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A Novel Molecularly Imprinted Polymer for the Selective Removal of Interfering Hemoglobin Prior to Whole Blood Analysis

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Abstract

A heavy red globular protein, hemoglobin, responsible for whole blood red pigmentation often interferes with the identification and quantification of disease associated biomarkers from whole blood, in field of molecular diagnosis. The main challenge is the direct introduction of whole blood as a sample into analyzing instruments because of its physiological complexity and `dirty` nature. For example, the red pigment in whole blood, which is characterized as 'dirt', usually co-elute with the biomarkers and masks them from easy chromatographic separation prior to their final detection. It also clogs the instrument's components such as the separating columns which are known to be sensitive, hence leading to imprecise and inaccurate results during bio-assaying. To address these challenges, our lab synthesised a novel, selective, effective and a robust hemoglobin imprinted polymer, in the form of a powder, through bulk, free-radical polymerization employing molecular imprinting technology, to selectively remove interfering hemoglobin from whole blood samples prior to instrumental analysis of disease associated biomarkers. From the results, the polymer powder effectively removed hemoglobin from whole blood sample as demonstrated by the ultraviolet-visible absorbance reduction from as high as 0.794 Au to lower values of 0.193 Au before and after polymer powder application, respectively. Experimentally, the powder had a high binding ability towards the targeted hemoglobin as demonstrated by the high percentage removal efficiency of 76% from hemoglobin standard solutions, when compared to its low binding ability towards an analogous species, (chlorophyll), at 32% from chlorophyll standard solutions. Furthermore, the polymer powder proved to be robust as it removed hemoglobin interference from the 'dirty' complex matrix of real human whole blood samples by up to 74% hemoglobin removal which was comparable to 76% hemoglobin removal from neat standards, thus, the polymer powder demonstrated that it can work effectively in diverse environments of clean and dirty matrix. Furthermore, the polymer powder presented itself as an efficient, selective and non-destructive whole blood clean-up pre-analytical tool that with further research may replace the destructive and non-selective conventional whole blood clean-up strategies such as the commonly employed centrifugation.

Keywords: Hemoglobin imprinted polymer; Molecular imprinting technology; Centrifugation; Molecular diagnosis; Sample preparation; Whole blood clean-up

Abbreviations: AIBN: Azobisisobutyronitrile; Au: Absorbance Units; EGDMA: Ethylene Glycol Dimethacrylate; FE-SEM: Field Emission Scanning Electron Microscopy; FTIR: Fourier Transform Infrared; GCB: Graphitized Carbon Black; GC-MS: Gas Chromatography Mass Spectrometry; H: Hours; Hb: Hemoglobin; Hb-MIP: Hemoglobin-Molecularly Imprinted Polymer; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; LLE: Liquid-Liquid Extraction; MAA: Methacrylic Acid; mg: Milligrams; min: Minutes; MIP: Molecularly Imprinted Polymer; ml: Millilitres; SPE: Solid Phase Extraction; UV-Vis: Ultra-Violet Visible; mM: Micrometres

Introduction

Bioanalytical assays are often affected by the presence of either exogenous or endogenous interfering matrix components [1], in samples assayed for the targeted analytes such as the disease associated biomarkers [2], in molecular diagnosis [3]. Whole blood, as one of the most preferred samples, is known for its complex matrix and sometimes characterized to be 'dirty' due to presence of the interfering hemoglobin found in higher levels than the targeted analytes that often exist at trace levels. In this case, hemoglobin as the interferent poses a challenge when whole blood is directly introduced in to sensitive analytical instruments such as gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) as sensitive and specific detection systems of choice [4]. On the other hand, hemoglobin usually masks the targeted analytes from being easily detected, thus, clog the instrument's 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, when not removed, hemoglobin generally leads to matrix effects caused by its co-elution with the biomarkers, which are the targeted analytes. Consequently, prior to employing sensitive instruments with low detection and quantification limits, whole blood as a sample must undergo extensive sample preparation to eliminate the matrix interferents as well as to achieve even more accurate and precise results.

Conventionally, sample preparation strategies such as Protein precipitation and centrifugation [5] have been extensively described in cleaning whole blood for a number of targeted analytes in several bioassays [6], with the aim of removing matrix interferents such as hemoglobin from whole blood as a sample. These strategies have been known not to remove the interferents up to the required level, hence, further sample preparation strategies are highly sought, before instrumental analysis. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have also been employed for whole blood preparation to obtain cleaner and purer extracts that are concentrated with targeted

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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. SPE employ a number of sorbents such as chemically modified silica gel, polymer sorbents with C8/C18 skeletal backbones [7], porous carbon and other newly developed extraction sorbents such as the restricted access sorbents and immunoaffinity extraction sorbents [8]. At times, these sorbents are known for their three dimensional capability, high porosity, rigidity and high surface areas for enhanced extraction power. However, some of the SPE sorbents are highly expensive and have low selectivity resulting in co extraction which makes the SPE technique not to be effective and efficient. In order to deal with these challenges, whole blood clean-up employing highly selective, cheap and robust extraction sorbents must be employed in removing only hemoglobin up to high removal efficiencies, thus, leaving behind the targeted analytes at detectable levels. Such extraction sorbents are referred to as molecularly imprinted polymers (MIPs). MIPs are man-made polymeric materials with functionality and selective recognition sites, due to template (targeted molecule) removal from the MIP matrix, after polymerization [9]. These polymers have been employed in various fields of science such as in chromatographic separation [10], biosensing [11], drug delivery [12] as well as in sample preparation [13] as suitable and selective extraction sorbents for improved SPE procedures.

This paper presents preliminary results for the synthesis, characterization and optimal application of a novel hemoglobin imprinted polymer (Hb-MIP), in a powder form, for the selective removal of interfering hemoglobin, prior to whole blood analysis.

Materials and Methods

Reagents and materials

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 µm 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 university. Commercially available green leaves (Spinach) were purchased from Spar (Palapye, Botswana).

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 and 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).

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. 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 the unreacted reagents. 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.

Characterization of Hb-MIP powder and NIP

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 is 10 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.

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.

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 m 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.

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.

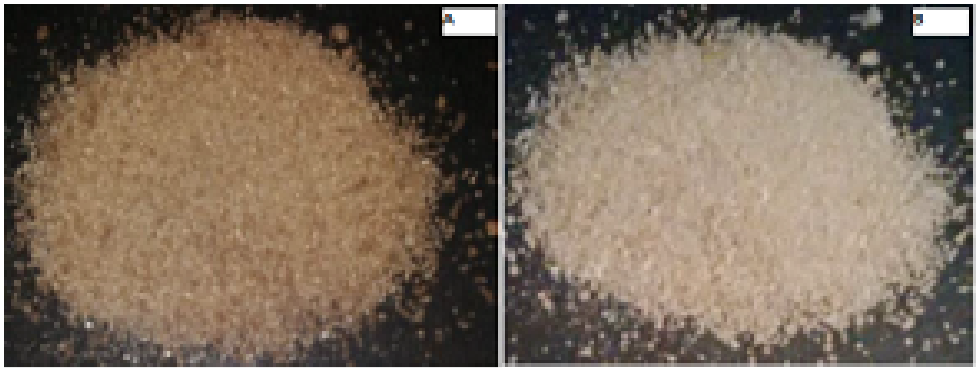

Figure 1: Hb-MIP before hemoglobin removal (a) and after hemoglobin removal (b).

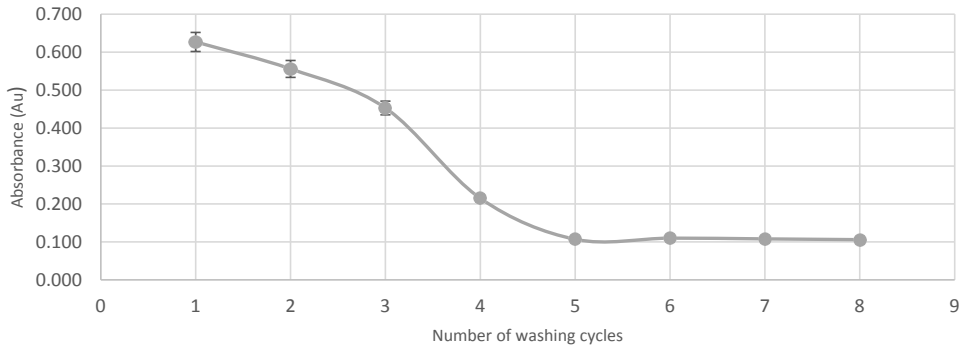

Figure 2: An absorbance plot to confirm hemoglobin template removal.

Optimization of time for maximum hemoglobin removal

With optimized MIP quantity, 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.

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.

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

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.

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 real 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.

Results and Discussion

Formation of Hb-MIP and template removal procedure

After polymerization, a brown monolith (Hb-MIP) obtained was ground to a brown powder (Figure 1a). The brown powder was refluxed to wash off the hemoglobin as the template resulting in an almost white powder (Figure 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.

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 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 Au. 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.

Characterization of Hb-MIP powder

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, (Figure 3, 4 and 5, respectively) were distinctively different from that of the finished product (Hb-MIP) (Figure 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} (Figure 3), and another peak at 1691 cm^{-1} in the MAA spectrum (Figure 4) were found to be absent as shown in the spectrum of the finished product (Hb-MIP) (Figure 6). The differences confirmed that polymerization occurred.

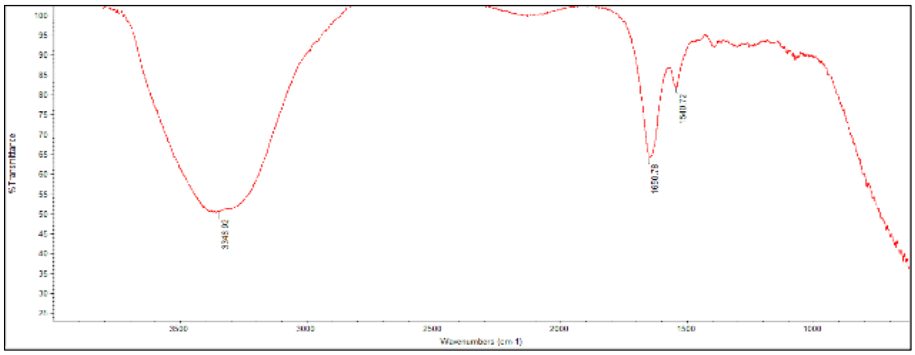

Figure 3: Hemoglobin FTIR spectrum.

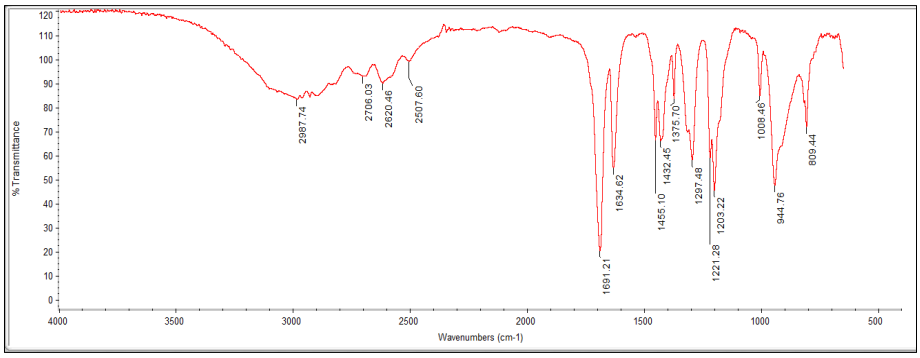

Figure 4: MAA FTIR spectrum.

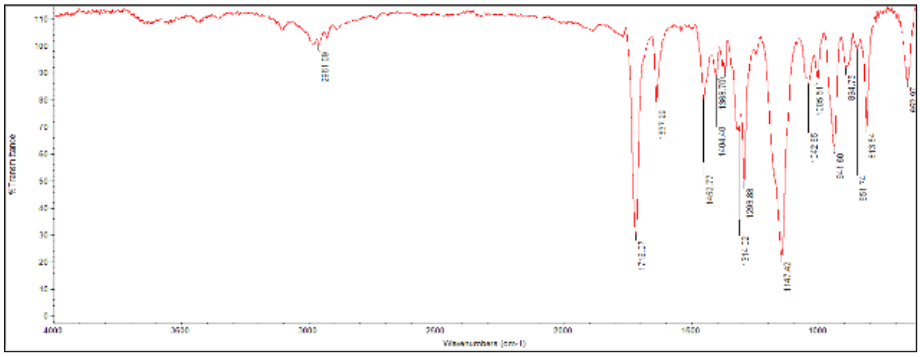

Figure 5: EGDMA FTIR spectrum.

Comparing the spectra of the unwashed Hb-MIP and the washed Hb-MIP, discernible peaks at 1636, 1320 and 1295 cm^{-1} that were present in the unwashed Hb-MIP spectrum (Figure 6), were found to have disappeared in the spectrum of the washed Hb-MIP (Figure 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.

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 a sorbent material, with conglomeration of fine particles on top of bigger particles (Figure 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 particle.

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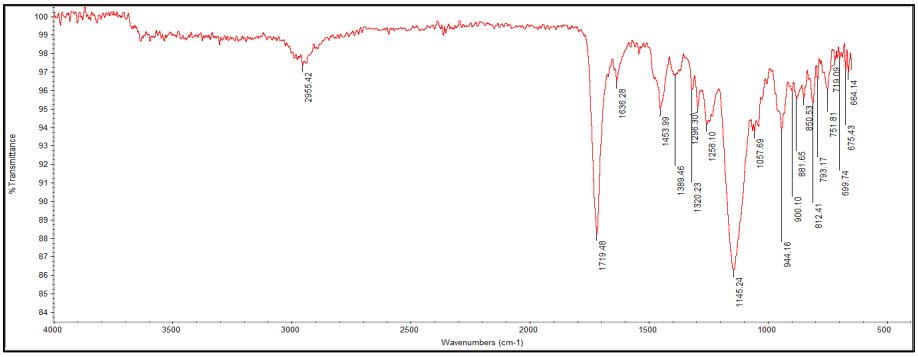

Figure 6: Hb-MIP FTIR spectrum before hemoglobin removal.

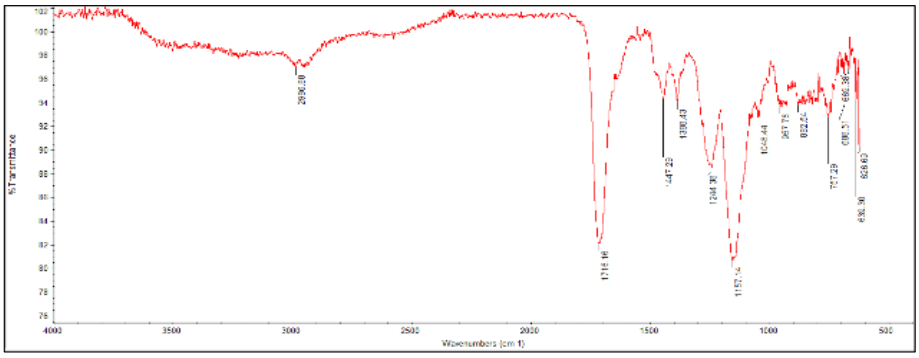

Figure 7: Hb-MIP FTIR spectrum after hemoglobin removal.

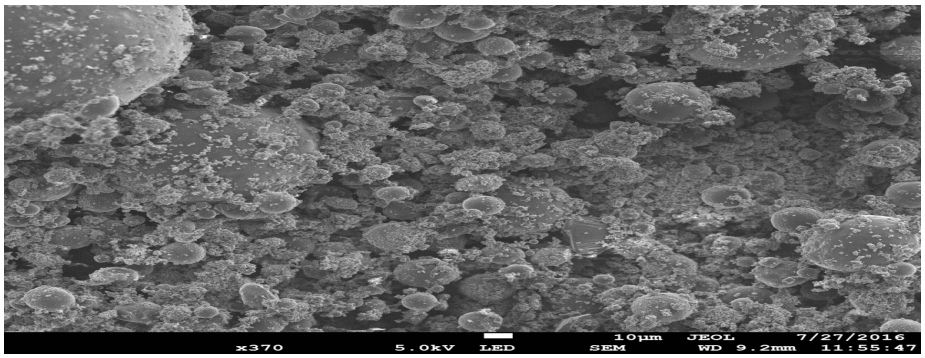

Figure 8: SEM image for the washed Hb-MIP powder.

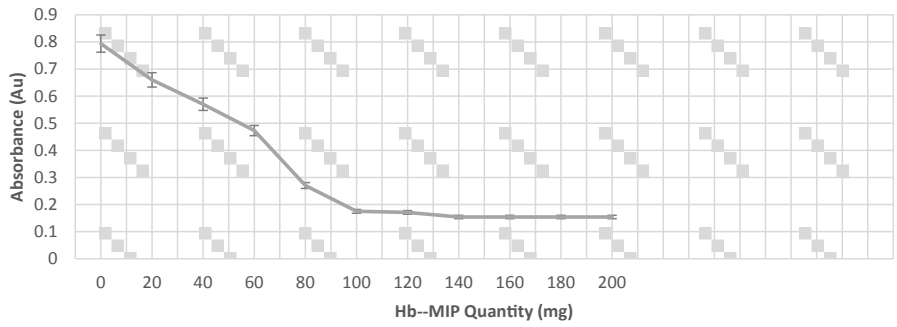

Figure 9: Hb-MIP powder optimization for maximum hemoglobin removal.

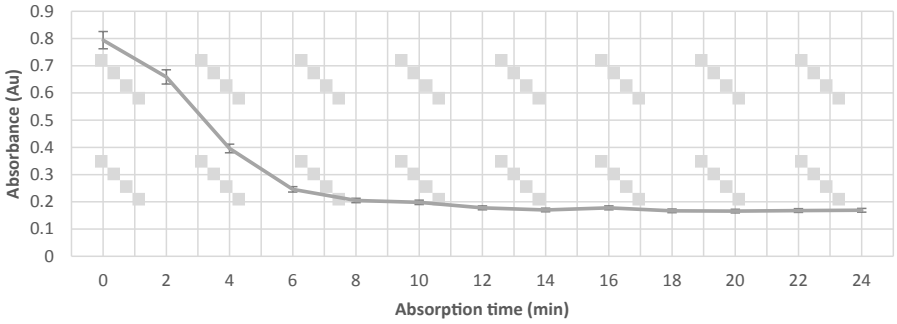

Figure 10: Time optimization for maximum hemoglobin removal.

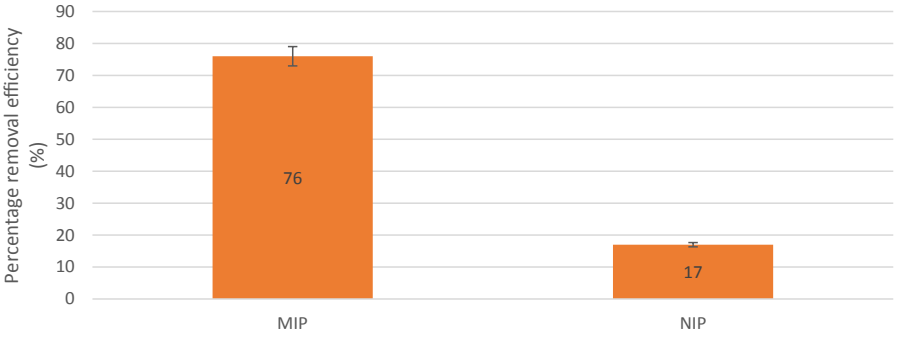

Figure 11: Percentage removal of hemoglobin from hemoglobin standard solution by Hb-MIP and NIP.

particles on top of bigger particles (Figure 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 particle.

Batch rebinding experiments

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 Au to as low as 0.154 Au. The hemoglobin absorbance decreased with increasing Hb-MIP quantity until it remained constant at an absorbance of 0.154 Au that was obtained from 140 mg addition of the MIP as well as with further increase beyond 140 mg (Figure 9).

From the absorbance versus time plot in Figure 10, there was a reduction in hemoglobin absorbance from 0.794 Au with increasing time down to 0.169 Au, 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 (Figure 10).

Percentage removal efficiencies: 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 (Figure 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

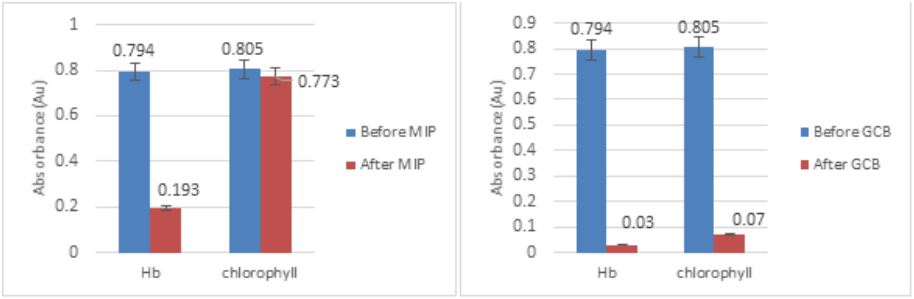

Figure 12: Selectivity on hemoglobin and chlorophyll employing Hb-MIP and GCB.

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 [14]. The result is usually template bleeding during rebinding experiments [15].

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 Au-0.193 Au (76%) when compared to 0.805 Au-0.773 Au (32%) reduction of a competing species (chlorophyll) solution absorbance (Figure 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 Au-0.030 Au (96%) and chlorophyll from 0.805 Au-0.07 Au (91%) (Figure 12). Hb-MIP powder showed great selectivity towards removing Hb from its standard solution by a reduction absorbance of 0.794 Au-0.193 Au (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 Au-0.773 Au (32%) (Figure 12).

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 Au before Hb-MIP application. After Hb-MIP application under optimal conditions, its absorbance was found to be 0.169 Au, 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 diagnosis.

Conclusion

Relative to the preliminary results in this work, 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 (disease associated biomarkers). Furthermore, the novel Hb-MIP powder demonstrated on the effectiveness, selectivity and the robustness as a cheap hemoglobin removal sorbent when applied to both clean hemoglobin standard solutions and 'dirty' real whole blood samples.

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