

**A NOVEL MOLECULARLY IMPRINTED POLYMER
FOR THE SELECTIVE REMOVAL OF INTERFERING
HEMOGLOBIN PRIOR TO WHOLE BLOOD ANALYSIS**



Department of Chemical and Forensic Sciences

Master of Science in Analytical Chemistry

College of Science

A thesis submitted to Botswana International University of
Science and Technology, Palapye, in fulfillment of the
requirements of the degree of Master of Science in Analytical
Chemistry

November 18, 2016

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Based on research carried out under the supervision of

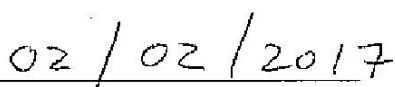
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Declaration

I hereby declare that this thesis is solely my work, in fulfillment of the requirements of Master of Science degree in analytical chemistry. All the research was carried out under the department of Chemical and Forensic Sciences, Botswana International University of Science and Technology (BIUST), Botswana.



Tumelo Hendrick Tabane



Date

Dedication

This thesis is dedicated to my late beloved sister, **Boikobo Tabane**, whom I will always remember for the rest of my life.

Acknowledgements

To my Supervisor, Dr. B. S. Batlokwa, whom I deeply owe my sincere gratitude, for believing in my abilities, ever since we started this journey. I thank you for all the knowledge in this field of science, the encouraging pieces of advice and the leadership you provided. I came to this university without any information about this research field. If it was not for you, I would not be able to understand the chemistry behind the imprinting technology, which I now have a greater passion to study it more. Thank you again for showing me the ins and out of how the research is carried out prior to submitting the end results. Furthermore, the whole study would not have been a great success without your greatest supervision.

To my colleagues that I worked with in our MIP laboratory, Mr Morlu Stevens, Mrs Oratile Semong, Miss Neo Balole, Miss Hawa Mukami, Miss Refilwe Keipidile and Miss Irene Maina, I thank you all for the assistance, the support and the encouraging words you gave me, since you all knew how hesitant I was when it came to finishing the work at hand on time. You always passed both negative and positive remarks regarding this project, hence, all that was to build up my research abilities up to this level. It has been a rough time for all of us but what mattered was the end results. I would also like to acknowledge all my family members for giving me the strength through thick and thin, while pursuing my studies.

To the department of chemical and forensic sciences staff members, more especially the chemistry technical team, thank you for the great support regarding the practical part of the project, you were a great team indeed when it came to the issue of laboratory glassware, the reagents and all the necessary tools to use while undertaking this project.

Lastly, I would like to show a great gratitude to BIUST, my full sponsor for the past two years during my studying. It has been a great pleasure to be part of this institution's community and I have learnt a lot, hoping to achieve more while I am still here to further my studies.

Abstract

A novel, selective, sensitive, cheap and robust hemoglobin imprinted polymer (Hb-MIP) in the form of a powder, was synthesised through bulk, free-radical polymerization employing molecular imprinting technology (MIT), for the selective removal of interfering hemoglobin from whole blood samples prior to instrumental analysis in molecular diagnostics and toxicological assays. Following solid phase extraction clean-up through batch rebinding experiments, the Hb-MIP powder effectively removed hemoglobin from whole blood samples as demonstrated by the UV-Vis absorbance reductions from as high as 0.794 Au to lower values of 0.193 Au. It also proved to be efficient by optimally removing hemoglobin within 18 min. Experimentally, the powder showed good selectivity towards hemoglobin as demonstrated by the percentage removal efficiency of 76%, even in the presence of analogous species such as chlorophyll with a negligible percentage removal efficiency of about 32%. Furthermore, the Hb-MIP powder was compared to a commercially available and expensive interferents removing material, graphitized carbon black (GCB) powder that is not selective, as shown by almost the same percentage removal efficiencies of 96% and 91%, for hemoglobin and chlorophyll, respectively. The synthesized Hb-MIP powder in this thesis presented itself as an efficient, selective, cheap and non-destructive whole blood clean-up pre-analytical tool that with further research may replace the conventional whole blood clean-up strategies that are currently employed in molecular diagnostics and toxicological assays such as the common, inefficient and destructive centrifugation.

Keywords: Hemoglobin imprinted polymer, Molecular imprinting technology, Centrifugation, Molecular diagnostics, Forensic toxicology, Whole blood clean-up

List of Abbreviations

AFM:	Atomic force microscopy
AIBN:	Azobisisobutyronitrile
ASP:	Ammonium sulphate precipitation
Au:	Absorbance units
BET:	Brunauer, Emmet and Teller
BJH:	Barret, Joyner and Halenda
CLB:	Clenbuterol
DRA:	Drug residue analysis
EDTA:	Ethylene diamine tetraacetic acid
EGDMA:	Ethylene glycol dimethacrylate
FE-SEM:	Field emission scanning electron microscopy
FI:	Freundlich
FTIR:	Fourier transform infrared
GCB:	Graphitized carbon black
GC-MS:	Gas chromatography mass spectrometry
GPC:	Gel permeation chromatography
h:	Hours
Hb:	Hemoglobin
Hb-MIP:	Hemoglobin-molecularly imprinted polymer
IAEs:	Immunoaffinity extraction sorbents
IASPEs:	Immunoaffinity solid phase extraction sorbents
ISs:	Immunosorbents

LC-MS/MS: Liquid chromatography tandem mass spectrometry

L-FI: Langmuir-Frundlich

LLE: Liquid liquid extraction

MAA: Methacrylic acid

MAX: Mixed-mode anion-exchange

MCX: Mixed-mode cation-exchange

mg: Milligrams

MIA: Molecularly imprinted adsorbents

min: Minutes

MIPs: Molecularly imprinted polymers

MIT: Molecular imprinting technology

mL: Milliliters

NIPs: Non-imprinted polymers

PBS: Phosphate buffered saline

PPT: Protein precipitation

PS-DVB: Polystyrene divinylbenzene

RBC: Red blood cells

RNAs: Ribonucleic acids

SPE: Solid phase extraction

TRIM Trimethylolpropane trimethacrylate

UV-Vis: Ultra-violet Visible

μm: Micrometers

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Chapter One Background

1.1 Introduction

Bioanalytical assaying [1] for clinical/molecular diagnostics [2] and forensic toxicology [3] purposes, employ human body fluids such as whole blood, urine and saliva as samples [4]. Amongst them, whole blood is usually the most preferred [5]. This is because it is highly concentrated with both endogenous and exogenous components that contain targeted analytes even when the blood sample is of low volume (e.g. a drop). Whole blood is known to connect all the bioprocesses in living systems' organs. Furthermore, it is highly homeostatic and largely unaffected by factors such as age, gender and diet, hence a better sample to provide crucial information concerning causes of impairment and death [6]. On the other hand, urine and saliva contain endogenous/exogenous components in lower concentrations even when larger quantities have been sampled, thus, relative to whole blood, they are less preferred [7].

The medical and forensic investigation authorities rely mostly on accurate and precise interpretation of subsequent measurements in modern fields of molecular diagnostics and forensic toxicology, which are concerned with the identification and quantification of biomarkers [8] and toxicants/drug residues from whole blood, respectively [9][10]. The challenge has always been direct introduction of whole blood as a sample into analytical instruments due to the complexity of its matrix which is usually characterized by 'dirt' from hemoglobin (the red part of the blood) and phospholipids [11]. The red part and/ or the phospholipids usually mask the targeted analytes from being easily detected, thus, clog the instrument components more especially the columns, which end up being plugged and inactivated during chromatographic separation, leading to interference, imprecise and inaccurate results after a few runs. Furthermore, the interferents within whole blood generally lead to matrix effects caused by their co-elution with the biomarkers, toxicants or drug residues, which are the targeted analytes [12]. Matrix effects usually occur when employing gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) as the sensitive and specific detection systems of choice for the

accurate determination of biomarkers, toxicants and drug residues in whole blood [13][14]. Thus, prior to employing sensitive instruments with low detection limits such as GC-MS and LC – MS/MS for analysis of biomarkers, toxicants and drug residues, whole blood as a sample must undergo extensive sample preparation to achieve even more accurate and precise results.

Hypothetically, a newly developed hemoglobin recognition material as a sample preparation/clean-up tool, in this thesis will help to minimise the level of interference caused by the existing hemoglobin within the ‘dirty’ whole blood sample prior to its analysis for the targeted analytes. Furthermore, this study tested the significance of employing the hemoglobin recognition material by investigating its effectiveness and selectivity in removing the hemoglobin interference, hence, exposing the targeted analytes such as the biomarkers, that are known to exist at trace levels within whole blood to improved detectable levels, during instrumental analysis.

1.2 Sample Preparation of Whole Blood

Extensive sample preparation [15][16] is key to perform in sample assaying when handling complex matrices including those of biological origin. The main purpose of sample preparation as the bottleneck of all analytical procedures is to eliminate potential interferents, pre-concentrate the targeted analytes and convert them into more suitable detectable forms, for accurate analysis employing instruments with low detection and quantification limits [17][18].

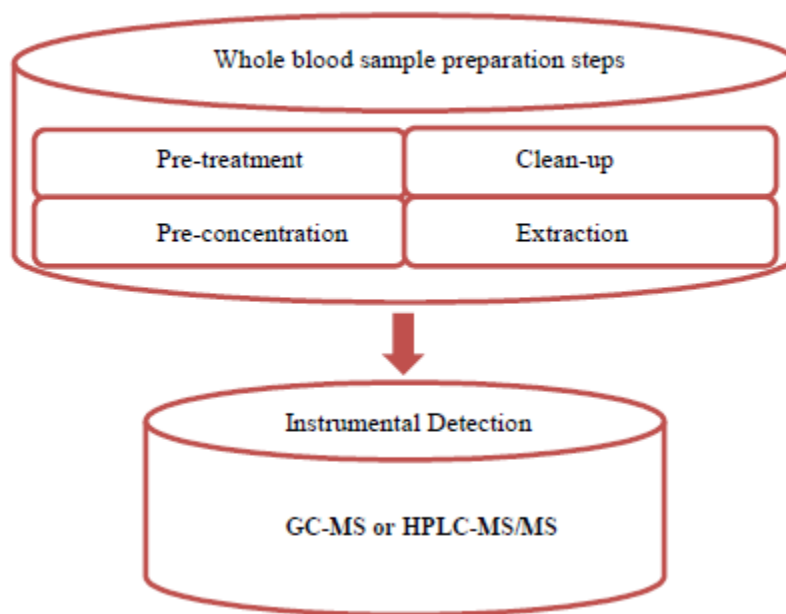


Figure 1.1: Whole blood sample preparation steps prior to instrumental detection

When analysing whole blood for biomarkers, toxicants and drug residues, sample preparation steps such as pre-treatment [19], extraction [20], clean-up and pre-concentration [21] (**see figure 1.1**) are usually employed as pre-analytical procedures that achieve cleaner samples with less or no interferents, as well as concentrated targeted analytes that are easily detectable. Furthermore, minimal destruction of some sensitive instrumental components such as the chromatographic columns, that are liable to column overloading and the injector ports that may become jammed, is another benefit that results from extensive sample preparation, thus reducing the cost of maintaining the analysing instruments that often is very expensive and stalls research. Protein precipitation [22] followed by centrifugation [23] have been extensively described both in pre-treating and cleaning whole blood for a number of targeted analytes in several bioassays [24][25]. Liquid-liquid extraction (LLE) [26] and the conventional solid-phase extraction (SPE) [26] have also been employed for whole blood preparation with the aims of obtaining cleaner and purer extracts that are concentrated with targeted analytes as well as free from potential interferents for easy separation and accurate detection. LLE is challenged as it is labour intensive and less reproducible [27], whilst the conventional SPE, which is more versatile than LLE, is known for its non-selective extracting power that results in poor analyte recoveries [28].

1.2.1 Whole Blood Pre-Treatment

Pre-treatment aims at conditioning the sample, thereby, turning it into a state that the targeted analytes are easily detectable [24]. It also facilitates further sample preparation steps such as extraction as well as removing undesired, obvious to the eye, large matrix constituents that would otherwise disturb the accurate determination of the targeted analytes by the instruments. Dilution is a well-known traditional method that is commonly employed as a sample pre-treatment step for a variety of biological fluids such as urine and whole blood [4]. Whole blood dilution has been described employing different solvents, depending on the targeted analyte within the matrix. For example, polar/nonpolar organic or aqueous solvents have been employed for whole blood dilution, for the purpose of isolating toxicologically aqueous drugs and their metabolites from the organic-based blood matrix during drug residue assaying [29]. It is mostly employed as a sample pre-treatment step in preparations of serial diluted blood solutions prior to high performance liquid chromatography (HPLC) based analysis. This usually minimises the risk of column overloading and detector saturation during analysis [30]. Shin *et al*, achieved whole blood pre-treatment by diluting the blood sample with hydrogen peroxide. They observed red blood cells' discolouration within the blood sample prior to immuno-chromatographic assays. The purpose for the study was to minimise the red colour of the red blood cells (RBCs) as it was found to interfere with the analysis. This pre-treatment method is unsafe since hydrogen peroxide as a strong oxidizer not only is capable of destroying the targeted analytes, but poses a human health hazard during handling [19].

Employment of whole blood collection devices coated with anticoagulants like heparin, EDTA and its derivatives to prevent coagulation and blood clotting is another way of pre-treatment. Sampling vacutainers with different coatings have been employed depending on the type of blood component to be isolated from whole blood. For example, EDTA-coated vacutainers were designed to allow for RBCs isolation from the rest of the matrix, thus, making it possible for easy plasma isolation free from cells [31]. To some extent, these chemicals can act as extra additives to the sample matrix and interfere directly with the targeted analytes, thus, changing their morphological nature and preventing them from being easily isolated and quantified [32]. Bowen and Remaley reported on chemicals such as anticoagulants, gel separators and clot activators that they were found to leach into blood

specimen, thus altering the targeted analytes stability and eventually contributing to pre-analytical errors in laboratory testing [32].

1.2.2 Whole Blood Clean-Up and Pre-Concentration

Sample clean-up refers to the process of eliminating undesired matrix components from the sample at hand including absorbed interfering matrix even at molecular level. Clean-up often results in simultaneous pre-concentration/enrichment and purification of the sample as a spinoff benefit [33]. This sample preparation step has proven to be very key and convenient in improving separation and instrumental detection of the targeted analytes from whole blood in several assays of molecular diagnostics and forensic toxicology. Employment of clean whole blood as a sample for analysis also reduces the chances of high down time of instruments due to clogging of chromatographic columns and sensitive detection compartments of the instruments, by the ‘dirty’ sample and interferents after several runs [34][35]. Furthermore, samples free from the ‘dirt’ and interferents greatly reduce the instrumental maintenance costs. Conventionally, clean-up strategies have been employed to remove interfering suspended large macromolecules from whole blood, including the red part of the blood (hemoglobin), and these include filtration [36], gel permeation chromatography [37], Freeze drying [38], protein precipitation [39] and centrifugation [40].

1.2.2.1 Filtration

Filtration is a sample preparation clean-up procedure based on particle size differences and selective segregation by dimensional pores with the benefit of improving reproducibility of analytical data [41]. It aims at cleaning blood samples by separating the plasma (rich with the targeted analytes) from suspended particulates like hemoglobin-based cells and phospholipids that are known to interfere and contribute to the “dirt” of the sample matrix during instrumental analysis. The need for these components to be removed is necessary since they mask specific biomarkers and toxicants/drug residues that exist in lower concentrations within whole blood as a sample [42]. Filtration by micro filters (micro-fluidic) and membrane filters have also been employed as improved clean-up devices, however, these devices also lacked selectivity [43][44]. Pham *et al* carried out a successful research on the filtration of hemoglobin-rich cellular components from whole blood employing nano porous membrane under hydrodynamic conditions to achieve clean plasma rich in the targeted analytes and free

from interferents. Despite the benefits of the method, it was found to be less cost effective and time consuming [41].

1.2.2.2 Gel Permeation Chromatography (GPC)

Employment of chromatographic systems as clean-up strategies is known in whole blood sample preparation. Practically, whole blood sample would simply be poured into a chromatographic column, packed with small porous polymer material of cross-linked polyacrylamide, with pore sizes controlled by the degree of cross-linking. This has been pronounced mostly when cleaning blood off its protein components to avoid protein binding on the targeted analytes, more especially during drug residue analysis (DRA) [45]. Protein binding has also been reported during quantification of metal ions like lead in blood samples, hence the need for those interfering large proteins to be eliminated [46]. For example, Kraak *et al* employed GPC as a sample clean-up step and achieved significant removal of suspended proteins from the blood plasma before analysis for the targeted analytes. Despite the benefits, this sample preparation step was found to be inefficient as a result of its co-extraction and non-selective performance of the column packing [47][45].

1.2.2.3 Protein Precipitation

Protein precipitation (PPT) is another sample preparation procedure that is usually employed for whole blood clean-up especially for mass spectrometry based analysis. This step aims at depleting large macromolecules like proteins (e.g. hemoglobin and lipo-proteins) that composite the complex and ‘dirty’ whole blood samples [22]. It was reported to assist in the prevention of protein-drug binding especially in forensic toxicology and drug residue analysis [9]. Most of the proteins in whole blood are characterised as heavy, with the ability to clog the instrument components, cause emulsions with liquid-liquid extraction (LLE) and contribute to restricted flow in solid-phase extraction (SPE) procedures, thus, negatively affecting the separation and detection steps during analysis of the targeted analytes. PPT has also been found not to produce proper sample extracts that are effectively clean since it only removes large proteins, leaving behind other interfering low lying micro proteins within the blood matrix [1]. Environmentally unfriendly and toxic protein precipitants employed for this procedure include: organic solvents like acetonitrile, methanol, acids and inorganic acids like

perchloric and trichloro acetic acids. The sample is usually mixed with a chosen solvent and subjected to a vortex for mixing, followed by centrifugation to sediment the precipitated proteins prior to their removal [48]. Raymond *et al* demonstrated that PPT as a sample clean-up was efficient in removing interferents from whole blood samples prior to instrumental analysis of pharmaceuticals in the same blood matrix. They further proved that PPT could be automated in a 96-well format, and the clean-up finally offered good recoveries of the pharmaceuticals [49]. Mahn and co-workers also reported on whole blood clean-up step employing ammonium sulphate precipitation (ASP), which consequently depleted the highly, abundant, unwanted proteins, prior to determining the lower abundant protein based biomarkers using gel-electrophoresis mass spectrometric based analysis. PPT does not always remove all the interferents like proteins, salts and phospholipids from the complex matrix. This is because some low lying proteins would remain unremoved within the matrix and still contribute to the interference, hence PPT as a clean-up step has limitations during sample preparation of whole blood [18].

1.2.2.4 Freeze Drying

Freezing with subsequent thawing stood out to be another whole blood clean-up strategy. It aims at removing unwanted molecules, particularly large macromolecules including blood cells, after subjecting the sample to lower temperatures and allowing it to thaw with increasing temperature [38]. This is normally performed in cycles to achieve effective sample clean-up. Ritu *et al* reported on the freeze drying/thawing of whole blood, followed by subsequent cycles of exposure to higher temperatures (90°C) in a thermostatic system. The method proved to be ineffective because of its harsh conditions, that could cause denaturation of targeted analytes in the process of whole blood clean-up [50]. Lee and co-workers on the other hand reported that clean-up employing repeated freeze-thawing strategy up to five cycles interfered with concentration levels of some of the circulating proteins within the blood plasma, thus , leading to imprecise and inaccurate analysis [51].

1.2.2.5 Blood Centrifugation

Generally, whole blood sample contains different blood constituents, both endogenous and exogenous [52]. These constituents possess different densities. In relation to the targeted

analyte like biomarkers and toxic chemicals/drug residues which often exist at trace levels within the blood matrix, highly concentrated constituents such as hemoglobin and fibrinogen act as interferents that need to be removed by centrifugation to eliminate the chances of co-extraction during bio-assaying [53]. Zheng *et al* achieved whole blood clean-up by centrifuging the collected blood sample, resulting in the elimination of interfering cellular debris from plasma, for the effective micro RNAs detection, as biomarkers in clean plasma specimen [40].

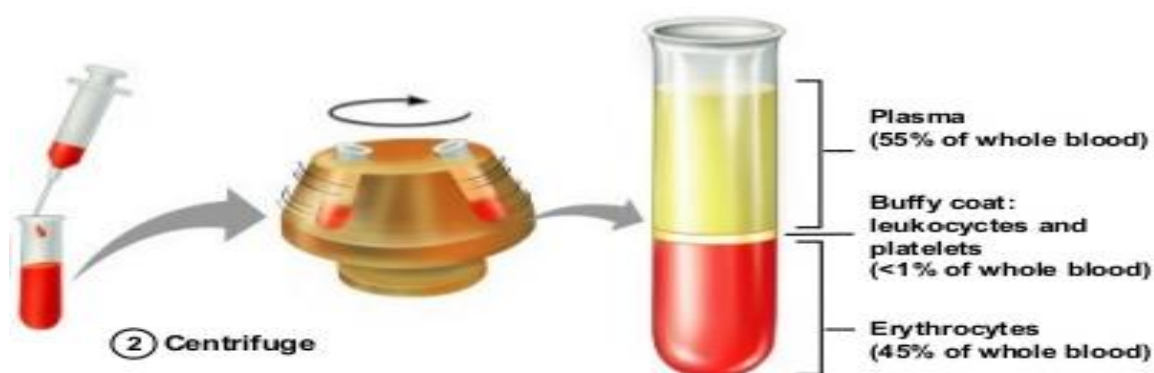


Figure 1.2: Whole blood clean-up employing centrifugation technique [54]

Principally, the sample at hand is placed in centrifuge tubes and spun at a high angular velocity measured in revolutions per minute (**see figure 1.2**) Whole blood is then separated into layers which vary in densities. The unwanted layer is then discarded from the targeted analyte layer. The technique suffers from challenges such as hemolysis because of high centrifugation forces, more especially in molecular diagnostics. Also, layer separation after centrifugation is a challenge because of the inefficient centrifugation forces that result in subsequent layers mixing up during decanting and consequently leading to targeted analytes being lost, depending on their distribution within the layers to be separated [55][56].

1.2.3 Whole Blood Extraction

Extraction of the targeted analytes from whole blood as a sample in bioanalytical assaying, often achieves simultaneous isolation, clean-up and enrichment of the targeted analytes [57] [58]. The procedure for extraction, typically involves dissolving the ‘dirty’ and complex

samples such as whole blood into a suitable solvent with comparable properties to the targeted analytes. The optimal and effective extraction will only be achieved if the chemical properties of both the sample matrix and the extracting phase (solid, liquid, gaseous or supercritical phase) are well known. Of recent, SPE has been reported as the most promising extraction strategy for whole blood sample preparation, with more advantages such as lower sample and organic solvents consumption, higher sample throughput, simpler, and more efficient than LLE in molecular diagnostics and toxicological assaying [59].

1.2.3.1 Solid-Phase Extraction

The main objectives of SPE are to reduce the level of interference through clean-up and minimise the final sample volume which results in highly concentrated targeted analytes. Consequently, sensitivity and detection during analysis is improved [60]. Caglar and co-workers reported on human blood clean-up employing SPE technique prior to MS-based toxicological analysis. They proved that SPE minimized the matrix effects because of the depletion of the undesired components from whole blood. Interfering endogenous components from the sample matrix were successfully removed resulting in high recoveries of the targeted analytes [61].

1.2.3.1.1 Principle of Solid Phase Extraction (SPE)

SPE is a form of solid absorption extraction technique, that employs similar principles such as hydrophilic, hydrophobic, ion exchange and affinity as used in chromatographic separations [62][63][64]. Principally, it involves partitioning between a liquid (sample matrix or solvent with the targeted analyte) and the solid (sorbent) phase, with the aim of extracting and retaining the targeted analyte into the solid sorbent while the interfering matrix components pass through, due to high affinities, selectivity and chemistries for the targeted analyte. A suitable solvent with more eluting strength will then be employed to recover the retained targeted analyte for analysis with the desired analytical instruments such as HPLC-MS/MS [63]. Sometimes the sorbent retains the interferents while the targeted analyte(s) is allowed to pass through for further analysis in a clean state, which is the case in this thesis [65][66][64]. The partitioning of the components between the liquid (sample matrix) and the solid sorbent depend on the partition coefficient, 'K', (**see equation 2**) that is usually calculated in relation to the concentrations of the analyte/interferent between the two phases

involved during the extraction procedure. Larger partition coefficients, of the magnitude greater than one (> 1) are known to favour extraction of the targeted analyte or the interferent to the sorbent phase than to the liquid phase [67].

1.2.3.1.2 SPE Sorbents

SPE sorbents with greater selectivities and affinities for the targeted analytes and/or the interferents are highly sought and have been reported [68][69] as they achieve high partition coefficients for greater extraction. Other considerations that are usually made during selection of the sorbents include the sample volume, nature of the targeted analyte and its concentration as well as the properties of the sorbents itself. The most frequently employed types of sorbents in SPE include chemically modified silica gel, polymer sorbents, porous carbon and other newly developed extraction sorbents such as the restricted access sorbents and the immunoaffinity extraction sorbents. Some of these sorbents are known for their three dimensional capability, high porosity, rigidity and high surface areas for enhanced extraction power [68]. However, some of the SPE sorbents have, low selectivity resulting, in co extraction which makes the SPE technique not to be effective and efficient.

1.2.3.1.2.1 Conventional SPE Sorbents

These are sorbents that have been used ever since the SPE technique was put to practice as a sample preparation technique. Examples of these conventional sorbents include polymeric materials with C8/ C18 skeletal backbones, silica based sorbents [70], hydrophilic-lipophilic balanced macro-porous copolymers, polystyrene divinylbenzene (PS-DBV), mixed-mode cationic-exchange (MCX), mixed-mode anion-exchange (MAX) and weak anion-exchange (WAX) sorbents [15]. Some of these sorbents are still in use and they have proved to be effective in different extraction areas for the purpose of clean-up and pre-concentration from biological matrices including whole blood [71]. However, new selective sorbents aimed at introducing high selectivity and good adsorptive/desorptive capacity have been reported [15].

1.2.3.1.2.2 Selective SPE Sorbents

Selective sorbents are based on molecular recognition mechanism. Examples include immuno-affinity extraction sorbents [72], restricted access sorbents as well as molecularly imprinted sorbents. The benefits of applying these sorbents have been reported to be highly specific and selective with enhanced partitioning power. They can be applied in diverse environments for the purpose of achieving clean extracts that are ready for instrumental analysis [69][73].

1.2.3.1.2.2.1 Immunoaffinity Extraction (IAE) Sorbents

After realizing that SPE conventional sorbents suffer from lack of selectivity and result in co extraction of targeted analytes with other matrix interferents, immunosorbents were developed to replace conventional sorbents. Immunosorbents (ISs) are based on molecular recognition and employ biological receptors such as antibodies or enzymes, immobilized on a solid support. The sorbents are usually referred to as immunoaffinity extraction sorbents (IAEs) or immunoaffinity solid-phase extraction sorbents (IASPEs) [72]. However, it has been reported that there are challenges in developing immunosorbents as acquiring antibodies or enzymes is not easy and they are expensive, as they are not readily available. The use of immunoaffinity extraction devices is internationally controlled to avoid the possibility of proliferation in using them as biological weapons [34] and this has made it even more difficult to widely employ them in analysis as there are delays when procuring them.

1.2.3.1.2.2.2 Restricted Access Material (RAM) Sorbents

RAMs are special selective extraction materials that have been used as extraction SPE sorbents since their introduction around the year 1991, for sample clean-up before instrumental analysis. Their employment allows direct injection of biological matrices into analysing instruments. They are known for their common property of excluding macromolecules like large blood proteins acting as interfering components, thus, leading to reduced matrix effects due to unnecessary protein binding. Mechanistically, these sorbents act by limiting the accessibility to interaction sites (**see figure 1.3**), hence allowing only small molecules like biomarkers and toxicants/drug residues found within whole blood to pass through the pores and reach the hydrophobic, ion-exchange or affinity areas for them to be extracted. Simultaneous isolation, clean-up and pre-concentration of the targeted analytes also result during the extraction process [74][75].

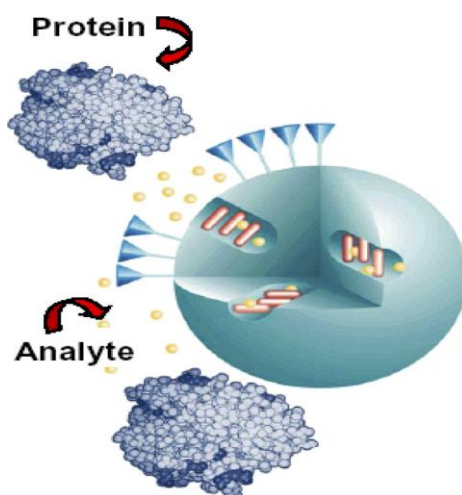


Figure 1.3: Schematic representation of a restricted access material [76]

RAMs can be classified based on protein exclusion mechanism. Macromolecules can be excluded by a physical barrier due to pore diameter or by a chemical diffusion barrier created by protein network at the outer surface of the particle [76][77]. RAMs have been explored but their challenge is that they could not be employed for selective enrichment of analytes of interest because of their limited selectivity.

1.2.3.1.2.2.3 Molecularly Imprinted Polymers (MIPs)

Molecularly Imprinted Polymers (MIPs), are man-made polymeric materials, with tailor-made recognition sites that are complementary in shape, size and functionality to the targeted analytes, during rebinding [68]. They are designed to mimic the interactions found in natural systems such as antigen-antibody or enzyme-substrate in binding targeted analytes. These man-made polymer materials are known for their versatility in recognising both biological and chemical molecules. MIPs became popular and have proved to be better SPE sorbents due to their cheap synthesis, chemical and physical stability, greater affinity and selectivity toward the targeted analytes [78][79].

Chapter Two Molecular Imprinting Technology (MIT)

2.1 Molecularly Imprinted Polymers (MIPs)

Molecular imprinting [79][78] evolved decades ago as an intriguing technology, for designing polymeric functional materials known as molecularly imprinted polymers (MIPs), that possess selective and specific molecular recognition sites for targeted analyte(s) at molecular level. They are also often referred to as synthetic receptors when compared to their natural counterparts; antibody-antigen, enzyme-substrate and the ligand-receptor system since they have been designed to work in a similar fashion of the ‘lock and key’ mechanism that has been described by Emily Fischer, a century ago [80]. MIPs have been reported to be better than their natural counterparts since they are inexpensive to produce, easy to employ, stable and can withstand various harsh conditions when applied in different fields of science [81].

2.2 The History of Molecular Imprinting Technology

Molecular imprinting technology dates back to the early 1930s, when a soviet chemist, Polyakov [82] investigated a series of silica materials for the purpose of chromatographic separations. His work entailed the polymerization of hydrophilic sodium silicate in the presence of additives such as benzene and toluene. The work resulted in the formation of silica gels with predetermined selectivity and preferential binding towards the additives [78][83]. Later in 1949, a lecture was conducted by Pauling, regarding the work by his student, Dickey, who studied the same silica materials that were imprinted with alkyl orange dyes employing the same approach like that of Polyakov. From his experiment, the silica materials were found to possess recognition sites complementary to the size and shape of the dye that was employed as the template molecule. Dickey’s method became widely employed in other studies [84][82]. Around 1950, Curti *et al* employed the imprinted silica materials as stationery phases for the selective chiral separation of enantiomers [82]. More applications of the imprinted silica materials in chromatographic separations continued up to 1960, despite their limited stability and reproducibility. In the early 1970s, more research pertaining to the imprinting technology continued as it was reported by Wulff *et al*, who synthesised the

imprinted organic polymers that selectively recognised the chosen ligands as target molecules. Furthermore, Shea, an organic chemist, extended the research regarding the imprinted organic polymers which led to a better understanding of the imprinting process and the recognition sites within the imprinted materials. Since then, more researchers became interested on the technology of imprinting. In 1981, Mosbach, a German chemist, published a paper describing work on imprinted materials, by then, referred to as, ‘molecularly imprinted adsorbents’ (MIA). The imprinted adsorbents in the form of gels, were found to possess selective binding sites that recognised small molecules. By then, the imprinting approach was termed ‘host-guest polymerization’. It was later in 1993 when the name ‘molecularly imprinted polymers’, abbreviated, ‘MIPs’, became popular and many researchers started to fabricate them in different formats such as monoliths, fibres and beads either at micro or nano level and applied them to a wide range of environments [85][86].

2.3 Types of Imprinting Strategies

Generally, there are three known imprinting strategies in designing the MIPs, namely; the covalent, the non-covalent and the semi covalent, [87]. The covalent strategy by Wulff, relies on forming revisable covalent bond linkages within the template-monomer complex during the imprinting process, [88]. After polymerization in the presence of the second monomer (the cross-linker), the template is extracted from the MIP structure, by cleaving the covalent bonds to expose the recognition sites which are of homogeneous nature. The covalent strategy is seldom applied because of the limited number of the monomers that are suitable for it. The non-covalent strategy, introduced by Mosbach, [88] is based on the formation of the template-monomer complex that relies on temporary non-covalent forces such as hydrogen bonding, ionic, dipole-dipole, hydrophobic and electrostatic interactions that are similar to those of the biological systems. The non-covalent interactions are also the ones responsible for rebinding. In comparison to the covalent strategy, the non-covalent is easier to practice and more versatile because of the large number of monomers that are available to interact with any type of template molecule chosen. However, the non-covalent strategy suffer from non-imprinting of recognition sites, due to the existence of excess monomers left unused in the template-monomer complexation during the imprinting process [89][90]. Recognition sites of heterogeneous nature are usually formed within the MIP structure. Furthermore, the MIPs designed with this strategy have been described to be ‘group-specific’

that is, selective not only to the template but also towards other structurally related compounds. With the aim of combining the advantages of both the covalent and the non-covalent strategies, Whitcombe and co-workers introduced a semi-covalent strategy for molecular imprinting [91][92]. It is covalent, during template-monomer formation and non-covalent, for rebinding the target molecule, hence making this strategy unique. Usually a spacer group between the template and the monomer is introduced in the process of imprinting in order to achieve easy non-covalent rebinding [90]. For example, Whitcombe *et al* reported on semi-covalent strategy for developing an imprinted polymer selective to cholesterol (4-vinyl) phenyl carbonate ester, which formed covalent linkages with the monomer. The carbonate group was then removed by chemical cleavage, thus, creating hydroxyl based-recognition sites for temporary hydrogen bonding during the rebinding of cholesterol [93].

2.4 Typical Synthetic Process for Molecularly Imprinted Polymers (MIPs)

MIP Synthesis typically entails complexing one or more chosen functional monomers with a suitable template, that results in the formation of a pre-polymerization complex. Radical initiated polymerisation in the presence of cross-linking monomers fixes the functional monomers around the template(s), thus, giving the polymer matrix its three-dimensional, rigid structure. Subsequent extraction of the template from the rigid polymer matrix results into an imprinted polymer with controlled functionality, structured pores, defined inner surface area and selective recognition sites for rebinding. Optimization of the template-monomer mole ratio, the template-cross-linker mole ratio and the volume of the porogenic solvents plays a key role in the synthesis of the MIPS [92]. Generally, the main relevant chemical reagents that are usually reacted in the synthesis of MIPs are; the template(s), functional monomer(s), cross-linking monomer(s), initiator and the porogen(s) [83], (**see figure 2.1**).

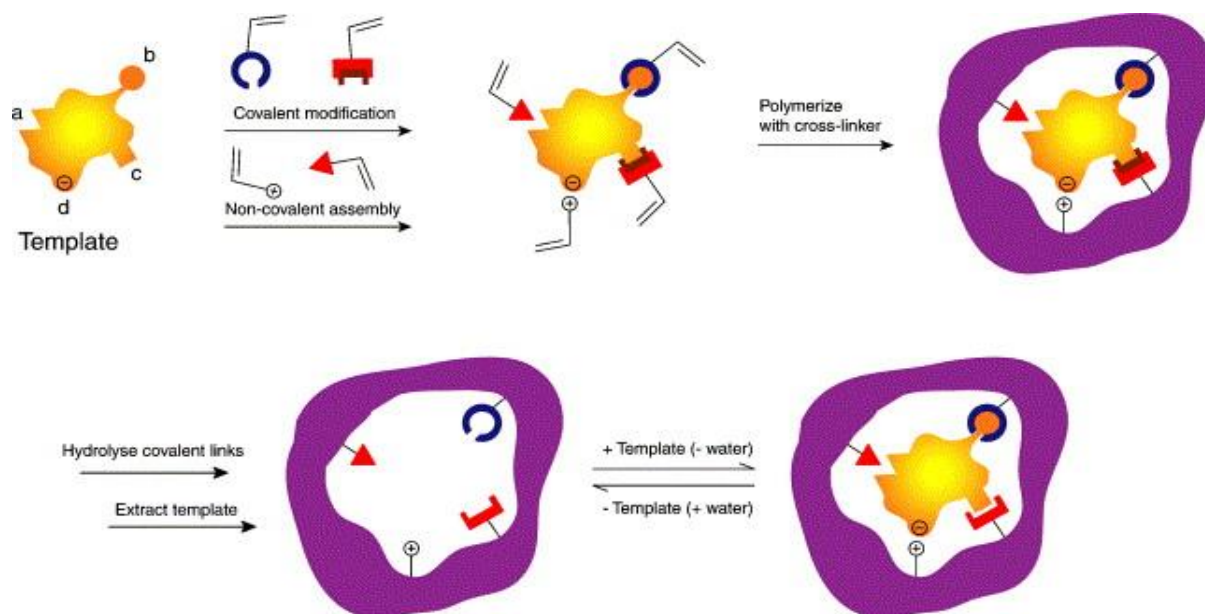


Figure 2.1: A typical imprinting process for MIP synthesis [94]

2.4.1 Template (Print) Molecules

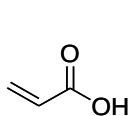
Organic molecules such as amino acids, drugs, carbohydrates, proteins, hormones, pesticides and metal complexes are usually employed as templates depending on the targeted analyte. These templates can either be of low or high molecular weight, but most significantly possessing functionality and complementary properties to allow for controlled recognition [95][96]. The presence of functional groups around these templates allow for direct self-organisation with functional monomer groups to attain a self-assembled pre-polymerized complex. Virtually any species can be targeted, hence any species can be a template [97][95].

2.4.2 Functional Monomers

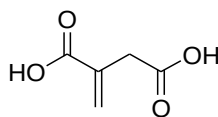
A series of functional monomers that are basic, acidic or neutral, (see **figure 2.2**) with active functional groups are known to play a significant role in the imprinting process [91][98]. Formation of the pre-polymerization complex during imprinting is guided by the best choice of the functional monomer(s) with reactive functional groups that are complementary to the reactive functional groups of the template molecules [98][86]. Furthermore, functional monomers are chosen based on hydrophilic and hydrophobic character, good bio-compatibility, better chemical and mechanical stability, and resistance toward microbial and enzymatic attacks. Most of the functional monomers reported herein are suited to be

employed in non-covalent imprinting because of their functionality to interact with the template molecules via weak forces of interaction such as H-bonding.

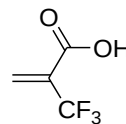
a. acidic functional monomers



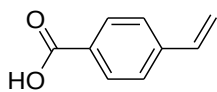
acrylic acid



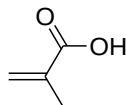
itaconic acid



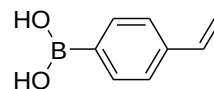
2-(trifluoromethyl)acrylic acid



p-vinylbenzoic acid

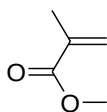


methacrylic acid

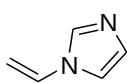


4-vinylbenzene boronic acid

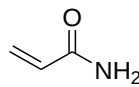
b. neutral functional monomers



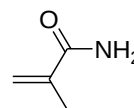
methyl methacrylate



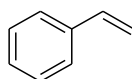
1-vinylimidazole



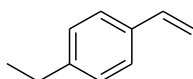
acrylamide



methacrylamide



styrene

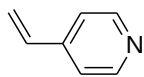


1-ethyl-4-vinylbenzene

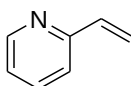


acrylonitrile

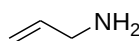
c. basic functional monomers



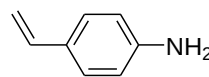
4-vinylpyridine



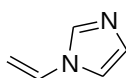
2-vinylpyridine



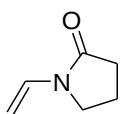
prop-2-en-1-amine



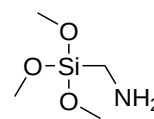
4-vinylaniline



1-vinylimidazole



N-vinyl-2-pyrrolidone



(trimethoxysilyl)methanamine

Figure 2.2: Examples of functional monomers for molecular imprinting

During pre-polymerization, functional monomers are commonly mixed with the template molecule in an optimized mole ratio of ($\geq 1:4$), to promote the self-organization step. Methacrylic acid (MAA) is by far the most widely available and used functional monomer and is suited for temporary bonding interactions with a wide range of template molecules. The choice of the functional monomers does not only depend on the ability to react with the template, but also on the availability and how cost effective they are. Due to some of these factors, more functional monomers are synthesised in relation to the MIP type and the template molecule. On the other hand, computational chemistry also known as ‘MIP dialling’ has also been employed as a form of selecting the best functional monomer at optimized levels, by comparing with other potential monomers [99].

2.4.3 Cross-linkers (Cross-Linking Monomers)

Cross-linking monomers [100] play a key role in successfully fixing the functional monomers around the template molecule, while promoting the formation of a three-dimensional, highly cross-linked polymer matrix with structured pores. They are known to define the morphology of the polymer matrix (MIP), whether it is in a form of a gel, a macro-porous or a monolith. Overall, they contribute to mechanical stability of the imprinted polymer [83][100]. A mole ratio of template to cross-linker of ($>1:4$) is commonly employed to obtain a highly stable cross-linked polymer matrix. Choosing the best cross-linking monomers is key as they contribute to the loading capacity and the selectivity of the MIP during rebinding experiments. Also, the cross-linker must have complementary functional groups such as multiple bonds to react with the functional monomer multiple bonds in the template-functional monomer complex to propagate further polymerisation. Consequently, the achieved cross-linking and morphology of the MIP will have an impact on the mass transfer, especially during template removal. This has been observed where large molecules such as proteins have been employed as print molecules, hence the highly cross-linked the MIP is, the slower the movement of the print molecule during removal and rebinding experiments. Ethyleneglycol dimethacrylate (EGDMA) [101] is the most available and employed cross-linker due to its versatility in polymerization. Some cross-linkers such as trimethylpropane trimethacrylate (TRIM) are known to be better candidates for cross-linking but also have the ability to act as functional monomers because of their functionalities (see **figure 2.3**) [101].

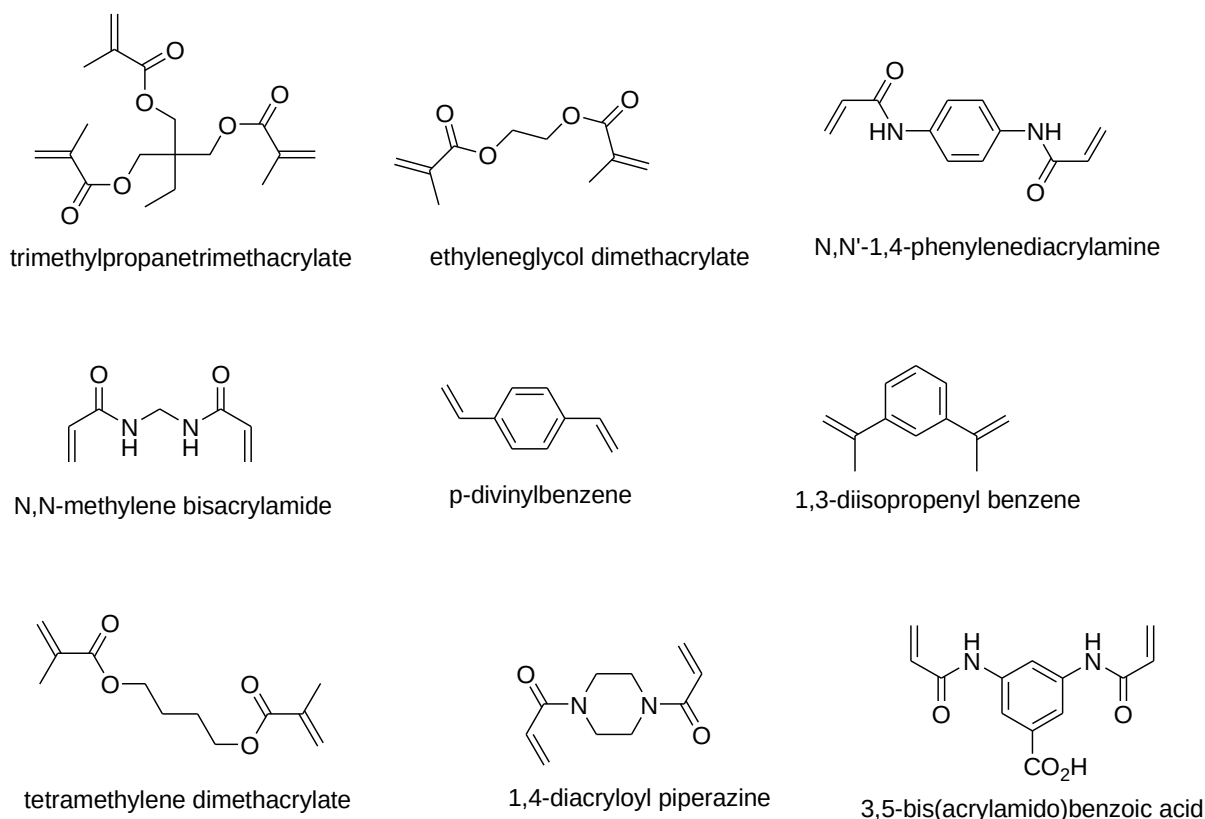


Figure 2.3: Examples of cross-linking monomers for molecular imprinting

2.4.4 Porogens

Various porogenic solvents (organic, inorganic or aqueous based) [102] have been employed in MIP synthesis for the purpose of dissolving monomers and other reagents during the imprinting process. The solvents play a significant role in defining the MIP porous structure, morphology and the surface area. Choosing a suitable solvent as a porogen depends on the reagents solubility and there are other aspects regarding its chemical and physical properties such as polarity, viscosity and the refractive indexes that are considered for better formation of the MIP particles. Most significantly, the porogen employed must not interfere with the interaction between the template and the functional monomer during pre-polymerization complex formation. For ionic interactions, apolar, non protic solvents such as benzene and toluene are employed in non-covalent imprinting. Highly polar solvents are most suited to be employed if there are more hydrophobic interactions during the imprinting process, whereas hydrogen bonding between the template and the functional monomer normally requires solvents that are poor in forming hydrogen bonds [103][104]. Sometimes, there is a need to

perform optimization by mixing solvents at appropriate ratios to vary the solubility, polarity and how the reagents would interact within the polymer solution.

2.4.5 Initiators

Polymerization in MIP synthesis is generally governed by the presence of reactive species known as radicals, which are spontaneously generated during the imprinting process, for the monomers to react. Consequently, initiators are radical sources that reactively induce monomer radical species for free radical polymerization to be propagated [105]. Radical formation is usually initiated by thermal induction or UV light irradiation. Azobisisobutyronitrile (AIBN) [106][69] is a widely employed initiator for kick-starting polymerization because of its wide decomposition temperature range and dissolution in a wide range of porogens. Other initiators (**see figure 2.4**) that readily undergo photolysis or thermolysis prior to the propagation step during polymerization have also been employed [106].

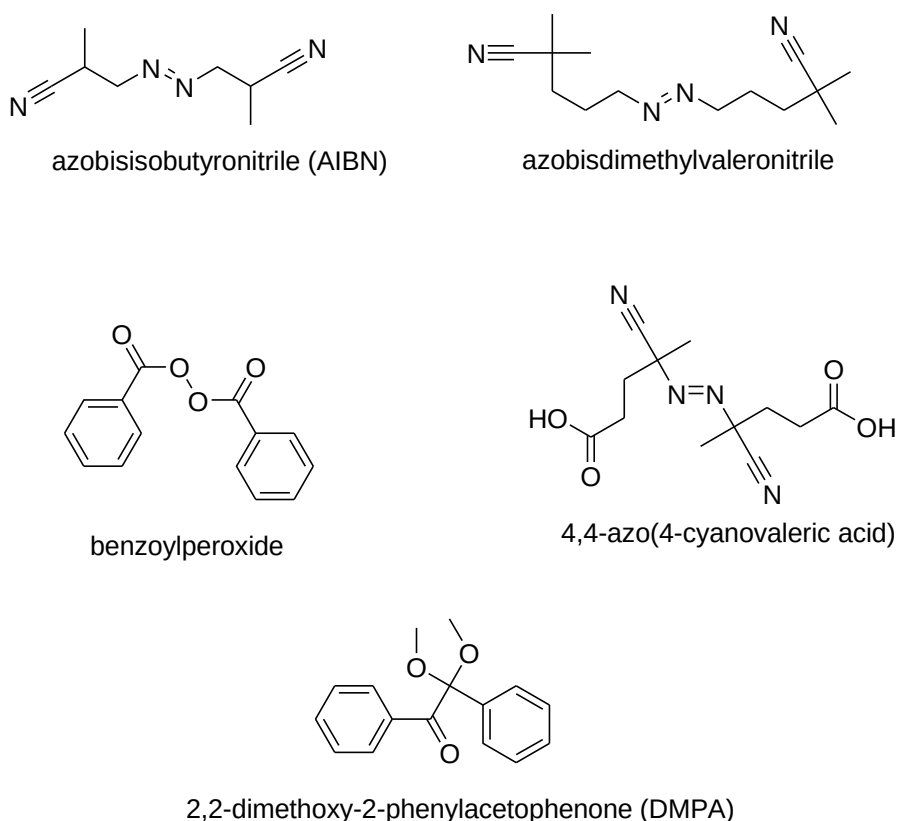


Figure 2.4: Examples of free radical initiators for molecular imprinting

2.5 Synthetic Fabrication Methods for MIPs

Molecularly imprinted polymers have been synthesized in various formats such as monoliths [60][107], micro/nano spheres [84][95] and hydrogels [108][109], depending on the synthetic or fabrication method employed. Suitable conditions for each method have been described with careful and optimal selection of imprinting reagents to fit the method employed. Examples of these fabrication methods include bulk [110][83], precipitation [84][111], suspension [112][83], In-situ [113] and multistep-swelling [114] polymerization as well as electrospinning [115].

2.5.1 Bulk Polymerization

Bulk Polymerization [83] also known as solution polymerization is the simplest method of synthesising MIPs. It requires less skills and employ cheap apparatus [101]. The mixing of all selected imprinting reagents (template molecule, monomer, cross-linker and initiator) all at once in a porogenic solvent defines the concept of bulk polymerization. The product is usually obtained in a form of a polymer lump known as a monolith [116]. The monolith is usually ground and sieved to achieve irregularly shaped particles of varying dimensions, in the form of powder. The grinding and sieving are known to be time consuming, and there is a great possibility of attaining a small polymer yield. Bulk polymerization has been employed for creating MIPs in which the template was of low molecular weight than high molecular weight, as template removal due to mass transfer proved to be a challenge and worsened with an increase in templates with high molecular weight. Also, the recognition sites for MIP polymer particles prepared via bulk polymerisation were found to be deeper in the structure of the particles, thus, making it difficult to react during rebinding [117][118]. Because of the drawbacks, more research on the synthesis or fabrication methods aiming at producing MIP particles of uniform shapes, recognition sites that are easily accessible and are closer to the surface are highly sought after. Such methods include precipitation, suspension, emulsion, multi-step swelling polymerization and electrospinning. These methods have the capability to produce nano-sized polymer particles.

2.5.2 Precipitation Polymerization

Precipitation polymerization is one of the imprinting methods that is usually employed for fabricating more regular and spherical monodispersed, particles at nano or micro level [111]. This is an advantageous method that produces polymer particles that are ready to be applied, due to the existing recognition sites closer to the surface in nano-sized particles. Template removal for this method is performed easily for rebinding purposes as the template molecules are easily accessible [119]. Principally, a homogeneous mixture with a suitable porogenic solvent containing monomers, template molecule(s) and the initiator is subjected to the polymerization conditions. The polymer particles then precipitate out of the polymerization solution, get isolated, dried and ready for application having removed the template molecules [110]. Precipitation polymerization is similar to bulk polymerization except that larger quantities of the porogen are employed (2-10 times higher) to promote polymer particle precipitation [120]. Additionally, no grinding and sieving of polymer particles is performed, hence precipitation is less labour intensive, less time consuming, more efficient and reproducible than the common bulk polymerization [121].

2.5.3 Suspension Polymerization

This is a type of heterogeneous polymerization which employs two non-immiscible phases during the synthetic process, with the same kinetic conditions as in bulk polymerization [114][122]. The two phases include a continuous aqueous phase and an organic phase with dispersion abilities. Synthetically, the organic phase with all the reagents is dispersed into the aqueous phase as liquid droplets, each of which behaves as a chemical reactor in which an independent polymerization reaction is kick-started in the presence of a soluble initiator, resulting in solid polymer particles in the form of beads. The sizes of the polymer beads are controlled by employing stabilizers such as poly vinyl-alcohol or salts such as magnesium or calcium carbonates. The salts improve the dispersion viscosity in the aqueous phase. The resulting polymer beads with uniform size and morphological existence would be ready for use without any grinding and sieving [123]. The drawback about this method is that it is costly, need skilled labour and also, the extra additives such as stabilizers which might alter the recognition sites by being absorbed to the surface of the polymer if left unremoved completely during washing the polymer particles are a challenge [124].

2.5.4 In-Situ Polymerization

In-situ polymerization [79][113] is an approach of fabricating MIPs of chromatographic applicability where the imprinting process is carried out in a suitable capillary or column tubes, without the need to grind, sieve or even pack it in a column. As in bulk polymerization, a similar monolithic type of polymer structure moulded into a shape that resembles the capillary or the column tube results. The idea behind this polymerization method is to create in simple, cheap and rapid manner, a high affinity and selective chromatographic column packing, usually employed in HPLC systems, for enantiomeric separations [125][126]. Amut *et al* reported on successful in-situ polymerization to produce a chiral MIP for the separation of racemic amlodipine, employing s-(-)- amlodipine as a print molecule [125]. On the other hand, Luo and co-workers demonstrated the importance of this method by creating a clenbuterol (CLB) molecularly imprinted monolithic stationary phase, employing in-situ polymerization and the MIP was applied for the pre-concentration of CLB in pig liver samples [116].

2.5.5 Multistep-Swelling Polymerization

Multi-step or two-step swelling polymerization [95] is another method suited for fabricating MIP based on regular and uniform particles that are monodispersed in size and shape. The particles obtained in this type of polymerization are also of great importance in the field of chromatographic separation as described by Hosoya *et al* [84]. It involves a few steps such as the initial synthesis of seed polymer beads/particles (polystyrene) by emulsion procedure in the presence of an emulsion activating solvent (for example, dibutyl phthalate), usually containing an appropriate stabilizer (for example, polyvinyl alcohol) and an initiator. For the second emulsion polymerization step, the seed particles will be equilibrated with a solution containing polymeric reagents (print molecule, monomer and a cross-linker) that will react to form a polymer matrix around the core [118]. Masci *et al* prepared and compared MAA and acrylamide- based clenbuterol-imprinted microbeads employing two-step swelling polymerization method and used them for the chromatographic separation of clenbuterol from other β -adrenergic molecules [127]. This method is more advantageous as the size and the number of polymer particles can be controlled but has shortcomings that include decreased selectivity and creation of non-specific binding sites with increased surface area as

a result of the aqueous suspensions used [128]. It is also labour intensive and time consuming even though the particles produced are not of irregular shapes, but of uniform design.

2.5.6 Electrospinning

Electrospinning is a technique for fabricating nanofiber structures by subjecting a spinnable polymer solution or melt to a high voltage [115][129]. Typically, the polymer solution or melt is allowed to flow through a capillary tube with a conducting tip (the spinneret) until the surface tension of an about to be ejected drop of the solution or melt at the tip of the capillary is overcome and stretched into fibres by an induced surface electrical charge that pulls and stretches a generated Taylor cone polymer jet formed at the spinneret to the second oppositely charged terminal referred to as the collector.

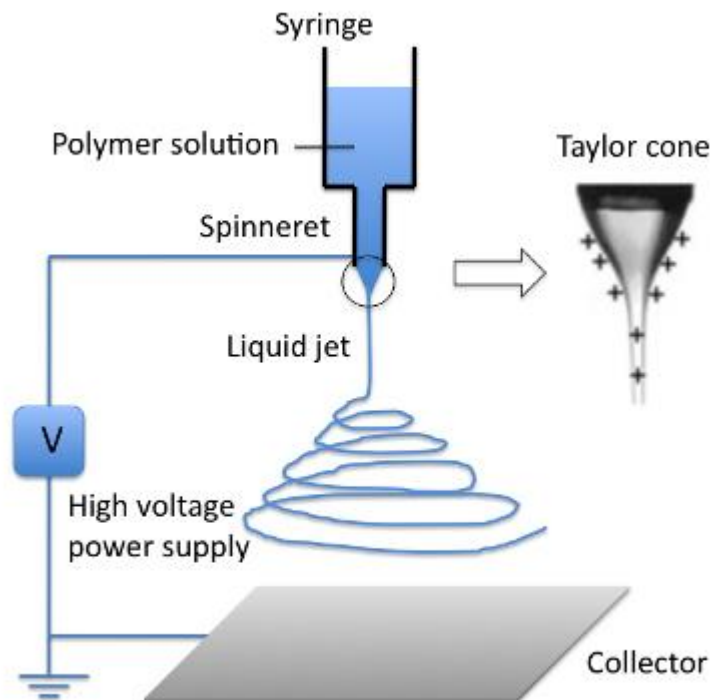


Figure 2.5: A typical electrospinning setup [130]

Optimizing ambient parameters (temperature, humidity), solution parameters (concentration, molecular weight and viscosity) and processing parameters (voltage, collectors and the distance between the tip and the collector), result in fibers of different morphology and sizes including nanofibers [130]. Nanofibers are sensitive materials owing to their one dimension with high surface area to volume ratio. It is these traits and their ability to be fabricated into

fiber mats that have led to their popularity and application in various fields of science such as conducting nanofiber mats (optical sensor, electrical circuit, displays and lighting device) developments,[129] tissue engineering[131], wound dressing, [132] and sample preparation especially as fiber based SPE sorbents[133]. Even though fiber based SPE sorbents boast unparalleled sensitivity [133], they are less selective. To deal with this challenge, molecular imprinting technology with its high selectivity has been combined with electrospinning technology with its high sensitivity to produce smart, functional nanofiber materials that are templated known as molecularly imprinted electrospun nanofibers [134].

2.6 Polymer Characterization

Morphological, physical and chemical characterization of the polymer is important to ensure a good match between the applicability and the features of the prepared polymer particles. Characterization is also important for monitoring the progress of the imprinting process, especially the monomer-template interaction [135].

2.6.1 Physical Characterization

Fourier-transform infrared spectroscopy (FTIR) [136][137] is usually employed in the MIP synthesis, mostly to assess the nature of recognition sites in molecularly imprinted polymer particles and to examine the extent of complex formation between the functional monomer and the print molecule during the imprinting process. Ideally, the presence or absence of functional groups within the MIP before and after the template molecule removal is usually revealed by the IR-spectra obtained, giving rise to specific IR peaks or lack of them due to the existence or inexistence of some functional groups, respectively [138]. Also FTIR is usually employed to confirm whether polymerization has occurred or not, by comparing the IR spectra of starting materials (template molecule and monomers) to that of the pre-polymerization complex solution or the finished polymer product [139]. Usually, the peaks found in the spectra of the starting materials, the products [MIP with and without the template and the non-imprinted polymer (NIP which is the control)], exhibit shifting in terms of the intensity and the wavenumber. Towards the lower wavenumbers, the spectra for all reactants and products exhibit similar fingerprint region indicating that the products were formed from the reactants since they possess similar backbone as supported by the fingerprint region in the spectra. Zhu *et al* demonstrated the use of FTIR as a characterization technique for methanol

gas sensor-based molecularly imprinted polymer and they obtained spectra for the polymers which were compared to those of the reagents. The spectra confirmed the MIP formation, and differentiated between the MIP with and without the methanol as was supported by the presence and absence of some peaks in the spectra of the MIP with and without the methanol template, respectively [140].

2.6.2 Morphological Characterization

Fabrication of MIPs have been reported to result in various formats such as irregular particles, solid beads and nano-spheres which are known to differ in terms of their shape, texture, specific pore volumes, pore sizes, pore size distributions, surface areas and overall morphology [135]. The difference normally emanates from the MIP fabrication or synthesis method employed or from the mechanical efforts of grinding especially after bulk polymerization. The scanning electron microscopy (SEM) is commonly used to examine the structure and the surface morphology of MIPs [141][142]. SEM images are usually obtained for the synthesized polymer particles. Their images normally differ by a very small difference since they are of the same polymerization reagents. However, the sponginess, shape and the surface texture is often distinctively visible from the images, with possible particle size calculation. For example, nano-spheres are characterized to have a rounded shape than the irregularly shaped particles of bulk method. Atomic force microscopy (AFM) [143][144] is another kind of microscopy with an added advantage of depicting the depth of the polymer particles obtained. Surface area and pore volume of polymers are the other features that are key for a good sorbent material. Brunauer, Emmet and teller (BET) and the Barrett, Joyner and Halenda (BJH) are the methods that are usually employed for measurements of surface area and pore volume, respectively [145].

2.6.3 Chemical Characterization

Chemical characterization is key for assessing the binding and selective capability of the recognition sites within the prepared polymers. This is achieved through rebinding experiments, either by batch or column based approach [146][147].

Batch rebinding studies provide the first insight into the binding properties of the prepared MIP powder. For example, in batch experiments, a known quantity of MIP sorbent is added

to a reaction vial containing the solution of the targeted molecule of a known concentration (initial concentration, C_i). The whole mixture is then stirred and left to equilibrate to allow for the absorption of the targeted material by the employed MIP. Usually the mixture is centrifuged or filtered to remove the MIP sorbent residue from the supernatant or the filtrate, respectively. The concentration of the supernatant/filtrate (final concentration C_f) is then determined employing an appropriate analytical instrument, for example using ultraviolet visible-diode array detector spectrophotometer (UV-Vis-DAAD) for analytes with absorbing chromophores such as hemoglobin in this thesis. The percentage of the targeted analyte removed from the supernatant/ filtrate or absorbed into the MIP sorbent is then calculated employing **equation 1**. The same experiments are usually performed using the non-imprinted polymer (NIP) for comparison and control purposes. The NIPs possess no recognition sites as no template molecules are included during their synthesis. Consequently, MIPs are always expected to have higher removal percentages for targeted analytes as they possess recognition sites and NIPs do not. In addition to the % removed, the effectiveness of the MIP material produced, in selectively targeting the intended species is often investigated. A parameter known as the imprinting factor (**IF**), is the one associated with the effectiveness of the synthesised MIP materials in selectively extracting the targeted analytes only. It is calculated based on **equation 3** through **equation 2**. **IF** is directly proportional to the distribution coefficient, K_d .

$$\% \text{ Removal Efficiency} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \% \quad \text{equation 1}$$

The selectivity imprinting factor (**IF**) is typically calculated to investigate by what factor would the targeted molecules be selectively extracted by the MIP than the competing species. The imprinting factor, largely calculated by employing **equation 3**, depends on the distribution coefficient, (K_d) calculated for each bound molecule, employing **equations 2**.

$$K_d = \frac{C_i - C_f}{C_i} \times \frac{V}{m} \quad \text{equation 2}$$

$$IF = \frac{K_d \text{ print/template molecule}}{K_d \text{ Competing molecule}} \quad \text{equation 3}$$

Where K_d is the distribution coefficient, C_i is the initial concentration of the targeted analyte, C_f is the final concentration of the targeted analyte found in the supernatant/filtrate, V , is the total volume of the solution mixture and m is the optimal quantity of the MIP or NIP employed in rebinding experiments.

Other methods have been employed to characterize binding sites especially in natural systems such as antibody-antigen and enzyme-substrate. According to the characterization methods, imprinting can yield distribution of binding sites of varying affinity and selectivity that are of homogeneous or heterogeneous type[148]. Several graphical methods such as scatchard plots have been employed to fit binding isotherms to models such as that of Langmuir (LI). The isotherms allow characterization of binding sites in order to be able to understand their distribution within the polymer matrix as homogeneous or heterogeneous [149]. Generally, MIPs have been proven to be characterized by a high degree of varying binding sites, (heterogeneous) when compared to the homogeneous ones found in natural systems [150]. MIPs with heterogeneous binding sites have been employed mostly in separation techniques [151]. Additional studies to provide more accurate description on binding behaviour of MIPs, especially those of non-covalent character, have been performed on several MIP structures and have led to the binding isotherm models such as Freundlich (FI) and Langmuir-Freundlich (L-FI) combined [152].

2.7 Applications of Molecularly Imprinted Polymers (MIPs)

MIPs have been employed as synthetic receptors in chromatographic separations [78], enzymatic catalysis [153][119], selective solid-phase extraction[154][155], sensor technology [99] and the drug delivery [123][125]. For example, they have been employed for the affinity-based separation with subsequent pre-concentration of enantiomeric chemicals[156][157]. Many articles outlining the applicability of MIPs in areas of food security [158], environmental monitoring [159][112], molecular diagnostics [160][92][85] and forensic sciences [161] are still being published. Currently, MIP research is focused on developing uniform MIP materials that possess recognition sites that are easier to

access[130]. Examples of such materials are the MIP nanofibers [162], the MIP nano particles [163] and the MIP quantum dots [164].

In this thesis, a synthesised hemoglobin imprinted polymer (Hb-MIP), in the form of a powder was employed for the removal of interfering hemoglobin from whole blood in a selective and efficient manner as a form of sample clean-up procedure prior to the instrumental analysis of biomarkers. This was performed to deal with the challenges faced by the current clean-up procedures in whole blood analysis for biomarkers such as centrifugation which is non-selective, and often end up removing the cells that contain the targeted analytes (biomarkers) during clean-up intended for accurate analysis. The objective was to come up with a novel, selective, sensitive and cheap material that can eventually replace or be employed in combination with conventional methods such as centrifugation for the removal of interferents associated with inaccurate and imprecise analysis of biomarkers and toxicants residues during molecular diagnosis and toxicological assaying of whole blood, respectively. All the reagents employed for the synthesis of the Hb-MIP were chosen on the basis of their availability. Furthermore, trial and error synthetic method was applied without any optimization that was performed during synthesis.

Chapter Three Experimental

3.1 Preparation of an Hb-MIP for the selective removal of interfering hemoglobin from whole blood prior to instrumental analysis

The main aim of this thesis was to prepare a novel hemoglobin imprinted polymer (Hb-MIP) in a powdered form, to be employed as a sorbent in the selective removal of interfering hemoglobin (the red part) from whole blood sample, as a form of sample clean-up prior to its instrumental analysis for biomarkers. The Hb-MIP powder was prepared by a bulk, free radical polymerization method that was thermally initiated. The extent of imprinting and the overall morphology of the Hb-MIP powder particles were characterized by employing FT-IR and FE-SEM, respectively. Optimized SPE rebinding batch experiments were employed to demonstrate the binding, selectivity and efficiency of the prepared Hb-MIP powder in selectively removing the interfering hemoglobin from whole blood instrumental assaying for biomarkers.

3.2 Chemicals and Materials Used

Reagents used were; Ammonium dihydrogen orthophosphate (99%), Sodium chloride (99%), Ammonium phosphate dibasic (98%), Orthophosphoric acid (98%), Acetic acid (99.8%), Acetonitrile (99.9%), Acetone (99.5%), Ethanol (99.9%), Methacrylic acid (MAA) (99%), α , α' -azoisobutyronitrile (AIBN) (98%), Ethylene glycol dimethacrylate (EGDMA) (98%), all of analytical grade and a 0.45 μ m pore sized ashless whatman filter papers (Circles 45 mm) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Fresh human whole blood was acquired from Sekgoma hospital (Serowe, Botswana), supported with an ethical letter from the Botswana international university of science and technology. Commercially available green leaves (Spinach) were purchased from Spar (Palapye, Botswana).

3.3 Analytical Instruments and Equipment Employed

Evolution 201 UV-Vis spectrophotometer employed for absorbance measurements for all hemoglobin and chlorophyll solutions in rebinding experiments, Scientific drying oven (TTM-J4) employed for drying the prepared polymer powders in hot air and a Nicolet iS10

FTIR spectrophotometer employed to acquire the FTIR spectra of the starting materials and the prepared products, were all purchased from Thermo Fisher Scientific (Johannesburg, South Africa). A field emission scanning electron microscope (FE-SEM) JSM-7100F, was purchased from JEOL (UK) Ltd (Welwyn Garden City, Hertfordshire) and was employed to obtain highly resolved SEM images for the prepared polymer particles to assess their size and structural morphology. Mortar & pestle and all glassware employed were purchased from Pyrex companies (Frankfurt, Germany). pH meter to determine the pH of all solutions, was supplied by Crison Laboratory (Liverpool, England). Benchmark hot plate for thermal polymerization was purchased from Benchmark Scientific (Sayreville, NJ, USA). Boeco GP Series micropipettes were from BOECO (Berlin, Germany). Centrifuge VWR (24/16) for separating the MIP solid particles from the supernatant, was purchased from VWR Catalyst (Philadelphia, PA, USA).

3.4 Whole blood Preparation to Acquire Hemoglobin as the Template

The acquisition of pure hemoglobin as the template in this work was a challenge due to high cost, ordering delays and critical procuring regulations of biological chemicals for research purposes. To address these challenges, fresh human whole blood was acquired from a certified medical laboratory in Sekgoma hospital (Serowe, Botswana). The blood sample was collected in 5 mL EDTA-coated vacutainer. The EDTA prevents the blood sample from coagulation and denaturing. Additionally, it allows for easy separation of the RBC layer (rich in hemoglobin) from the top plasma layer in the vacutainer. The vacutainer was kept in a cold environment (4 °C) for further sample preparation. 0.05 mL of the sampled fresh blood was centrifuged for 15 min at 6000 rpm. The top plasma layer was discarded leaving behind a rich hemoglobin RBC layer. To acquire hemoglobin as the template, a portion of the RBC layer was treated with distilled water for cell osmotic shock, thus, exposing the hemoglobin out of the cells. The mixture was then centrifuged, and the supernatant containing hemoglobin, free from cell debris was collected, then treated with phosphate buffered saline (PBS) and kept in a refrigerated environment (4 °C).

3.5 Preparation of Hemoglobin Imprinted Polymer

The Hb-MIP powder was prepared as follows; 0.01 mL of freshly prepared hemoglobin supernatant, 5 mmol of MAA, 30 mmol of EGDMA, 1 mmol of AIBN and 25 mL phosphate buffered saline (PBS), were continuously stirred for 30 min in a 250 mL round-bottom flask at room temperature. After degassing, the flask was sealed then polymerisation was initiated at 37°C. After 48 h of polymerization, the monolith was crushed and treated with a ((1:1 v/v) acetonitrile (99.9%)/acetone (99.5%)) solvent mixture, to remove unreacted reagents. The same procedure was followed for the synthesis of the control polymer, Non imprinted polymer (NIP), but in the absence of hemoglobin as the template. Successful template removal was performed by refluxing for a total of 18 h at 2 h intervals, employing a fresh solvent mixture containing ((1:1 v/v) acetonitrile (99.9%)/acetic acid (99.8%)) for each for all the 9 cycles. The obtained washed MIP polymer particles were then dried in hot air oven at 35°C for 5 h. To confirm template removal, the absorbance for each filtrate was measured, employing a UV-Vis spectrophotometer until no further change in the hemoglobin absorbance was observed with further subsequent washings. A plot of all the absorbance values against the number of cycles was constructed.

3.6 Characterization of Hb-MIP Powder and NIP

3.6.1 FTIR Spectra of Washed Hb-MIP, Unwashed Hb-MIP, NIP and the Starting Materials

FTIR spectra of the washed Hb-MIP, unwashed Hb-MIP, NIP, hemoglobin, MAA and EGDMA were obtained employing a Nicolet iS10 Thermo Scientific FTIR, and recorded in the wavenumber range 500-4000 cm^{-1} , to confirm complete polymerization as well as to assess the template removal.

3.6.2 SEM Images of the Prepared Hb-MIP Powder Particles

To evaluate the morphology and particle size of the prepared Hb-MIP powder particles, a field emission scanning electron microscope (FE-SEM) JSM-7100F, was employed. Finely powdered carbon-coated Hb-MIP particles were supported on a sample holder (1 cm tall), that was inserted into the SEM system for 3 h before SEM image acquisition.

3.7 Batch Rebinding Experiments

Optimization studies were carried out via SPE batch rebinding experiments that were performed in triplicates employing the washed Hb-MIP, the NIP and commercially available graphitized carbon black (GCB) powder in 10 mL graduated tubes. 0.01 % (v/v) of hemoglobin stock solution prepared from the fresh hemoglobin supernatant (RBC layer), and phosphate buffered saline (PBS) was employed for all the batch experiments.

3.7.1 Optimization of MIP Powder for Maximum Hemoglobin Removal

Increasing quantities of the washed Hb-MIP powder were mixed with 5 mL of 0.01 % (v/v) buffered hemoglobin solution in 10 mL graduated tubes at 20 mg increments. Each mixture was left to equilibrate for 24 h, filtered, then the filtrate was analysed in triplicates by employing a UV-Vis spectrophotometer to obtain the absorbance of hemoglobin. This was repeated until there was a constant absorbance obtained with further 20 mg increments. A plot of absorbance (concentration) measured against the increasing quantities of the Hb-MIP powder was constructed.

3.7.2 Optimization of Time for Maximum Hemoglobin Removal

With optimized MIP quantity in (3.7.1), optimal time needed for Hb-MIP to extract maximum hemoglobin from standard hemoglobin solutions was investigated. Optimized quantities of the washed Hb-MIP powder were mixed with 5 mL of 0.01 % (v/v) buffered hemoglobin solution in separate 10 mL graduated tubes. Each mixture was left to equilibrate for a specific time at 2 min intervals starting from time zero. Hemoglobin absorbance was obtained for each filtrate from the equilibrated mixtures until constant absorbance was recorded. A plot of absorbance (concentration) measured against the increasing time at 2 min intervals was constructed.

3.7.3 Percentage Removal Efficiencies

To assess the binding capability of the prepared Hb-MIP, the quantity of the target analyte (hemoglobin) absorbed by the MIP from a standard solution of a known hemoglobin concentration (absorbance) was calculated as a percentage by subtraction of the concentration (absorbance) obtained after application of the MIP at optimal conditions from the initial

concentration (absorbance) before the MIP application. The **equation 1** below was employed to calculate the % removal efficiencies for the prepared MIP, NIP and GCB. The experiments were performed in triplicates.

$$\% \text{ Removal Efficiency} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \% \quad \text{equation 1}$$

3.7.4 Percentage Removal Efficiencies for Selectivity

Under optimal conditions, the prepared Hb-MIP sorbent and a commercially available GCB powder sorbent, were compared in selectively removing only Hb from prepared solutions. This was achieved by mixing 5 mL of 0.01 % (v/v) buffered hemoglobin solution with optimal quantity of Hb-MIP sorbent and GCB sorbent in separate 10 mL graduated tubes for optimal time. This was repeated with 5 mL of 0.01 % (v/v) buffered chlorophyll solution which was chosen to act as a competing species to hemoglobin. The procedures were performed in triplicates and percentage removal efficiencies were calculated from the absorbance values obtained. Corresponding comparative bar graphs were also constructed for further elucidation of selectivity.

3.7.5 Hb-MIP Application to Real Whole Blood Sample

Under optimal conditions, the effectiveness of the prepared Hb-MIP in removing interfering hemoglobin, was assessed by applying the Hb-MIP powder to a fresh human whole blood sample, that was diluted by a factor of two with phosphate buffered saline in 10 mL graduated tube. After Hb-MIP application at optimal conditions, the absorbance of the supernatant was determined in triplicates employing a UV-Vis-DAAD spectrophotometer. Absorption spectra of fresh human whole blood before and after application of the prepared Hb-MIP powder were obtained. Furthermore, the % removal efficiency of the MIP was calculated using **equation 1**.

Chapter Four Results and Discussion

4.1 Formation of Hb-MIP and Template Removal

Procedure

After polymerization as described in section 3.5, a brown monolith (Hb-MIP) obtained was ground to a brown powder (**see figure 4.1a**). The brown powder was refluxed to wash off the hemoglobin as the template resulting in an almost white powder (**see figure 4.1b**). This indicated that the Hb template that is known to be reddish brown and as such responsible for the resultant reddish brown monolith/powder was removed from the polymer structure resulting in an off-white powder without the reddish brown Hb template.

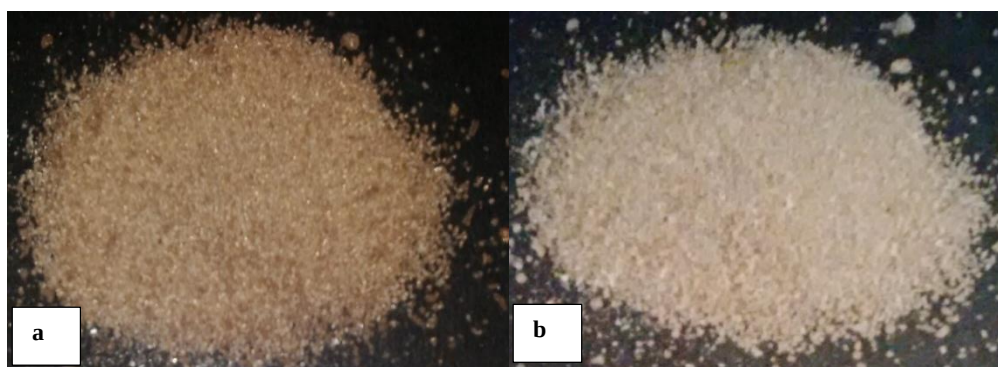


Figure 4.1: Hb-MIP before hemoglobin removal (a) and after hemoglobin removal (b)

The reflux washing procedure was repeated until there was no further hemoglobin removal which was marked by no further change in the recorded absorbance despite further refluxing with fresh solvent. In the plot of hemoglobin absorbance versus the number of washing cycles (**figure 4.2**), this is marked by a flat, horizontal line from the 5th cycle marking the constancy in the absorbance at the lowest of 0.107 a.u. It should be noted that, the constancy did not mean that the entire hemoglobin template was totally removed by the washing procedure at the point that the plot started to flatten, but meant that the employed template removal procedure could only go that far in removing the Hb template from the prepared MIP powder. To confirm complete template removal, further work is needed to be performed in investigating exhaustive template removal which is known to improve efficiency and reduce template bleeding [93].

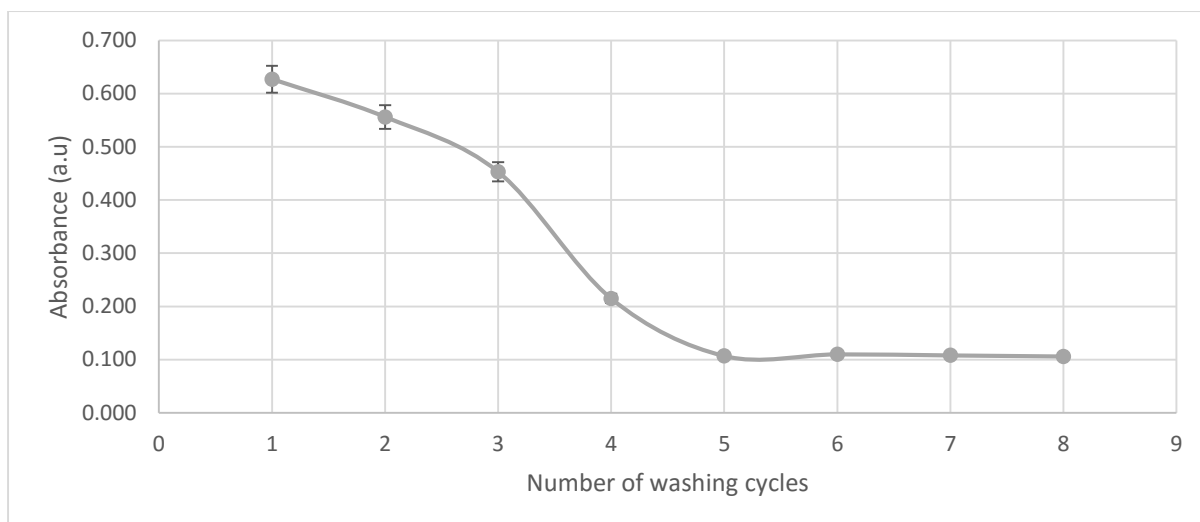


Figure 4.2: An absorbance plot to confirm hemoglobin template removal

4.2 Characterization of Hb-MIP Powder

4.2.1 Confirmation of the Formation of Hb-MIP Powder and the Removal of Hemoglobin Template from Hb-MIP by FTIR

The FTIR spectra of the starting materials; hemoglobin molecule, MAA and EGDMA, (see **figure 4.3, 4.4 and 4.5, respectively**) were distinctively different from that of the finished product (Hb-MIP) (see **figure 4.6**) confirming that a new compound (product) different from the starting materials was formed. For example, a peak in the hemoglobin spectrum at 3346 cm^{-1} indicating the N-H bond for hemoglobin amide linkage, (see **figure 4.3**), and another peak at 1691 cm^{-1} in the MAA spectrum, indicating the carbonyl stretching bond of the carboxylic group (see **figure 4.4**) were found to be absent as shown in the spectrum of the finished product (Hb-MIP) (see **figure 4.6**). The differences confirmed that polymerization occurred.

Comparing the spectra of the unwashed Hb-MIP and the washed Hb-MIP, discernible peaks at 1636 cm^{-1} , 1320 cm^{-1} and 1295 cm^{-1} that were present in the unwashed Hb-MIP spectrum (see **figure 4.6**), were found to have disappeared in the spectrum of the washed Hb-MIP (see **figure 4.7**). Another peak at 1258 cm^{-1} present in the unwashed Hb-MIP spectrum was shifted down to 1244 cm^{-1} in the spectrum of the washed Hb-MIP, further confirming template removal.

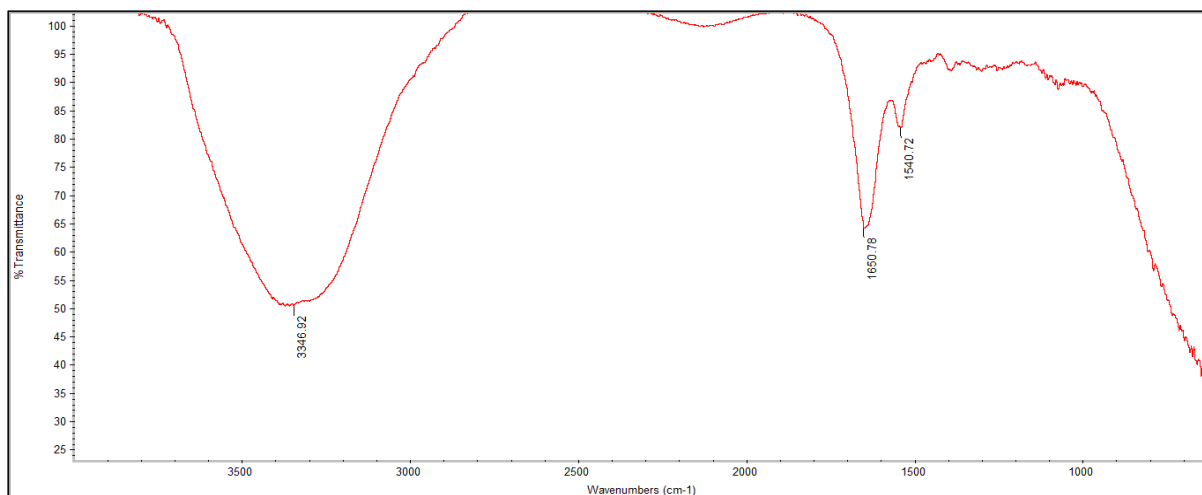


Figure 4.3: Hemoglobin FTIR spectrum

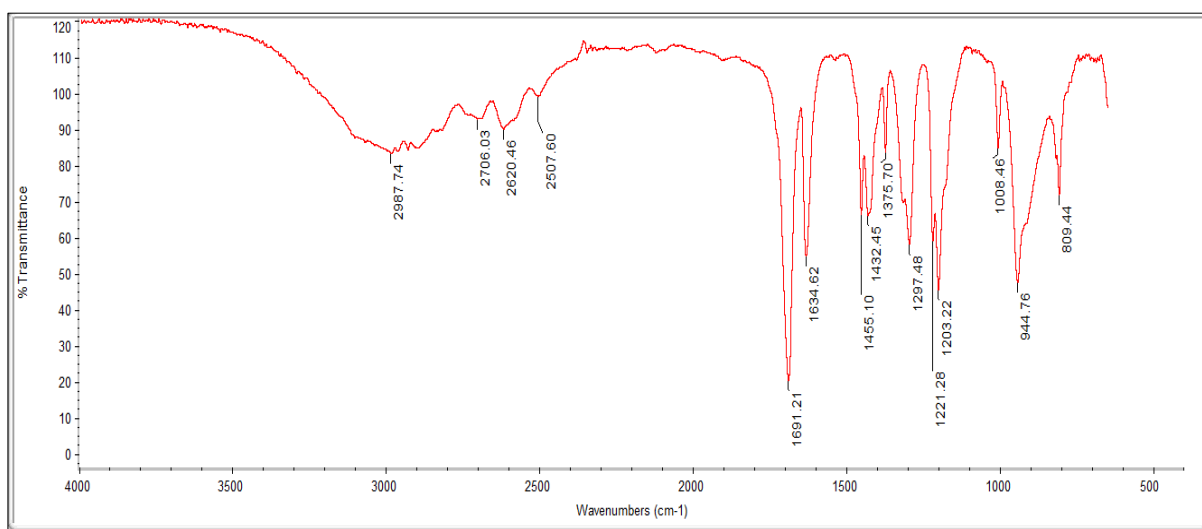


Figure 4.4: MAA FTIR spectrum

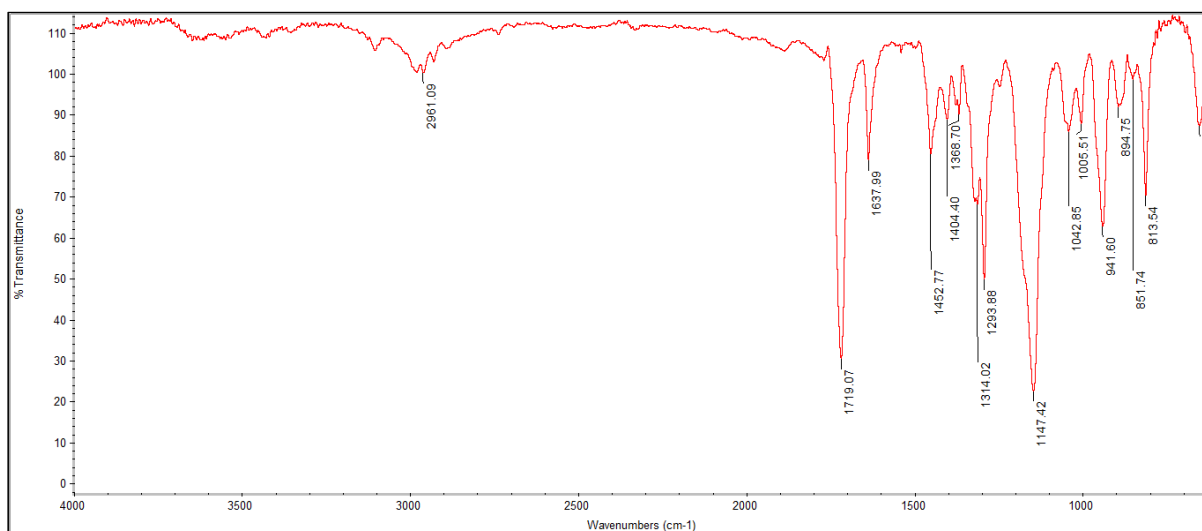


Figure 4.5: EGDMA FTIR spectrum

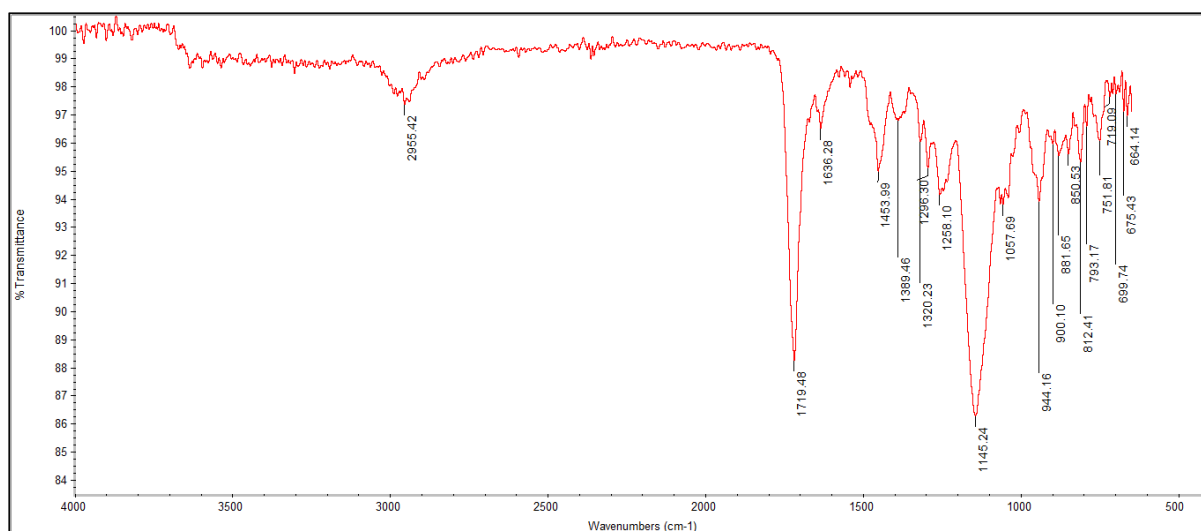


Figure 4.6: Hb-MIP FTIR spectrum before hemoglobin removal

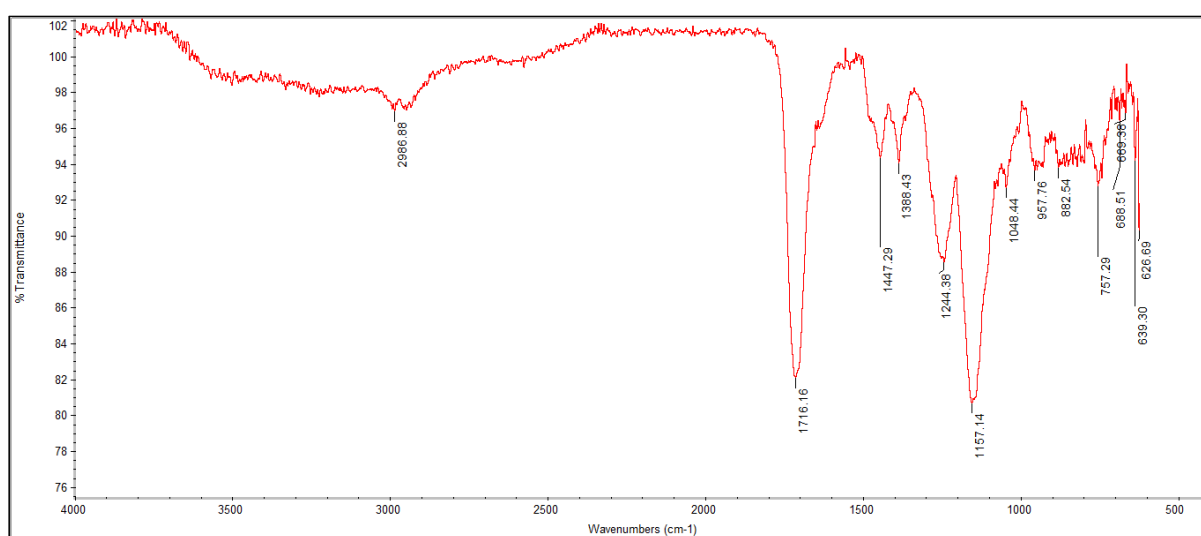


Figure 4.7: Hb-MIP FTIR spectrum after hemoglobin removal

4.2.2 SEM Images for the Washed Hb-MIP, Unwashed Hb-MIP and NIP

SEM images for the washed Hb-MIP powder, unwashed Hb-MIP powder and the NIP powder obtained did not show any notable difference, hence, only the SEM image for the washed Hb-MIP powder was reported. From the image, particles were found to be spherical, a good geometry for sorbent material [134], with conglomeration of fine particles on top of bigger particles (**see figure 4.8**). The particles were conglomerated because no sedimentation was performed. These particles were found to be of size range from 5 μm -70 μm , excluding the conglomerated particles.

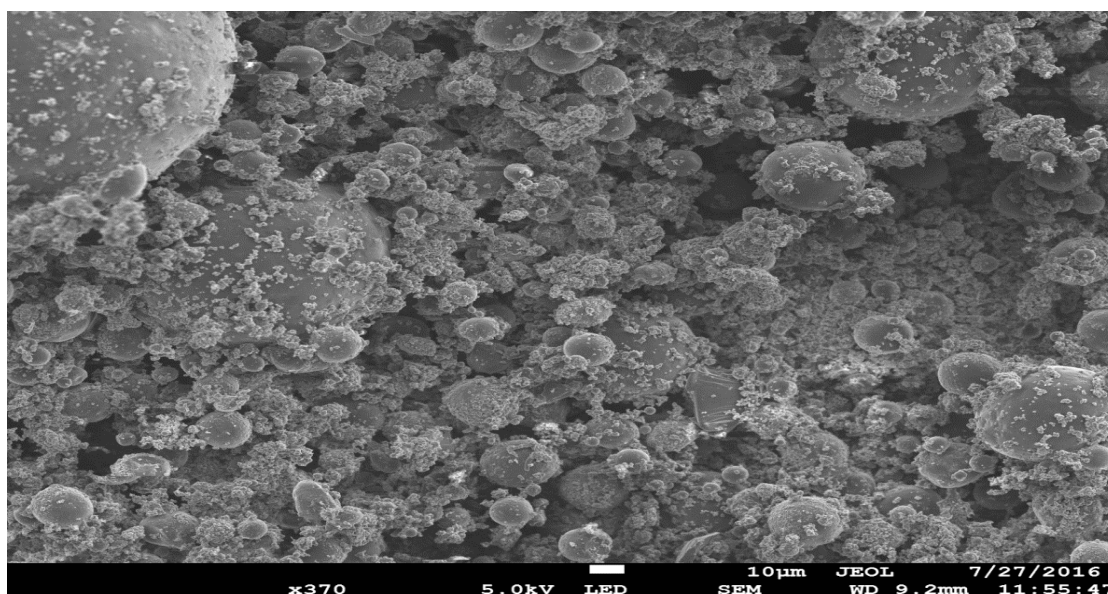


Figure 4.8: SEM image for the washed Hb-MIP powder

4.3 Batch Rebinding Experiments

4.3.1 Optimization of Hb-MIP Powder for Maximum Hemoglobin Removal

From the plot of Hb-MIP quantity versus absorbance, it was observed that 140 mg of Hb-MIP powder was the optimal quantity needed to reduce an absorbance of a 5 mL 0.01 % (v/v) buffered hemoglobin standard solution, from a high value of 0.794 a.u to as low as 0.154 a.u. The hemoglobin absorbance decreased with increasing Hb-MIP quantity until it remained constant at an absorbance of 0.154 a.u that was obtained from 140 mg addition of the MIP as well as with further increase beyond 140 mg (see figure 4.9).

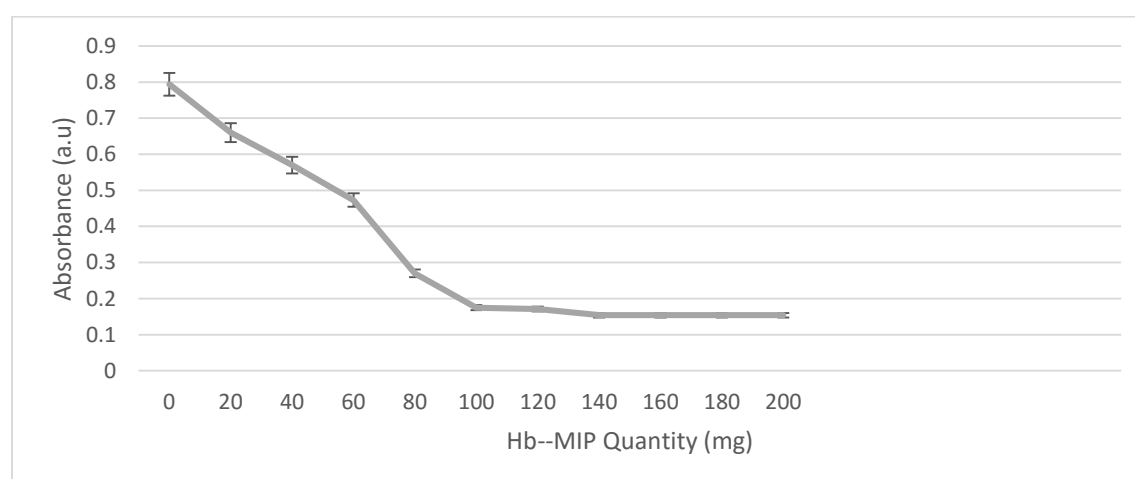


Figure 4.9: Hb-MIP powder optimization for maximum hemoglobin removal

4.3.2 Optimization of Time for Maximum Hemoglobin Removal

From the absorbance versus time plot in figure 4.10, there was a reduction in hemoglobin absorbance from 0.794 a.u with increasing time down to 0.169 a.u, as the experiment was monitored at 2 min intervals. This was continued up to a point where there was no more reduction but a constant trend from 18 min to 24 min, thus making 18 min to be the optimal time needed for maximum Hb removal by the optimized Hb-MIP (see figure 4.10).

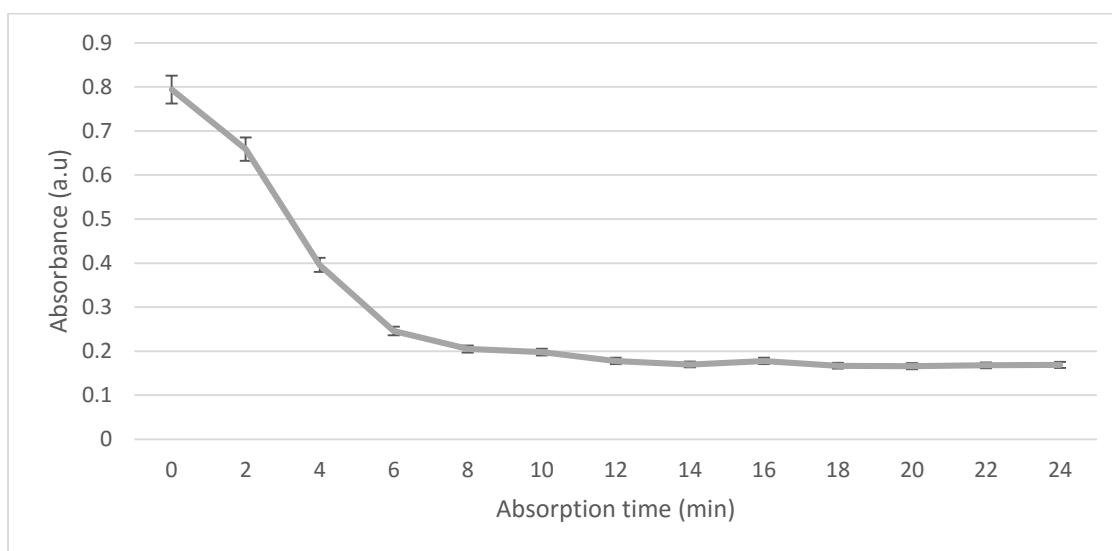


Figure 4.10: Time optimization for maximum hemoglobin removal

4.3.3 Percentage Removal Efficiencies

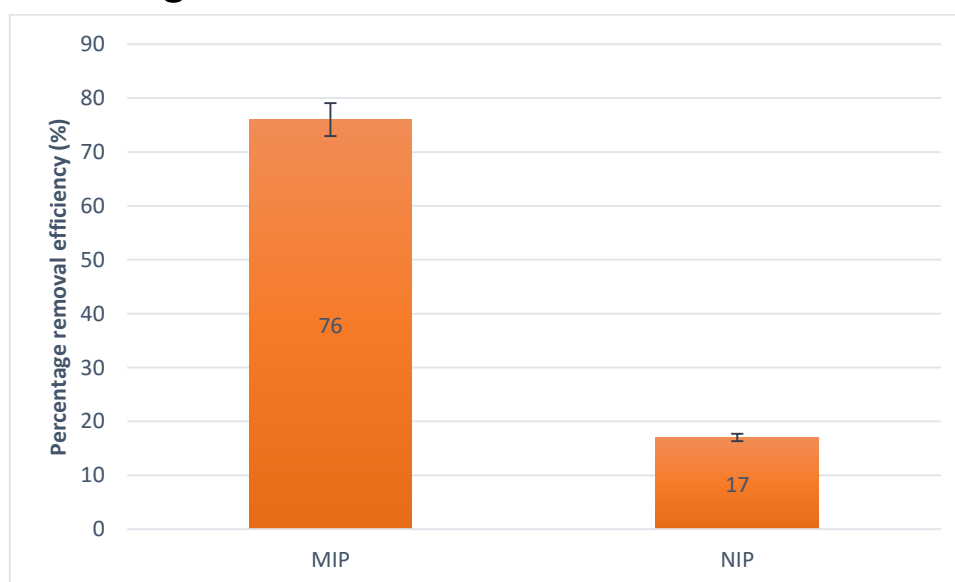


Figure 4.11: Percentage removal of hemoglobin from hemoglobin standard solution by Hb-MIP and NIP

The Hb-MIP powder removed Hb from the hemoglobin standard solution up to a percentage removal efficiency of 76 %, compared to a low percentage removal efficiency of 17 % that was removed by the NIP powder, the control (**see figure 4.11**). The NIP had a low % removal efficiency of 17 % because it did not have the recognition sites due to lack of imprinting. The prepared Hb-MIP powder, could not achieve very high % removal efficiencies of greater than 80 % because few recognition sites were formed during the synthetic process due to a very small quantity of hemoglobin template been employed during polymerization resulting in few recognition sites. It has been reported that imprinting with templates that are of large molecular weights such as proteins, is a challenge, when compared to templates of smaller molecular weights been employed. This is due to synthetic limitations in MIP preparation brought about by the need to employ large quantities of the functional and cross-linking monomers, which lead to permanent entrapment of large templates in the MIP polymer 3-D matrix [117]. The result is usually template bleeding during rebinding experiments [73][146].

4.3.4 Selectivity

Application of Hb-MIP powder to a solution of hemoglobin, and to a solution of a competing species, chlorophyll, separately, showed that the Hb-MIP powder had a greater affinity to the hemoglobin standard solution as the Hb-MIP powder reduced the Hb solution absorbance from 0.794 a.u-0.193 a.u (76 %) when compared to 0.805 a.u-0.773 a.u (32 %) reduction of a competing species (chlorophyll) solution absorbance (**see figure 4.12**). This showed that the prepared Hb-MIP powder had a greater selectivity for Hb than competing species such as chlorophyll. On the other hand, the selectivity of the prepared Hb-MIP powder was compared to the selectivity of a commercially available powder, GCB in removing Hb from hemoglobin standard solution and chlorophyll from a prepared chlorophyll solution. The results demonstrated that GCB is non-selective as it removed Hb and chlorophyll from their solutions to the same extent. Hb absorbance was reduced from 0.794 a.u-0.030 a.u (96 %) and chlorophyll from 0.805 a.u-0.07 a.u (91 %) (**see figure 4.12**). Hb-MIP powder showed great selectivity towards removing Hb from its standard solution by a reduction absorbance of 0.794 a.u-0.193 a.u (76 %) when compared to a low selectivity towards the competing molecule (chlorophyll) that it was not prepared for, with a low absorbance reduction of 0.805 a.u-0.773 a.u (32 %) (**see figure 4.12**).

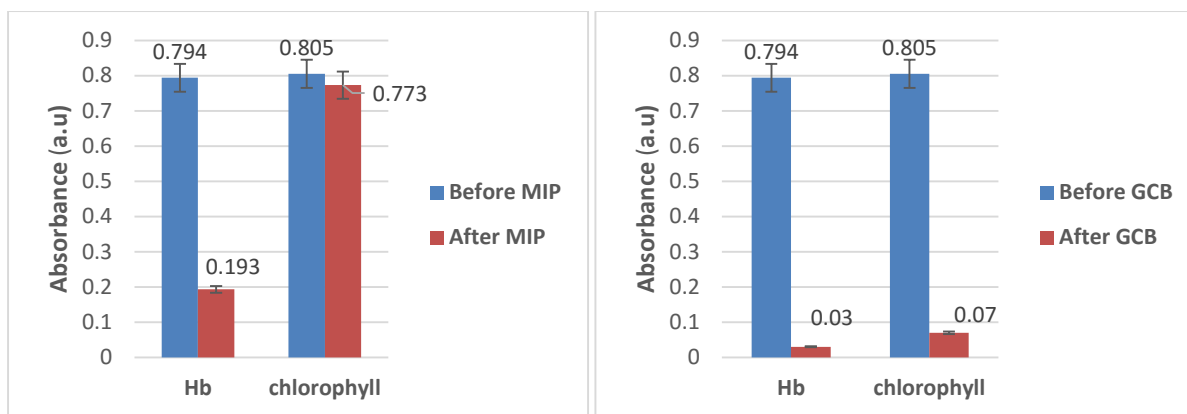


Figure 4.12: Selectivity on hemoglobin and chlorophyll employing Hb-MIP and GCB

4.3.5 Hb-MIP Application to Real Whole Blood Sample

The absorbance of real whole blood sample that was diluted by a factor of two with phosphate buffered saline was found to be 0.650 a.u before Hb-MIP application. After Hb-MIP application under optimal conditions, its absorbance was found to be 0.169 a.u, thus, a 74 % removal efficiency by the prepared Hb-MIP powder. The results demonstrated the robustness of the Hb-MIP by achieving statistically the same Hb % removal efficiencies of 76 % in ‘clean’ Hb standard solutions without interfering matrix and 74 % Hb removal in the ‘dirty’ real whole blood samples with interfering matrix, prior to instrumental analysis in molecular diagnostics and toxicological assays.

Chapter five Conclusions

5.1 Conclusions

This thesis presented the preparation of a novel Hb-MIP powder that was found to be selective, effective and robust as a clean-up sorbent in removing the interfering hemoglobin (Hb) from whole blood which is usually characterized by a 'dirty' and complex matrix, that poses a challenge when the sample is directly introduced into the sensitive analytical instruments without a proper sample clean-up. The prepared Hb-MIP powder demonstrated to be a potential alternative in dealing with challenges faced by the current sample clean-up procedures in whole blood analysis especially centrifugation, which is commonly employed but is not selective and ends up removing the interferents such as hemoglobin together with the targeted analytes, biomarkers and toxic residues. From the results, the Hb-MIP powder selectively removed Hb interference up to comparable % removal efficiencies of 76 % and 74 % for hemoglobin standard solutions and real whole blood samples, respectively, which confirmed the robustness of the clean-up procedure by working perfectly well in both 'clean' samples of standard solutions as well as in the 'dirty' real whole blood samples. The not so high % removal efficiencies of less than 80 % were largely due to the trial and error choice of the starting materials especially the choice of the functional and the cross-linking monomers that were paired with the targeted analyte (template).

For improved % removal efficiencies of greater than 80 % in future work, superior monomers must be chosen based on reliable selection methods such as MIP dialling [165]. To further improve the efficiencies and sensitivities of the prepared clean-up SPE sorbents, better polymerization or fabrication methods that yield smaller and uniform MIP sorbent materials such as electrospinning fabrication method may be employed. Also, much research must be undertaken on effective template removal which is a key synthetic step in MIP preparation for unlocking the recognition sites that are highly needed for rebinding the targeted analytes. This would also help to minimise template bleeding that usually results during template removal. To further improve on sample clean-up of whole blood prior to its analysis in molecular diagnostics and toxicological assaying, the developed sample clean-up method in this thesis can be combined with mild centrifugation which has been tried and tested in removing the interfering Hb from whole blood, to achieve cleaner extracts hence precise and accurate analysis. On a larger scale, our finished product (Hb-MIP) could be fabricated into

disk-shaped miniaturized Hb-nano samplers that are sensitive, selective and can be incorporated in automated systems during hematology assays.

5.2 References

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