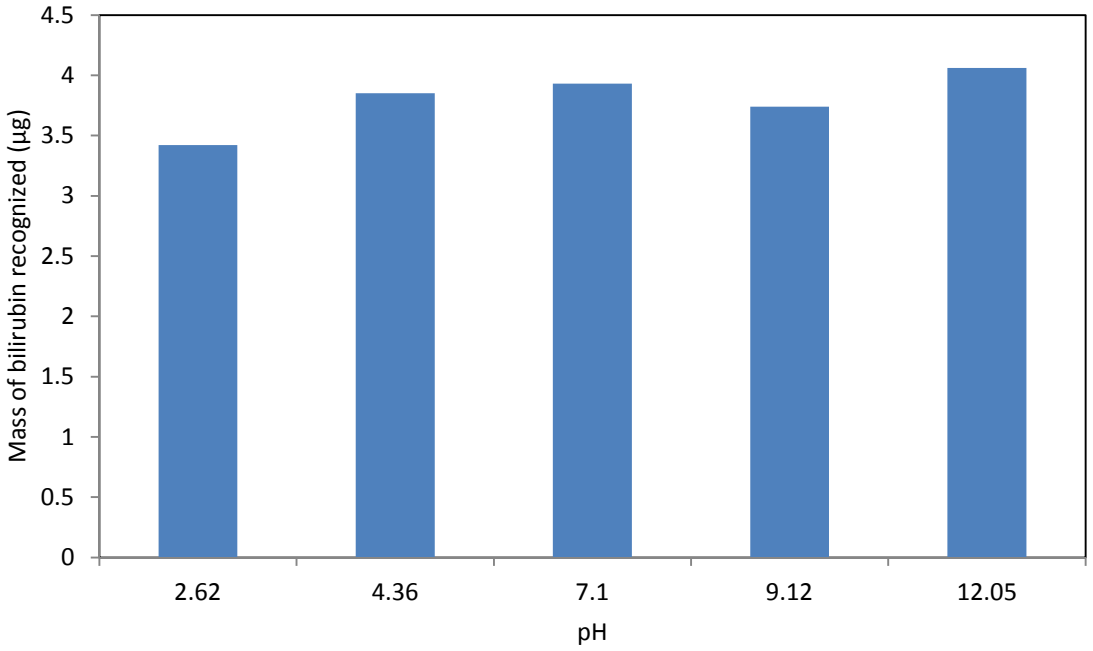

Figure 4.22. Effect of pH on the amount of bilirubin recognized by 1:32 ratio MIP crystal (0.615 mg/dl)

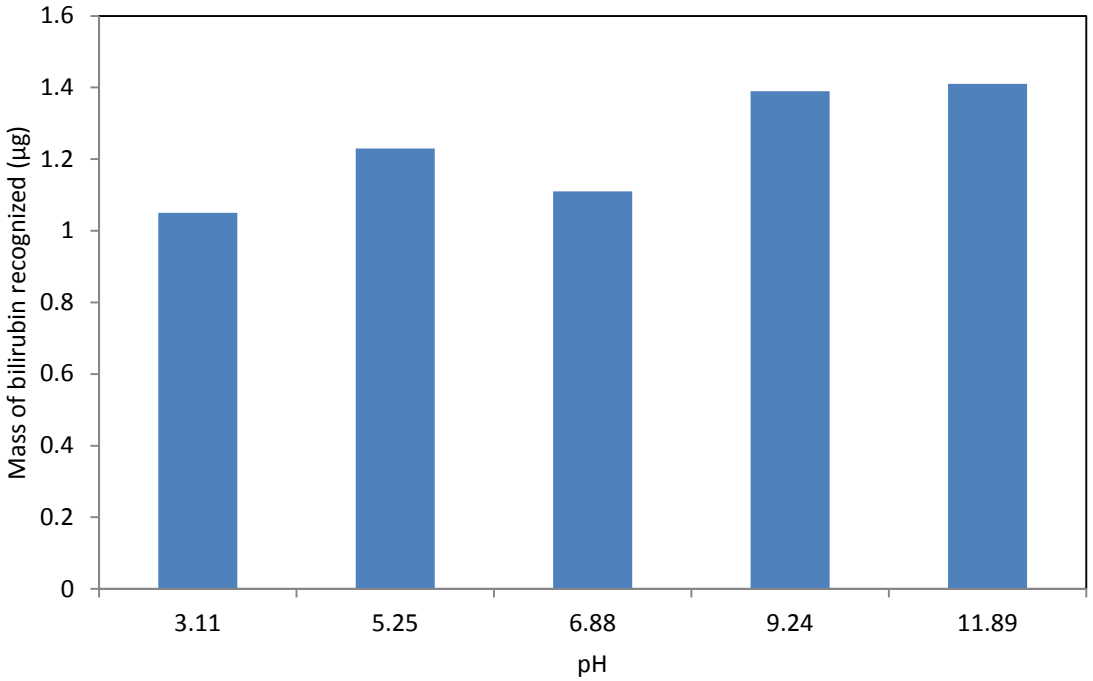

Figure 4.23. Effect of pH on the amount of bilirubin recognized by 1:8 ratio MIP crystal (0.1025 mg/dl)

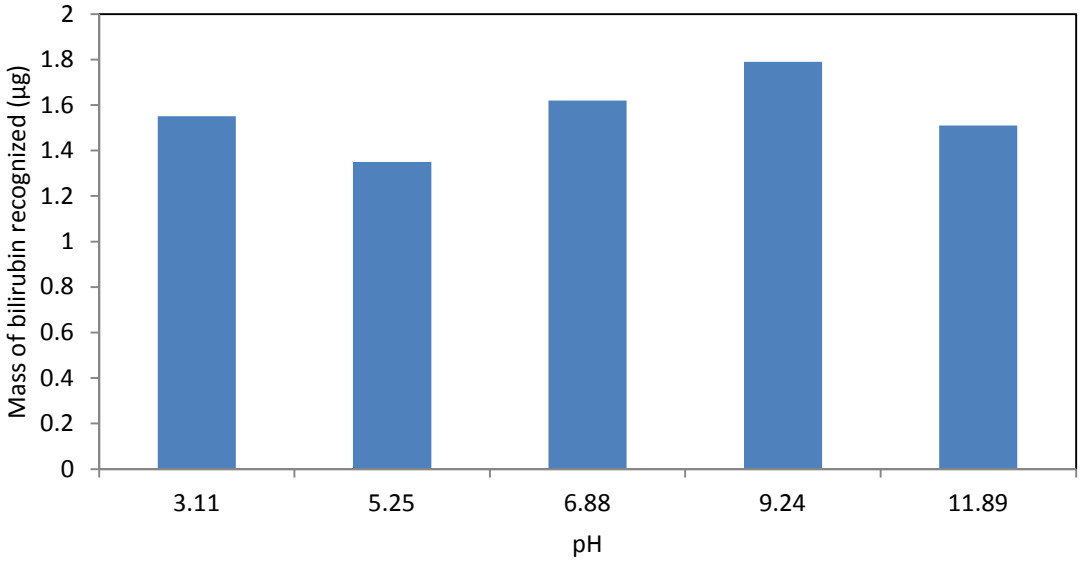

Figure 4.24. Effect of pH on the amount of bilirubin recognized by 1:16 ratio MIP crystal (0.1025 mg/dl)

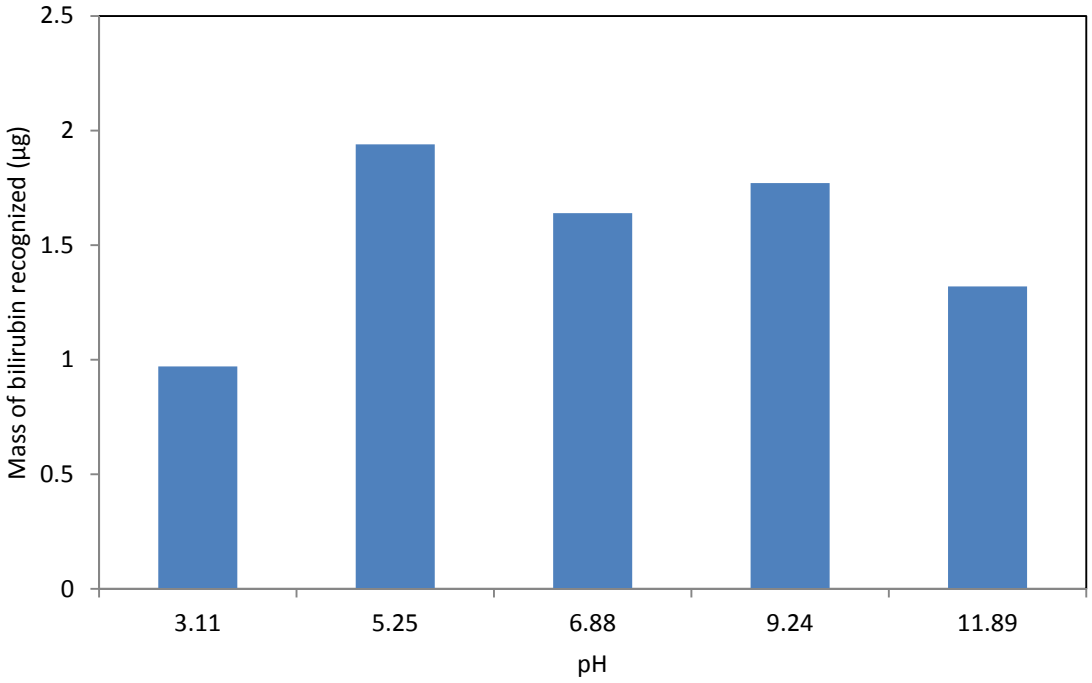

Figure 4.25. Effect of pH on the amount of bilirubin recognized 1:32 ratio MIP crystal (0.1025 mg/dl)

4.8 Effect of Ionic Strength on Recognition

Ions compete for imprinted sites in MIPs. The effect of ionic strength on recognition was investigated. NaCl solutions of various concentrations (0.05 M, 0.10 M and 0.2 M) were used as solvent to prepare bilirubin solution (1.23mg/dl). The presence of NaCl in the bilirubin solution did have an effect on the recognition of the template by the MIP. Upon the introduction of NaCl, there was a significant decrease in recognition of template in the MIP. Interesting, increasing the salt concentration actually increased the amount of bilirubin recognized as can be seen from Figures 4.26 and 4.28. However this amount was relatively less than bilirubin solution without NaCl present. The 1:16 ratio MIP in Figure 4.27 showed a slight deviation from the other two ratios (1:8 and 1:32). In general, the presence of the salt affected the recognition of the template by the MIP. It is believed that the salt present blocks some of the imprinted sites. This inhibits bilirubin molecules in solution to effectively bind to the imprinted sites.

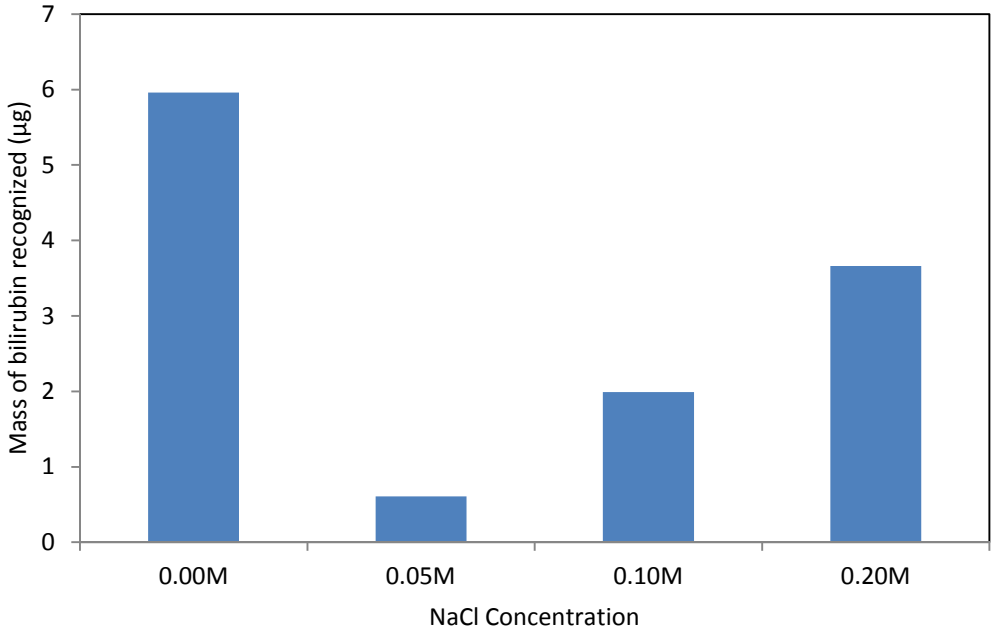

Figure 4.26. Effects of ionic concentration on the amount of bilirubin recognized 1:8 ratio MIP coated crystal

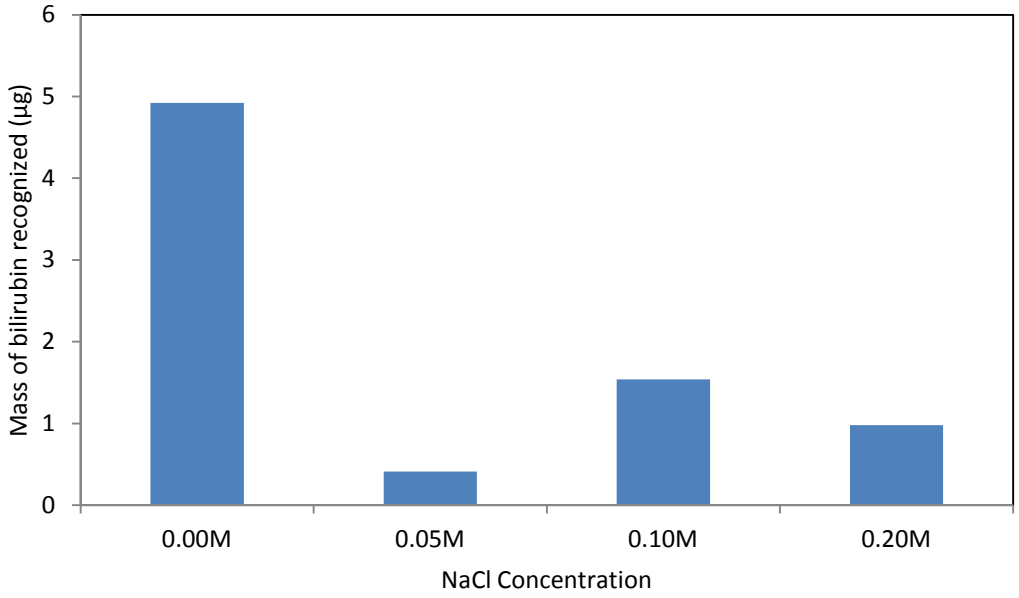

Figure 4.27. Effect of ionic concentration on the amount of bilirubin recognized by 1:16 ratio MIP coated crystal

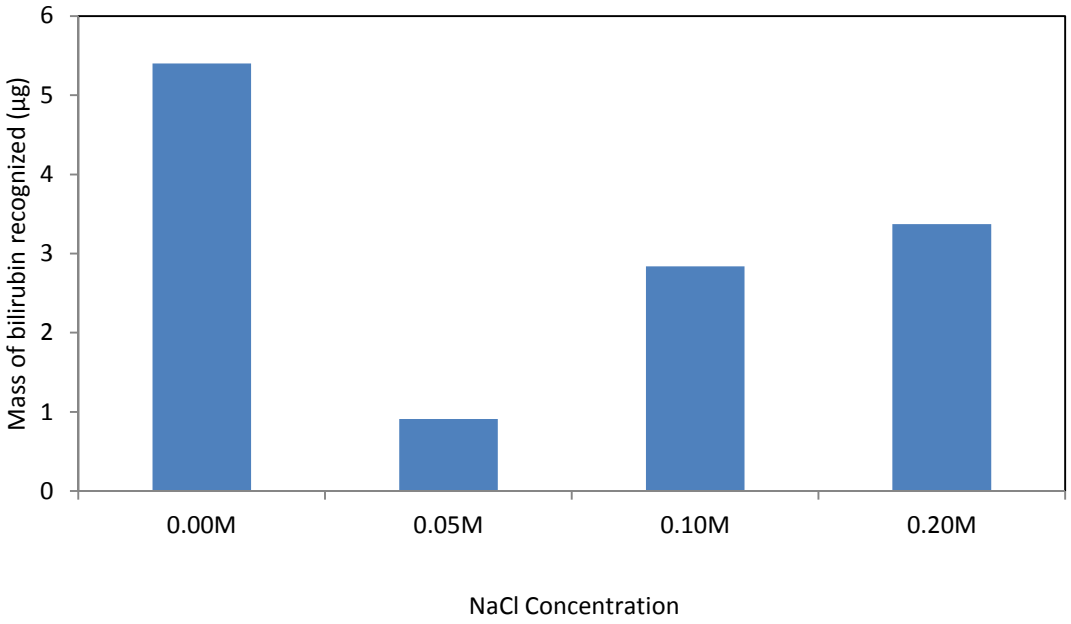

Figure 4.28. Effect of ionic concentration on the amount of bilirubin recognized by 1:32 ratio MIP coated crystal

4.9 Specific Recognition Studies (Selectivity of Imprinted Polymers)

The essence of a biosensor is its ability to detect a specific target molecule in a pool of similar sized and structured molecules. In the selectivity study, biliverdin recognition by the MIP was followed by the QCM system. Biliverdin is a green tetrapyrrolic bile pigment, and is a product of heme catabolism. It is the pigment responsible for a greenish color sometimes seen in bruises (Mosqueda, Burnight, & Liao, 2005). Bilirubin and biliverdin have similar structures and molecular weights. That was the reason biliverdin was chosen for the selectivity study. Figures 4.29, 4.30, and 4.31 show the detection results for bilverdin recognition compared to that of bilirubin. Figures 4.32, 4.33, and 4.34 compare the amount of analyte recognized by the QCM system. From the figures below, one can admit that MIP synthesized has a very high affinity for bilirubin (template used in synthesis). For each concentration of bilirubin and biliverdin compared for all test crystals, more bilirubin than biliverdin was detected. This confirms the fact

that molecular recognition is indeed a complex phenomenon. It is not based upon size alone, but combines size, specific polymer-template complex and a weak form of bonding such as hydrogen bonding.

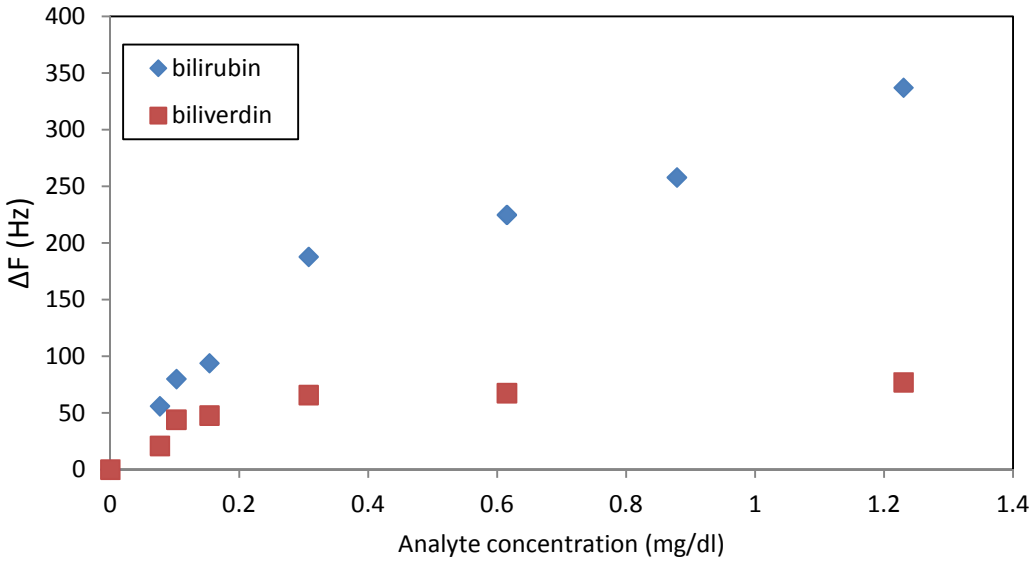

Figure 4.29. Comparison of detection frequency given by bilirubin and biliverdin (1:8)

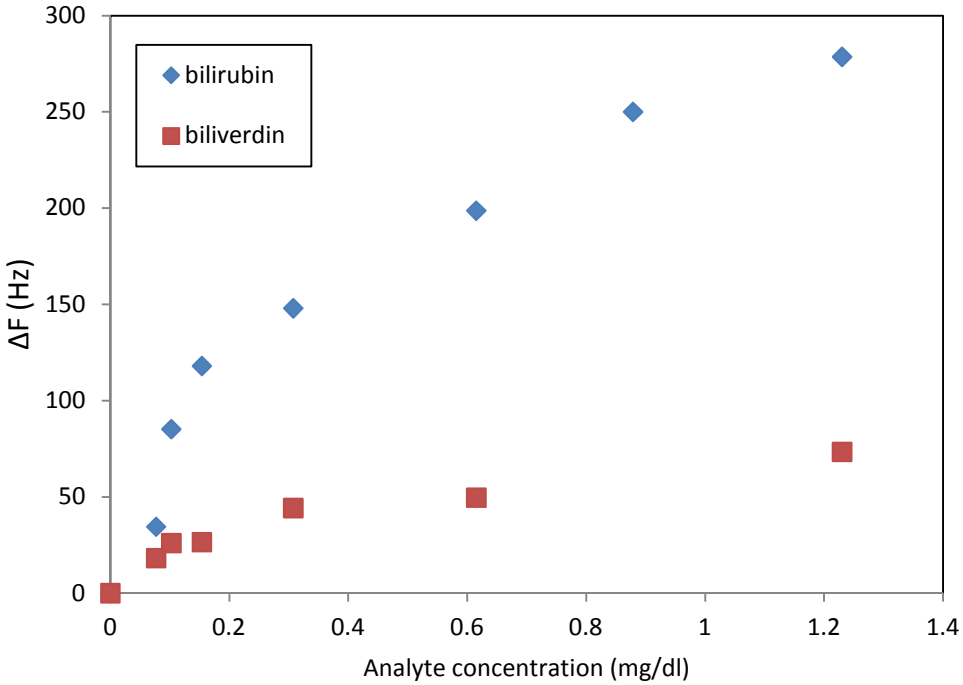

Figure 4.30. Comparison of detection frequency given by bilirubin and biliverdin (1:16)

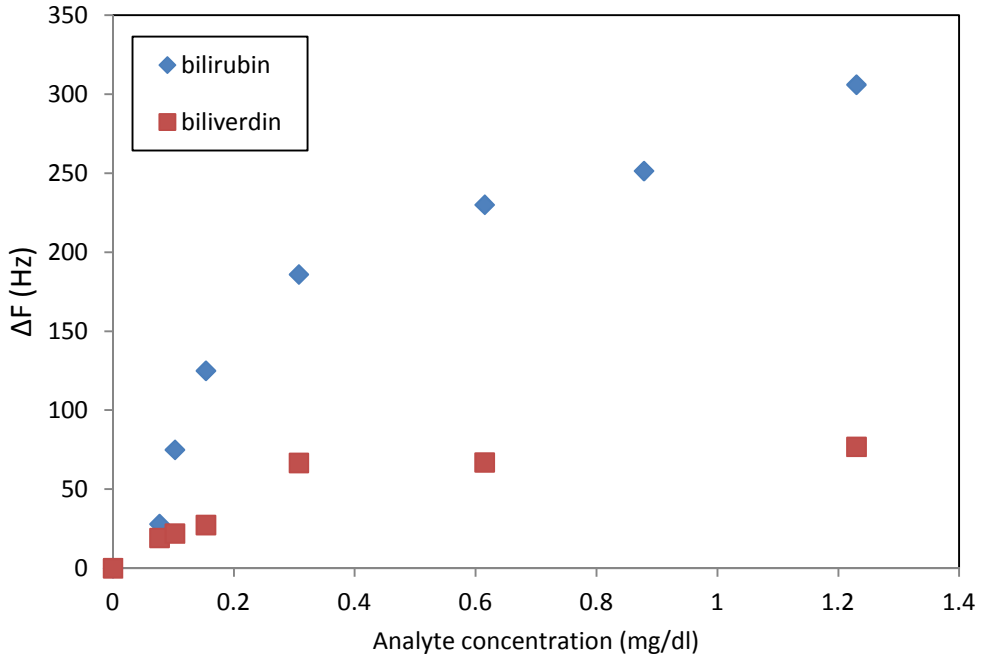

Figure 4.31. Comparison of detection frequency given by bilirubin and biliverdin (1:32)

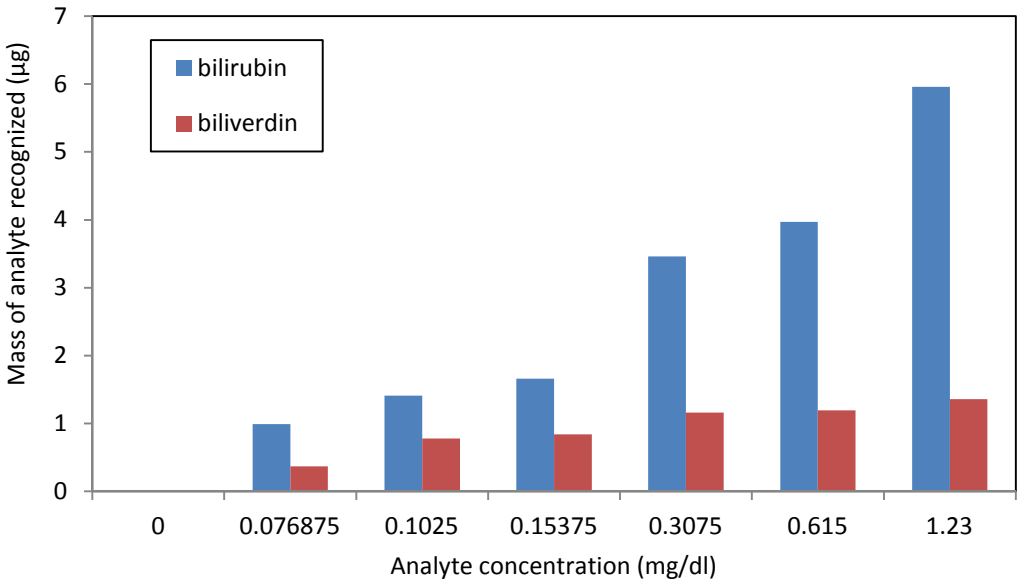

Figure 4.32. Comparison of amount of bilirubin and biliverdin recognized by 1:8 ratio MIP coated crystal

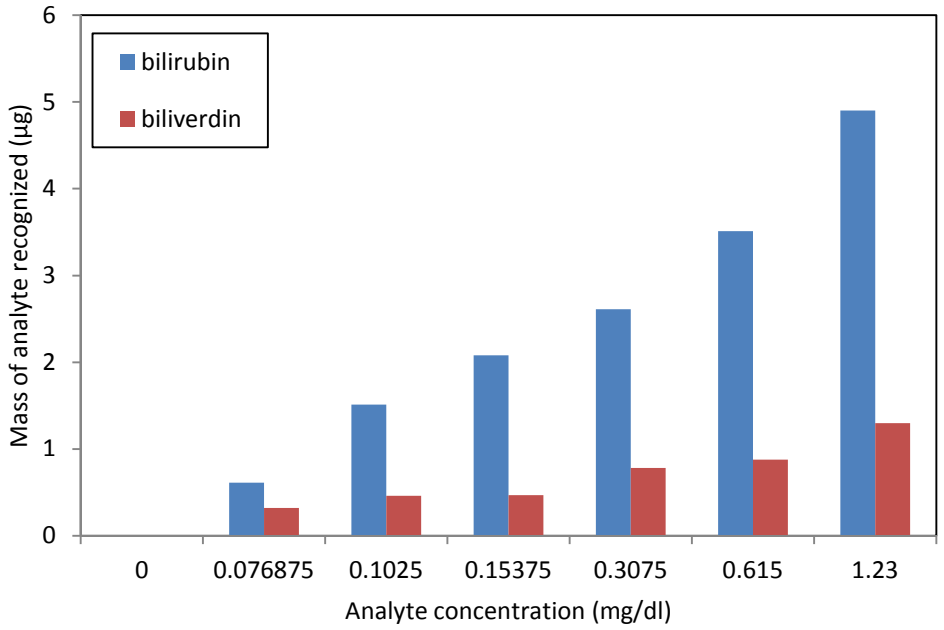

Figure 4.33. Comparison of amount of bilirubin and biliverdin recognized by 1:16 ratio MIP coated crystal

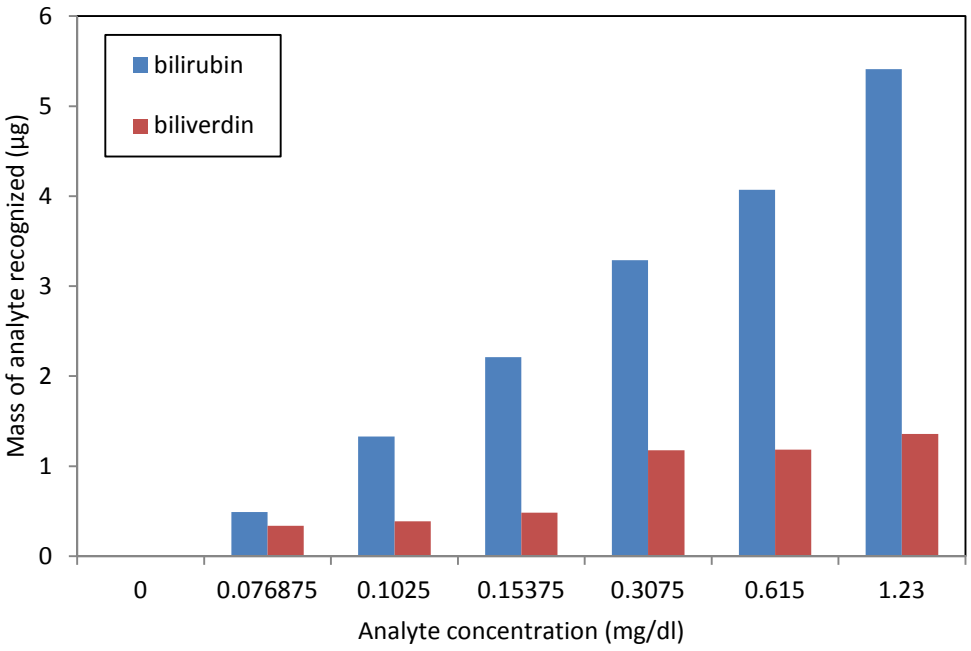

Figure 4.34. Comparison of amount of bilirubin and biliverdin recognized by 1:32 ratio MIP coated crystal

4.10 Reproducibility of Results

To ensure the reproducibility (precision or repeatability) of the measurement system under unchanged conditions, the binding experiment was duplicated. Relative standard deviation analyses were done to determine the precision of the collected data. Table 4.2 shows the mean, standard deviation, and relative standard deviation of detection frequency from a QCM crystal. From Table 4.2, it can be seen that the relative standard deviation ranged from 0.04% to 11.29%. The average standard deviation was 5.89%. This shows that the measurement system was quite precise.

Table 4.2

Mean, standard deviation and relative standard deviation from the (1:8) ratio MIP coated crystal

Bilirubin concentration	**1**	**2**	**Mean**	**Standard Deviation**	**Relative Standard Deviation**
0.076875	56.00	51.80	53.90	2.969848	0.055099
0.102500	80.00	75.70	77.85	3.040559	0.039057
0.153750	93.90	106.60	100.25	8.980256	0.089579
0.307500	187.80	186.60	187.20	0.848528	0.004533
0.615000	224.80	263.80	244.30	27.577160	0.112882
0.878570	257.90	286.70	272.30	20.364680	0.074788
1.230000	337.10	320.10	328.60	12.020820	0.036582

CHAPTER 5

Conclusion

MIP films for the specific recognition of bilirubin in solution have been synthesized on the surface of a QCM crystal. Five template to polymer ratios were studied. Three of these were successful (1:8, 1:16, and 1:32). The polymer was synthesized using 4-vinyl pyridine as functional monomer and divinylbenzene as cross linking agent. The initiator used was benzophenone. A calibration curve was obtained for bilirubin concentration using the QCM system to study the detection frequency of various concentrations of bilirubin solutions. Comparison of the MIP coated crystal and the non MIP crystal showed that the former had a higher affinity to rebind the target molecule. The selectivity of the MIP was also investigated. Biliverdin which has similar size and molecular weight as bilirubin was studied. When detection frequencies from both bilirubin and biliverdin were compared, it was observed that the MIP had a stronger interaction with bilirubin than with biliverdin. The effects of pH and ionic strength on recognition of target molecule by the polymer were also studied. From the promising results obtained, the sensor can be developed as a low cost alternative method in diagnosing diseases in remote parts of the world where quality healthcare is not easily accessible.

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