Development of magnetic molecularly imprinted polymers for the extraction of salivary pepsin prior to analysis by a novel HPLC-SEC method

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Abstract

Salivary pepsin is a very important and selective biomarker for gastroesophageal reflux disease and high concentrations in the saliva of potential patients can provide a very useful and non-invasive diagnostic tool. Therefore, this study focused on the development of new and highly selective magnetic molecularly imprinted polymers (MIPs) for the extraction of pepsin from human saliva samples. The developed MIPs provide a stable, cost-effective, and reusable synthetic method of extraction for pepsin. Surface imprinting technique was applied for the synthesis of MIPs to provide a wider surface area and faster binding/release of pepsin, since all the binding sites are allocated on the surface of iron oxide nanoparticles. Methacrylic acid was used as functional monomer, ethylene glycol dimethacrylate as cross linker, and deionized water as a porogen. The imprinting procedure was meticulously optimised to reach the highest possible binding indicated by the value of binding capacity Q which reached (190 mg g^{-1}) and an imprinting factor of 1.34, calculated in comparison to non-imprinted polymers (NIPs). Moreover, the surface morphology, thermal stability as well as the

binding properties were characterised to ensure validity. The developed MIPs were applied successfully for dispersive solid phase extraction of pepsin from saliva samples. Consequently, a novel, highly sensitive and simple HPLC-SEC method was developed for the quantitation of pepsin fragments extracted using MIPs. The method was optimised and validated according to ICH guidelines where the linearity ranged from 5 to 150 μg mL^{-1} in saliva samples with LOD reaching down to 0.6 μg mL^{-1} which is comparable to the low concentrations of pepsin expected to be found in real saliva samples. The developed system enables the accurate extraction and determination of salivary pepsin with recoveries reaching to 98%. Therefore, this system shows potential as an efficient and reliable testing method for pepsin.

1. Introduction

Pepsin enzyme is a digestive enzyme from the family of endoproteinases which has a molecular weight of approximately 34.5 kDa and isoelectric point of 3.24 [1]. Pepsin plays a major physiological role in our daily life to help in the digestion of proteins found in different foodstuffs. Pepsin also plays a significant role in the pathogenesis of peptic ulcers. It has been found that pepsin combined with gastric acid causes more severe damage to the gastric mucosa than the acid alone [2]. Moreover, it was recently affirmed that salivary pepsin can be used as a biomarker for gastroesophageal reflux disease (GERD) [3]. These discoveries suggest a need for the development of a highly selective, cheap, and easy method for the extraction of pepsin from saliva prior to its analysis. However, due to its size, nature, and presence in low concentrations in the saliva, pepsin presents a challenge in its extraction [1]. In addition, alongside pepsin, there are other salivary enzymes such as amylase and lipase that can interfere with a clean extraction process. Therefore, the need for a highly selective method to extract pepsin from saliva without interference from other salivary enzymes is a necessity.

Molecularly imprinted polymers (MIPs) are a type of synthetic polymer designed to mimic antibodies in their selectivity to a specific target molecule. Since their recent discovery, MIPs have

found many applications in the fields of pharmacy and drug delivery [4], catalysis [5], analytes' extraction [6], and chromatography [7,8]. Moreover, there are many methods developed for synthesis of MIPs each with their own advantages and applications [9,10]. One of the most popular synthetic techniques is surface imprinting. In this type of molecular imprinting, the polymer is formed as a thin layer on a solid support. Core shell imprinting is a subtype of surface imprinting technique which has many advantages that includes restricting all the binding sites on the surface of the polymer and increasing the exposed surface area for target binding, which, in turn, increases the sensitivity. In addition, core shell imprinting enables a precise and reproducible control over the shape, size and physical qualities of the resulting polymers [11]. Magnetic MIPs are a class of core shell imprinted polymers in which the polymeric layer is grafted on the surface of magnetic nanoparticles. Polymerisation on the surface of magnetite (Fe_3O_4) imparts a magnetic quality to the resulting polymers, which enables any target molecule to be extracted from a complex matrix with ease and without a need for centrifugation or filtration [12].

Proteins present a challenge of their own as targets for molecular imprinting because of their limited solubility in organic solvents which are commonly used in molecular imprinting procedures. Moreover, proteins are sensitive to pH, temperature, and ionic strength of solution, which, in turn, can change their folding upon any variation in physiological conditions. In addition, the large size of proteins makes it difficult for them to diffuse through the cross-linked polymeric mass of MIPs, resulting in slow binding kinetics and incomplete template removal upon washing [13]. Core shell imprinting provides a solution for protein imprinting since all binding sites of the polymer are on the surface. This, in turn, eliminates the need for the proteins to diffuse through the polymer and enables rapid binding and release. Moreover, the large surface area provided by core shell imprinting is very suitable for the low concentrations of proteins usually found in biological samples, thus contributing to enhanced sensitivity [11].

In our literature survey, some papers describing the development of MIPs for pepsin came to our knowledge. In 2013, MIPs were developed by a mini emulsion imprinting technique for the binding of pepsin [15]. The application of this type of polymerisation resulted in a very small particle size as illustrated by the authors (400 – 600 nm) and increased surface area available for binding (30 – 65 m^2). Nonetheless, this study lacked a complete optimisation profile for all the elements involved in the synthesis procedure, where the authors only tested four monomers to choose the optimum one. There was no optimisation for the cross linkers, initiators, porogens and other factors that can affect imprinting. Later in 2015, the authors published another paper showing some in-depth binding assays to highlight the binding performance of their developed polymers, yet still, an optimisation profile was missing. Moreover, there was no application to biological samples to indicate the suitability of the developed MIPs as extraction media [16].

Two methods were developed for the detection of pepsin utilizing MIPs in an enzyme linked immunosorbent assay (ELISA) format in which MIPs replace antibodies to bind the target. The first method was published in 2018, where magnetic MIPs for pepsin were immobilised to the walls of the wells of a microtitre plate via magnetic inserts [17]. The sample solution containing pepsin was mixed with polystyrene beads (PSB) as labels and subsequently added to the wells. As a result, pepsin with the attached PSB would be drawn to the magnetic MIPs resulting in a decrease in the central fluorescence. Despite the authenticity of this method, it had many drawbacks that we previously reviewed [14]. A very recent method that adopted the same principle of abiotic ELISA format came to our knowledge. This method also adopted the use of a microtitre plate modified with magnetic inserts which were used to immobilise pepsin that is covalently bound to magnetic iron (II, III) oxide nanoparticles. The method was novel and efficient, however, there was no application for the detection of pepsin in saliva or gastric fluid and no detailed optimisation profile [18].

Herein, we introduce the development of magnetic MIPs for binding and extraction of pepsin. A complete optimisation profile of all the reagents and reaction conditions involved in the synthesis was

conducted to ensure the production of the highest possible selectivity. Moreover, a detailed characterisation profile including morphological, thermal, and functional characterisation is introduced to assess the validity and usefulness of the developed polymers. Furthermore, the developed MIPs were applied successfully to bind and extract pepsin in dispersive solid phase format prior to its quantitation via a novel high performance liquid chromatography- size exclusion chromatography (HPLC-SEC) method. Finally, the developed polymers proved efficient for the extraction of pepsin from real saliva samples with minimal interference from other salivary enzymes and the extracted pepsin was detected and quantified via HPLC-SEC.

2. Experimental

2.1. Materials and instrumentation

Methacrylic acid 99% (MAA), acrylamide 99% (Am), acrylic acid 99% (AA), N-isopropylacrylamide (NIPAm) 99%, *N,N'*-methylenebisacrylamide 99% (MBA), ethylene glycol dimethacrylate 98% (EGDMA), phosphate-buffered saline tablets (PBS) , sodium acetate 99%, glacial acetic acid 99%, Tris base 99.9%, hydrochloric acid 36%, phosphoric acid 85%, ammonium persulphate 98% (APS), *N,N,N',N'*-tetramethylethylenediamine 99.5% (TEMED) and tetraethyl orthosilicate for synthesis (TEOS) were all purchased from Sigma-Aldrich, UK. HPLC grade water, deionised water, methanol 99%, ethanol 99%, sodium dodecyl sulphate 99% (SDS), oxalic acid 98%, sodium chloride 99.5%, sodium bisulphite 95%, potassium persulphate 99%, 2-aminoethylmethacrylate hydrochloride 90%, dimethylaminoethyl acrylate 99%, human pepsin, human amylase, human lipase, ferrous chloride 98%, ferric chloride 98%, and (3-aminopropyl)trimethoxysilane 98% (APTMS) were all purchased from Thermo Fisher Scientific, UK. Neodymium magnets were purchased from Amazon, UK. All reagents were used as received, without further purification.

UV spectra were collected using a Cary UV-Vis Compact, running on Cary UV Workstation™, software version 1.0.1284 (Agilent, UK). IR spectra were collected using a Thermo Scientific Nicolet

iS5 Fourier transform infrared spectroscopy (FTIR) running on OMNIC™ software. For thermogravimetric characterisation a Mettler Toledo TGA/DSC 1 Series – running on STARe™ software Version 10.00 was used. As for differential scanning calorimetry (DSC), it was performed on TA Instruments DSC25 Series, running on Trios™ software, v5.4.0.300. Visualization of the developed polymers was carried out via surface electron microscopy using a Zeiss Evo-50 electron microscope, operating on Smart SEM™ software

The HPLC-SEC method was performed on a LC Agilent 1260 Infinity ii (Agilent, UK) equipped with: 1260 Vial Sampler G7129A, Variable Wavelength Detector 1260 G7114A, Quat Pump VL 1260 G7111A, and a Biozen 1.8 µm dSEC-2, 200 Å LC column with the dimensions of 150 x 4.6 mm. The system control and data acquisition of the HPLC were performed by Chemstation™ C.01.10 software. Filtration of buffer solutions and samples was carried out using 0.20 µm cellulose nitrate membrane filters (Whatman Laboratory Division, Maidstone, UK). Adjustment of the pH of the mobile phase was performed using a Jenway™ 3510 pH meter (UK)

Data analysis and calculations was performed using Origin™ 8.5 software (OriginLab Corporation, North Hampton, USA).

2.2. Prepolymerisation study

The aim of a prepolymerisation study is to find a functional monomer capable of forming stable non-covalent bonds with the target analyte either through hydrogen bonds, Van der Waals, or hydrophobic interactions. To be able to choose the appropriate monomer for the synthesis of highly selective MIPs, especially in water as a solvent, the stability of the template-monomer adduct had to be estimated. UV spectrometry was applied in a reported method to estimate possible interactions between the template and suggested functional monomers [19]. Therefore, UV spectrometry was applied in this work to measure any interactions between pepsin and a number of monomers through measuring the change on the original UV spectrum of pepsin. Pepsin solution (0.5 mg mL^{-1}) in water

was titrated with 10 mmol L^{-1} solutions of different monomers including MAA, Am, AA, 2-aminoethylmethacrylate hydrochloride, dimethylaminoethyl acrylate and NIPAm. Those monomers were chosen due to their potential ability to form hydrogen bonds with either amino or carboxylic groups in the structure of pepsin. The collected UV spectra were compared to determine the monomer with the strongest interaction, identified by a noticeable shift on the original spectrum of pepsin.

2.3. Synthesis of functionalised iron oxide nanoparticles

2.3.1. Synthesis of iron oxide (magnetite) nanoparticles

Synthesis of magnetite (Fe_3O_4) was achieved by a chemical coprecipitation method, as reported previously, with some modifications [20]. Briefly, in a 100 mL deionised water, 1.5 g of ferrous chloride and 3.0 g of ferric chloride were dissolved and heated in a water bath under a continuous nitrogen stream with vigorous stirring. After reaching a temperature of 80 °C, 10 ml of 25% w/v ammonium hydroxide was added dropwise until reaching a pH of 10 to initiate the precipitation of the black iron oxide nanoparticles. After the complete precipitation, the obtained nanoparticles were collected by a neodymium magnet and washed with deionised water multiple times to remove excess ammonia and chloride ions.

2.3.2. Functionalisation of magnetite

In order to direct the polymer chains to gather around the surface of magnetite nanoparticles, they had to be surface modified with specific functional groups that would draw the polymerisation mixture to their surface. The first step was the process of silanisation which introduces a thin layer of silica around the surface of magnetite. Silanisation was carried out via TEOS, according to a reported method, with some modifications [21]. Briefly, the resulting magnetite nano particles from the previous coprecipitation reaction were redispersed after washing into 380 mL of ethanol, 80 mL of deionised water, and 5 mL of 25% w/v ammonium hydroxide via ultrasonication. The mixture was heated to 30°C and 4 mL of TEOS were added dropwise. The mixture was left to react for 6 hours under

continuous vigorous stirring. The resulting silanised magnetite nanoparticles (Fe_3O_4-SiO_2) were collected via a neodymium magnet and washed with ethanol and deionised water 4 times, then dried under vacuum at 70°C.

The next step was the further functionalisation of Fe_3O_4-SiO_2 to introduce amino groups on the surface via APTMS [21]. 2.5 g of the silanised magnetite were dispersed in 200 mL of ethanol via ultrasonication followed by heating the solution to 70°C. APTMS (4 mL) was then added dropwise over a period of 15 minutes. The mixture was allowed to react under a nitrogen gas atmosphere for 12 hours. The resulting magnetite nanoparticles (Fe_3O_4-SiO_2-NH_2) were collected by a neodymium magnet and washed with ethanol and deionised water 4 times, then dried under vacuum at 70°C.

The last step of functionalisation was the introduction of carboxylic groups via maleic anhydride [21]. 1.2 g of Fe_3O_4-SiO_2-NH_2 nanoparticles were dispersed in 50 mL of N,N-dimethylformamide containing 400 mg of 4-dimethylaminopyridine and 5 g of maleic anhydride. The reaction was left overnight at 60°C and with continuous stirring. The product was collected by a neodymium magnet and washed with deionised water and ethanol 4 times and then freeze dried.

2.4. Preparation of magnetic MIPs

The functional monomers that were assessed in the prepolymerisation studies were tested in a series of prepared MIPs either alone or in a combination of two monomers. Moreover, different cross linkers, initiators, and porogens were tested as well, in order to optimise the synthesis procedure to reach the MIPs with the highest possible selectivity. Overall, the optimal procedure is as follows: 250 mg of magnetite were dispersed in 15 mL of deionised water via ultrasonication followed by the addition of 14 mmol of MAA and 8 mmol of EGDMA. The solution was stirred for 1 hour to allow for preassembly with continuous nitrogen sparging to remove dissolved oxygen. Finally, 25 mg of potassium persulphate and 50 mg of sodium bisulphite were added to initiate a free radical polymerisation reaction. After the complete formation of the brown polymer particles, they were

collected by a neodymium magnet and washed with water 4 times to remove unreacted monomers and oligomers.

To remove pepsin from the imprinted binding sites, MIPs were washed with a solution of 1% w/v SDS/10% v/v acetic acid. MIPs were stirred with this solution for 4 hours to ensure complete template removal followed by washing with deionised water 5 times. UV spectrometry was used to detect pepsin in fractions of the washing solution to confirm complete washing.

In order to compare the performance of MIPs and ensure their selectivity, control non-imprinted polymers (NIPs) were prepared using the same procedure without the addition of pepsin. Both MIPs and NIPs were dried under vacuum overnight at 70°C.

Figure 1 illustrates the functionalisation of magnetite and the core shell imprinting process that was carried out on the surface of the functionalised magnetite nanoparticles in the presence of pepsin as a template.

2.5. Protein adsorption experiments

A batch adsorption method at the room temperature (25°C ± 2°C) was used to assess the binding capacity of all the developed polymers. Briefly, 50 mg of MIPs or NIPs were incubated with 20 mL of 0.5 mg mL^{-1} pepsin solution in water for 2 hours on a mechanical shaker. Afterwards, polymers were collected by a neodymium magnet and the concentration of pepsin remaining in solution was determined by UV spectrometry to calculate the amount in milligrams bound per gram of polymer (Q). In the case of incomplete template removal upon washing of MIPs, a problem known as template bleeding can occur, in which case the template is released upon using the developed MIPs resulting in erroneously high results [14]. To manoeuvre the probability of template bleeding, a blank rebinding experiment was conducted simultaneously in which 50 mg of MIPs or NIPs were incubated with 20 mL of deionised water for 2 hours and the supernatant after magnetic separation was used as a blank for the UV measurements of pepsin.

Q (mg g^{-1}) was calculated using the following equation:

$$Q = (Ci - Ct).V/m$$

where, Ci (mg mL^{-1}) is the initial concentration of pepsin, Ct (mg mL^{-1}) is the remaining concentration of pepsin after incubation time (t), V (mL) is the volume of solution, and m (g) is the mass of the polymers used in the experiment. All the tests were conducted in triplicates to ensure precision of the results.

2.6. Characterisation of magnetic MIPs

The morphology of the synthesised magnetite, amino functionalised magnetite, MIPs and NIPs was investigated using scanning electron microscope (SEM) which enabled visual detection of the polymeric layers and an estimate of the particle size to confirm surface imprinting.

A batch rebinding assay at the room temperature (25°C ± 2°C) was performed to determine the binding kinetics order and the time of binding equilibrium for both MIPs and NIPs. The same mass of both polymers (50 mg) was incubated with 20 mL of 0.5 mg mL^{-1} of pepsin solution for different time intervals (t). The amount of pepsin bound per gram (Q) of MIPs or NIPs was computed for each time interval.

A similar batch adsorption experiment was conducted however using different concentrations of pepsin during a fixed time interval to determine the binding isotherm model for both MIPs and NIPs. 50 mg of MIPs or NIPs were incubated for 2 hours with different concentrations of pepsin and the amount of pepsin bound per gram (Q) of MIPs or NIPs was calculated for each concentration.

Binding selectivity was validated through comparing the binding of MIPs and NIPS to competitive analytes that can coexist with pepsin in human saliva. Amylase and lipase were chosen for selectivity testing in which 20 mL of 0.5 mg mL^{-1} solution of each enzyme were incubated with 50 mg of MIPs or

NIPs for 2 hours and the amount bound per gram (Q) was computed for each analyte and compared to that of pepsin.

Thermal analysis was used to provide useful information about the relative mass ratio of the polymeric layer to the mass of magnetite in its core, the amount of adsorbed moisture, and the presence of any unreacted monomers or crosslinkers. The developed MIPs and NIPs along with functionalised magnetite were analysed using thermogravimetric analysis (TGA) with a temperature program set from 25 to 750 °C at a heating rate of 10 °C min^{-1} and a nitrogen gas flow rate of 50 mL min^{-1}. In addition, differential scanning calorimetry (DSC) was conducted at a temperature range from 25 to 350 °C at a heating rate of 10 °C min^{-1}.

2.7. Development of dispersive solid phase extraction of pepsin via MIPs

The developed MIPs were used as an adsorbent for the extraction of pepsin from aqueous solutions and saliva samples prior to quantitation. The eluting solvent and elution time were thoroughly optimised to achieve the highest recovery of pepsin. Herein, 50 mg of MIPs were incubated with different concentrations of pepsin standard solutions from 0.5 to 150 mg^{-1} mL on a mechanical shaker to extract the target. After two hours, MIPs were collected by a neodymium magnet and eluted using PBS buffer for one hour, and the collected fractions were analysed by HPLC-SEC.

2.8. Development and validation of a bioanalytical HPLC-SEC method for pepsin

The use of conventional reversed phase columns for the analysis of proteins is not always a straightforward process [22]. As a result, it was not useful for the analysis of pepsin as it caused denaturation and unfolding of the protein causing very irregular and unreproducible peak shape and sometimes many split peaks. Consequently, the size exclusion chromatography (SEC) was chosen for the analysis of pepsin enzyme due to its ability to separate large biomolecules. In addition, the use of SEC would enable the determination of the efficiency of the extraction procedure and the presence of any other proteins in the extracted samples.

In order to obtain a good peak shape, the method was methodically optimised by testing different buffer compositions, pHs, ionic strengths and concentrations. A working solution of pepsin with the concentration of 100 µg mL^{-1} was used throughout the entire optimisation phase. Phosphate buffer, acetate buffer and Tris buffer covering a pH range from 2.5 to 7.5 were tested as mobile phases to give the best peak. In addition, different concentrations of the optimum buffer including 0.01, 0.02, 0.05 and 0.1 mol L^{-1} were applied to investigate their influence on the resulting peak. 1 mol L^{-1} of sodium chloride was added to the optimum buffer as ionic strength modifier to study its contribution in the separation efficiency. Furthermore, to ensure the reproducibility of the results, different compounds were tested as internal standards such as bovine thyroglobulin and human γ-globulin to select a compound with a suitable retention time and a good separation from the peak of pepsin.

In addition, the developed method was validated according to the ICH guidelines [23]. Linearity was verified at eight different concentrations of pepsin with a fixed concentration of the internal standard (2.5 µg mL^{-1}) through triplicate injections of each concentration level and applying the linear regression equations to the resulting peak area ratios. Accuracy and precision were evaluated across three different concentration levels of pepsin including high, medium, and low. Triplicate injections of each concentration were measured within the same day for accuracy and intraday precision and within three consecutive days for inter-day precision. Herein, accuracy was expressed as percentage of recovery from regression equation and precision was expressed as relative standard deviation percentage (RSD%). To estimate the sensitivity of the developed HPLC-SEC method, the limit of detection (LOD) and the limit of quantitation (LOQ) were practically tested through injecting very low concentrations of pepsin (0.05 to 0.2 µg mL^{-1}) and calculating the signal to noise ratio (S/N). LOD and LOQ were then defined as the concentrations giving S/N value of 3 and 10 respectively. In addition, to ensure the robustness of the developed method, minor changes in the chromatographic conditions such as flow rate, pH and concentration of buffer were applied to assess their influence on the peak area ratio and method performance. Finally, the system suitability parameters were calculated for a

representative chromatogram which includes retention factor, separation factor, resolution, number of theoretical plates, height equivalent to theoretical plates and tailing factor.

2.9. Development of a MIPs assisted dispersive solid phase extraction method for the extraction of pepsin from saliva samples

This study was conducted in agreement with the declaration of Helsinki principles and under the rules of UK Human Tissue Act (HTA) 2004 and received full ethical approval from Kingston University Ethics Committee under Ethics Code 2895. Saliva samples were collected from the subject after rinsing the mouth three times with water, centrifuged at 4500 rpm for 30 minutes and then used immediately. 150 µL aliquots of saliva were then spiked with different volumes of standard pepsin solution and vortex mixed for 1 minute. To extract the spiked pepsin, 50 mg of the developed magnetic MIPs were added to the spiked saliva samples followed by addition of deionised water to make a final volume of 10 mL. Samples were then incubated on a mechanical shaker for 2 hours. A neodymium magnet was used to separate the magnetic MIPs from solution and 5 mL of PBS buffer were used to elute pepsin from MIPs for 1 hour. After magnetic separation, triplicate injections of 20 µL of each of the extracted fragments were injected into the HPLC-SEC system after filtration through a 0.20 µm cellulose nitrate membrane filter and the addition of the internal standard (IS). Peak area ratios were plotted against the concentration in µg mL^{-1} to obtain a calibration curve and calculate the linear fit equation.

3. Results and discussion

3.1. Prepolymerisation study

The results of the prepolymerisation study are shown in **Figure 2** which reveals a hypochromic shift on the spectrum of pepsin with all the tested monomers. However, the strongest shift was observed with MAA which can indicate the formation of a strong and stable bond with pepsin.

Nevertheless, since all the tested monomers caused a hypochromic shift, they were further tested in the optimisation phase to verify the selectivity of their corresponding MIPs towards pepsin.

3.2. Synthesis of functionalised magnetite

Synthesis and surface modification of magnetite were performed according to previously reported methods [20,21]. However, in the reported method for silanisation of magnetite, dry magnetite nano particles were weighed and dispersed in ethanol prior to the reaction with TEOS. Upon repeating the same procedure, it was noted that the magnetite nanoparticles were severely aggregated after drying which resulted in very poor dispersibility prior to silanisation even with prolonged sonication. As a result, an *in situ* silanisation reaction was adopted in which silanisation followed the synthesis of magnetite nanoparticles without an intermediate drying step. Using this approach, the resulting nanoparticles (Fe_3O_4-SiO_2) had a very good dispersibility and could be further modified with ease. Functionalisation of magnetite was confirmed by FT-IR spectroscopy for bare magnetite, silica, and amino modified particles. **Figure 3** shows the IR spectrum of bare magnetite in which a band at 580 cm^{-1} corresponding to the vibration of the Fe-O bonds is prominent. In addition, two more peaks at 1633 and 3400 cm^{-1} can be attributed to the stretching of the hydroxyl groups on the surface of magnetite. These results are in concordance with the IR spectrum for the reported method of synthesis [20]. Further IR spectrum was collected for magnetite after silanisation in which an additional peak at ~ 2900 cm^{-1} corresponding to the stretching of the Si-O bond appeared. Moreover, the IR spectroscopic analysis of the amino functionalised magnetite showed one more peak at ~ 3600 cm^{-1} corresponding to the stretching of the N-H bond. The two new peaks after silanisation and amination provided proof of successful surface modification of bare magnetite nanoparticles.

The surface modification of magnetite should be with functional groups that attract the template-monomer adduct to its surface. Since the isoelectric point of pepsin is relatively low (3.24), this means that at the working pH of the polymerisation solution (6 – 8), pepsin would be negatively charged.

Therefore, amino functionalisation of magnetite would result in the presence of positively-charged amino groups on the surface that would attract the negatively-charged pepsin molecules. Hence, amino functionalised magnetite nanoparticles were preferable for the synthesis of magnetic MIPs for pepsin. Meanwhile, carboxyl surface modification would result in negatively-charged carboxylic groups that would repel pepsin molecules resulting in poor selectivity. This hypothesis was further confirmed by comparing the binding performance of MIPs prepared using (Fe_3O_4), (Fe_3O_4-SiO_2-NH_2), and (Fe_3O_4-SiO_2-COOH). It was found that MIPs prepared with amino functionalised magnetite had the highest binding indicated by the value of Q, as shown in **Figure 4**.

3.3. Synthesis and optimisation of magnetic MIPs for pepsin

Functional monomers are the key factor responsible for the selectivity of MIPs. It is very important to select a monomer capable of forming a strong and stable bond with the template, yet this bond should be readily broken using an appropriate solvent. Since MAA showed the strongest interaction in the prepolymerisation studies, it was tested alone or in combination with other monomers. MIPs and their corresponding NIPs were prepared from MAA, MAA with AA, MAA with Am, MAA with NIPAm, MAA with 2-aminoethyl methacrylate hydrochloride, and MAA with dimethylaminoethyl acrylate using MBA as crosslinker and deionised water as porogen. The mixture of MAA with either 2-aminoethyl methacrylate hydrochloride or NIPAm did not polymerise under the specified polymerisation conditions. The mixture of MAA with dimethylaminoethyl acrylate produced hydrophobic polymers that had very poor dispersibility in water which would compromise the application in aqueous media. Finally, **Figure 5** shows the difference in binding performance indicated by (Q) for the three MIPs prepared with MAA and their corresponding NIPs. It was concluded that the mixture of MAA with Am was the optimum combination of monomers.

Cross linkers are responsible for the structural rigidity of the produced polymers, which, in turn, allows MIPs to preserve their memory for a specific target. Moreover, not only the type but also the

concentration of cross linker is a parameter that can directly impact the selectivity. Hence, two cross linkers were tested, namely, MBA and EGDMA in different concentrations. From **Figure 6**, it is obvious that EGDMA provides a better binding than MBA. Nonetheless, when EGDMA was tested with MAA and Am as functional monomers, the binding was significantly reduced. Therefore, MAA was used alone as functional monomer with EGDMA as crosslinker which gave an unprecedent binding capacity for MIPs. Moreover, the concentration of EGDMA was optimised, as shown in **Figure 7**. The 4 mmol concentration gave a higher Q value for MIPs, however the 8 mmol concentration provided a better selectivity difference between MIPs and NIPs, therefore it was chosen as the optimum concentration.

Due to the limited solubility of pepsin in organic solvents, only water and aqueous buffers were tested as porogens. However, polymerisation in water is difficult since water molecules can disrupt the hydrogen bonds between the monomers and the template. Nevertheless, the careful choice of monomer, the presence of useful functional groups in both the monomer and the solid core, along with the appropriate preassembly time can help alleviate the problem. Upon testing deionised water and PBS buffer (pH 7.4) as porogens, deionised water gave slightly better rebinding results, so it was selected as the optimum solvent.

There are different types of initiators that can be used to start a free radical polymerisation, these include mainly azo initiators and oxidising agents [24]. Since the application of heat (mostly required for azo initiators) can affect the conformation of pepsin, oxidising agents with low activation energy were chosen for initiation. Mixtures of APS and TEMED, and potassium persulphate and sodium bisulphite were tested in different ratios. APS and TEMED mixtures required a much longer time to initiate the reaction in comparison to a persulphate and bisulphite mixture, therefore the latter was used in this work. Different ratios of bisulphite to persulphate were tested including 1:1, 2:1, and 1:2. The optimum ratio was 2:1 which initiated the reaction at reasonable speed and produced MIPs with the strongest binding, as shown in **Figure 8**.

The concentration of pepsin was also optimised as it is important to achieve an optimum ratio of monomer to target that would not compromise the selectivity. **Figure 9** shows that the optimum amount of added pepsin is 50 mg (~ 1.4 µmol). Moreover, the preassembly time between pepsin and the monomer is a critical factor that also needed to be estimated. Experiments with 0, 1, 2, and 3 hours of preassembly, showed that 1 hour was sufficient to achieve the strongest assembly, which was reflected in the binding of the corresponding MIPs, as shown in **Figure 10**.

Removal of template after polymerisation is a very important step to free the binding sites for further rebinding of target analyte in different samples. Incomplete template removal can result in reduced sensitivity of MIPs due to the limited availability of the binding sites. In addition, these trapped template molecules can cause template bleeding [25]. Therefore, it is important to ensure complete template removal though the selection of the best washing solvent, optimisation of the washing time and number of washing cycles. Solutions of oxalic acid (0.5 mol L^{-1}), PBS (0.02 mol L^{-1}, pH 7.4), sodium chloride (0.5 mol L^{-1}) and a mixture of 1%w/v SDS/10%v/v acetic acid were assessed for complete template removal. It was found that the mixture of SDS and acetic acid was the most successful in removing the template, indicated by the UV measurements of the washing solutions and the high selectivity of MIPs washed using this solution in comparison to the same MIPs washed with the other solutions. The optimum washing procedure was using ~ 75 ml of SDS/acetic acid solution with vigorous stirring for 4 hours followed by washing with deionised water 5 times to completely remove any traces of the SDS and acetic acid.

3.4. Characterisation of magnetic MIPs

3.4.1. Physical characterisation of magnetite and magnetic polymers.

SEM was applied to determine the surface morphology and relative size range of the developed magnetite nanoparticles, MIPs and NIPs. From the images shown in **Figure 11,** the formation of the polymeric layer on the surface of magnetite nanoparticles can be visually detected in **Figures 11c**

and 11d. This is further confirmed by the determination of particle size of magnetite, MIPs and NIPs. the measured diameter of amino functionalised magnetite was 75 nm, while that of NIPs and MIPs was 134 nm and 169 nm respectively. The increase in diameter of the polymer proves the surface imprinting on magnetite nanoparticles. Furthermore, the slightly bigger diameter of MIPs can be attributed to the presence of wider imprinted cavities in MIPs compared to NIPs.

3.4.2. Functional characterisation

a) Binding kinetics

From the graphical representation in **Figure 12a,** we can estimate that the maximum binding for MIPs occurs after 2 hours which can also be considered as the equilibrium time. The fast-binding kinetics can be attributed to the application of surface imprinting technique which confines all the binding sites on the surface to enable rapid binding and release. To be able to understand the binding mechanism of pepsin to the imprinted polymers, both pseudo first order and pseudo second order kinetics' models were computed to fit the adsorption data. From **Table 1,** we can depict from the value of R^2 that pseudo second order is a much better fit for the experimental data, which suggests that binding is ruled by a chemical adsorption mechanism.

Table 1. Binding kinetics parameters for the adsorption of pepsin on MIPs and NIPs applying two binding orders.

Pseudo first order parameters					
MIPs			**NIPs**		
K_1 (min $^{-1}$)	Qe (mg g^{-1})	R^2	K_1 (min $^{-1}$)	Qe (mg g^{-1})	R^2
0.00425	24.6	0.0426	0.00228	44.9	0.111
Pseudo second order parameters					
MIPs			**NIPs**		

K_2 (g mg^{-1} min^{-1})	Qe (mg g^{-1})	R^2	K_2 (g mg^{-1} min^{-1})	Qe (mg g^{-1})	R^2
0.0396	152	0.997	0.0520	127	0.992

K_1 and K_2 are the first and second order rate constants respectively, Qe is the amount of pepsin adsorbed per gram of polymer at equilibrium, and R^2 is the linearity coefficient.

b) Binding isotherm

The results of the batch adsorption experiment are shown in **Figure 12b** in which a linear relationship between the concentration of pepsin and Q was observed. This linear relationship indicates that the adsorbed amount increases with increasing the concentration until a maximum further concentration after which all the binding sites will be saturated. The binding isotherm data was fitted into Langmuir and Freundlich isotherm models to determine if the adsorption of the target is in monolayer or in multiple layers and if the binding sites are homogenous or heterogenous in nature. From **Table 2** the experimental data fits better with the Langmuir adsorption isotherm suggesting that the adsorption of pepsin on the surface of MIPs is in a monolayer form on homogenous binding sites.

Table2. Adsorption isotherm parameters of MIPs and NIPs applying two models.

Langmuir isotherm							
MIPs				**NIPs**			
K_L (L mg^{-1})	Q_{max} (mg g^{-1})	RL	R^2	K_L (L mg^{-1})	Q_{max} (mg g^{-1})	RL	R^2
5.45	770	0.234	0.981	17.2	355	0.132	0.963
Freundlich isotherm							
MIPs				**NIPs**			
n	K_F		R^2	n	K_F		R^2
2.37	509		0.935	1.69	937		0.941

K_L and K_F are the Langmuir constant and Freundlich constant respectively, Q_{max} is the theoretical maximum adsorbed concentration, RL is the separation factor (1/1+C_{eq}.KL), n is the variation trend coefficient for the adsorption isotherm, and R^2 is the linearity coefficient.

c) Binding selectivity

In order to confirm the selectivity of the developed MIPs to pepsin, the binding of optimum MIPs was compared to the binding of the corresponding NIPs to calculate the imprinting factor (IF).

$$IF = \frac{Q_{MIPs}}{Q_{NIPs}}$$

The value of IF at the concentration of 0.5 mg mL^{-1} of pepsin was 1.34 indicating a significant difference in selectivity between MIPs and NIPs.

However, for a further confirmation of the selectivity of MIPs, it is better to compare their binding with the target to the binding with other competitive analytes. Amylase (58.4 kDa) and lipase (48 kDa) were chosen as competitive analytes as they are naturally occurring enzymes in the human saliva. The selectivity factor (α) for every competitor analyte was calculated using the following equation.

$$\alpha = \frac{Q_{MIP.target}}{Q_{MIP.competitor}}$$

The values of α were 3.17 and 9.52 for amylase and lipase respectively, indicating higher selectivity towards pepsin.

d) Reusability study

The developed MIPs were tested for the number of times they can be used to adsorb and desorb pepsin with no loss in the binding capacity or selectivity as well as the magnetic quality. From **Figure 13** it can be observed that the binding of MIPs remains almost the same during the first three cycles of usage, however, it starts to decline after the fourth cycle, reaching a much lower value on the fifth cycle. Moreover, the developed polymers did not show any reduction in the magnetic properties and

could still be completely separated from solution using neodymium magnet. Therefore, MIPs can be used safely for three consecutive cycles, which contributes to the economic value of the developed MIPs and make them suitable for commercial applications.

3.4.3. Thermal characterisation

TGA thermographs represented in **Figure 14a** for both MIPs and NIPs, do not show any noticeable difference between both curves, which is expected as there is no chemical structural difference between the two materials. Decomposition occurs at almost 430 °C accompanied by almost 75% mass loss followed by a plateau corresponding to the mass of the residual magnetite. As for DSC analysis, there are two endothermic peaks observed for both MIPs and NIPs, again with no noticeable difference, as illustrated in **Figure 14b**. The first peak at 90 to 100 °C corresponds to evaporation of moisture, while the second peak corresponds to the melting of the polymeric layer at around 257 °C. The curve for amino functionalised magnetite shows only one endothermic peak representing the evaporation of moisture similar to the peak observed for NIPs and MIPs.

3.5. Dispersive solid phase extraction of pepsin from aqueous solutions

The conditions for extraction and elution of pepsin using the developed magnetic MIPs were studied wisely. Different eluting solvents were tested for their ability to completely elute pepsin adsorbed to MIPs. Water was tested alone and with application of ultrasound, however in both cases the elution was not satisfactory. This can be attributed to the fact that it is very difficult to completely extract an analyte from water to water. Ultrasonic waves may have improved the extraction, yet it was still not high enough and pepsin molecules were still trapped inside the MIPs' binding sites. 0.01 mol L^{-1} HCl (pH ~2.4) was tested as an eluting solvent, but the elution was worse than that obtained by water. Finally, PBS (0.02 mol $L^{-}1$, pH 7.4) buffer was tested and here the recovery of pepsin reached ~ 85%, which was greater than all the obtained values from the other tested solutions and hence it was selected as the eluting solvent. In addition to trying different eluting solvents, different elution

times (0.5, 1, 2 and 3 hours) were tested to choose the time corresponding to the complete release. It was found that the optimum time corresponding to maximum release was 1 hour. As a result, the optimum elution process was to stir MIPs with PBS (0.02 mol $L^{-}1$, pH 7.4) for 1 hour, after which all the bound pepsin is released. The short time of release is considered an advantage for the developed MIPs which again highlights the benefit of applying surface imprinting technique.

3.6. Optimisation and validation of a bioanalytical HPLC-SEC method for pepsin

Due to the use of SEC column for HPLC analysis of pepsin, the tested mobile phases were limited to aqueous solutions only. It was noted that the peak of pepsin became very distorted at high pH values in the range (4.0 to 7.5) however, the shape of the peak got significantly better at lower pH values (2.5 to 3.5) with pH 3.0 being the optimum value. This could be attributed to the unfolding of the protein structure at higher pH values compared to the lower pH in which it normally exists in the stomach (~pH 2.0). Addition of ionic strength modifiers such as 1 mol L^{-1} sodium chloride to the buffer solution as well as the use of higher or lower concentrations of the buffer solution itself (0.01, 0.05 or 0.1 mol L^{-1}) did not contribute much to peak shape improvement. Therefore, after careful optimisation and testing, the optimum parameters for the HPLC-SEC were the use of 0.02 mol L^{-1} phosphate buffer (pH 3.0) at a flow rate 0.35 mL min^{-1}, injection volume was 20 µL, UV detection was performed at 210 nm, and the total run time was 11 minutes in which pepsin eluted at 6.9 minutes while the internal standard eluted at 9 minutes.

ICH guidelines [23] were followed in the validation of the method regarding linearity, accuracy, precision, and robustness with some limitations to avoid damage to the packing of the column that can result from higher flow rates or the use of very low or very high pH values. All statistical analysis was performed using Origin™ 8.5 software at a 5% significance level. Linearity was determined by plotting peak area ratio (peak area of pepsin to that of internal standard) versus concentration in the range of (0.5 to 150 µg mL^{-1}). Accuracy at three different concentrations along the calibration range

with triplicates of each concentration was expressed as percent recovery of pepsin from the linear fit equation. RSD% was used as indicative of precision along the same three concentration levels injected within the same day (intra-day) and within three different days (inter-day). Minor changes applied to the chromatographic conditions such as flow rate, pH and concentration of mobile phase did not affect the method performance as indicated by calculating the percentage of recovery obtained from each minor change from the linear fit equation. Sensitivity of the developed method was determined practically by measuring LOD and LOQ from the signal to noise (S/N) ratio as conducted before in one of our previous works [26], where LOQ was found to be 0.25 µg mL^{-1} and LOD was 0.10 µg mL^{-1}. The collective values for method validation are tabulated in **Table 3**.

Table 3. Validation parameters of the proposed HPLC-SEC method in standard solutions of pepsin.

Linearity range (µg mL^{-1})	**Intercept (a)± SD[a]**	**Slope (b)± SD[b]**	**S_{yx}[c]**	**R[d]**	**LOD (µg mL^{-1})[e]**	**LOQ (µg mL^{-1})[f]**
0.5 - 150	0.103± 0.0350	0.0406± 0.001	0.0770	0.9989	0.10	0.25
Accuracy as percentage recovery of pepsin and precision as relative standard deviation (RSD)						
Concentration (µg mL^{-1})	**%Recovery ± SD**	**Intra-day RSD % of recovery**	**Inter-day RSD % of recovery**			
5	100.22 ± 2.68	0.027	2.01			
50	98.04 ± 1.21	1.26	0.88			
100	99.95 ± 2.17	2.17	1.55			
Robustness as percentage recovery of pepsin at 50 µg mL^{-1}						
Experimental parameter		**%Recovery ± SD**				
pH of buffer 2.8 3.2		 100.46 ± 1.61 99.51 ± 0.38				
Conc. of phosphate buffer (mol L^{-1}) 0.01		 102.27 ± 0.51				

0.03				100.41 ± 1.97		
Flow rate (mL min^{-1}) 0.30				99.82 ± 0.45		
System suitability						
Analyte	**k[g]**	**α[h]**	**Rs[i]**	**N[j]**	**HETP[k] x 10^{-3}**	**T$_f$[L]**
pepsin	2.45	1.42	3.80	1009.90	14.85	1.75
IS	3.50			22898.98	0.65	0.16

[a] Standard deviation of the intercept, [b] Standard deviation of slope, [c] Sum of square errors, [d] R is the correlation coefficient, [e] LOD is limit of detection, [f] LOQ is limit of quantitation, [g] Retention factor, $k = t_R - t_0/t_0$, [h] Separation factor (α) between pepsin and IS peaks = $t_{R2} - t_0 / t_{R1} - t_0$, [i] Resolution factor (Rs) between pepsin and IS peaks = $2(t_{R2} - t_{R1})/ (w_1 + w_2)$, [i] Number of theoretical plates (N) = $16(t_R/w)^2$, [k] Height equivalent to theoretical plates = length of column/ N and [L] Tailing or asymmetry factor, Tf = (a + b)/2a. Where t_0 is the dead time, t_{R1} and t_{R2} are the retention times and w_1 and w_2 the baseline peak width of successive peaks.

3.7. Application to human saliva samples

The developed MIPs were successfully applied for the extraction of pepsin from the spiked saliva samples prior to quantitation via the developed HPLC-SEC method. **Figure 15** shows a representative chromatogram of pepsin (25 µg mL^{-1}) and the internal standard (2.5 µg mL^{-1}) in a saliva sample. A calibration curve was constructed for spiked pepsin in saliva after subtraction of blank saliva readings and the linearity parameters were computed and presented in **Table 4**. Moreover, to verify the applicability and selectivity, percentage of recovery for three different pepsin concentrations from saliva were computed from the linear fit equation using MIPs and NIPs as extraction media and presented as well in **Table 4**. From the results in table 4, it is obvious that MIPs provide much higher recovery compared to NIPs.

Table 4. Linearity parameters of the proposed HPLC-SEC method and percentages of recovery of pepsin in saliva samples using MIPs and NIPs.

Linearity range (μg mL^{-1})	Intercept (a)± SD[a]	Slope (b)± SD[b]	S_{yx}[c]	R[d]	LOD (μg mL^{-1})[e]	LOQ (μg mL^{-1})[f]
5 - 150	3.940 ± 0.006	0.035 ± 0.001	0.068	0.9990	0.598	1.813

Recovery of spiked pepsin from saliva samples

Concentration (μg mL^{-1})	Average total amount found (μg mL^{-1})		Average% recovery ± SD		RSD %	
	MIPs	NIPs	MIPs	NIPs	MIPs	NIPs
10	9.74	4.90	95.74 ± 0.67	49.01±1.42	0.70	2.91
50	49.33	15.51	98.66 ± 1.32	31.03±0.64	1.32	2.08
100	97.31	33.16	97.31 ± 0.19	33.16±1.26	0.19	3.80

[aa] Standard deviation of the intercept, [b] Standard deviation of slope, [c] Sum of square errors, [d] R is the correlation coefficient, [e] LOD is limit of detection, [f] LOQ is limit of quantitation.

The developed method is compared to previously published methods for the analysis of pepsin in **Table 5**. From the data shown in the table we can emphasis the comprehensiveness of our method. Among all the previously reported methods, our system is the only one that provides a complete extraction and quantitation profile of pepsin which is further applied in human saliva samples. Moreover, our method is very simple, made from cost effective starting materials and can be easily applied for accurate determination of pepsin.

Table 5. Comparison of the developed method against previously reported ones.

Technique of imprinting	Composition of MIPs	Linear range	LOD	Application	Ref.
Mini-emulsion polymerisation	(3-acrylamidopropyl)-trimethylammonium	N/A	N/A	The method was only applied to extract	[15]

	chloride as a functional monomer, EGDMA as crosslinker.			pepsin from standard solutions to study their binding behaviour.	
Surface imprinting on magnetic nanoparticles	NIPAm was used as main functional monomer and MBA as crosslinker.	10 – 50 μg mL^{-1}	Not mentioned.	The method was applied to extract pepsin from synthetic gastric juice samples.	[17]
Solid phase synthesis with fluorescent dyes.	NIPAm was used as main functional monomer and MBA as crosslinker.	1 – 100 μmol L^{-1}	1 μmol L^{-1}	The method was applied to extract pepsin from standard solutions and compare cross reactivity.	[18]
Surface imprinting on magnetic nanoparticles	MAA was used as functional monomer and EGDMA as crosslinker.	0.5 – 150 μg mL^{-1}	0.1 μg mL^{-1}	The developed MIPs were applied to extract pepsin from standard solutions and human saliva samples followed by quantitation via HPLC-SEC method.	Our method

1

2 3.8. Potential application of magnetic MIPs in treatment of GERD and ulcers

As we mentioned before, pepsin contributes to the damage to the gastric mucosa in the cases of peptic ulcers [2]. Therefore, denaturing excess pepsin can help with the symptoms, reduce the pain, and have a great therapeutic value. Herein, magnetic MIPs can be very useful due to the presence of magnetic iron oxide in their core. Magnetic hyperthermia is a property that relies on the use of alternating magnetic field (AMF) to induce magnetic nanoparticles to release heat. Hence, due to the presence of magnetite in the core of MIPs, applying AMF after binding with pepsin can cause heat denaturation of excess pepsin in the stomach in a very simple, non-invasive and easily controlled process. This procedure has been proven effective before to denature green fluorescent protein with great success [27]. Therefore, the combined use of MIPs to selectively target pepsin, and magnetite in their core to inhibit it, can be a very promising tool to efficiently help patients with GERD after appropriate toxicity testing.

4. Conclusion

In this study, new magnetic MIPs were developed for the selective binding and extraction of pepsin enzyme. The produced magnetic polymers provided the advantages of being selective, reproducible, reusable, easily synthesized, and cost effective. Thorough optimisation of all synthesis parameters was conducted to ensure maximum selectivity and highest efficiency of separation. In addition, magnetic MIPs were characterised by different methods to prove their validity including morphological, functional, and thermal characterisation methods. Moreover, as a proof of function, magnetic MIPs were applied for selective extraction of pepsin from standard solutions and saliva samples via dispersive solid phase extraction. Besides, the presence of magnetic core allowed for very easy and rapid separation after extraction. Furthermore, to quantify the extracted pepsin, a novel, highly sensitive, HPLC method was developed for the quantitation of pepsin, which along with the application of size exclusion chromatography allowed for clean and reproducible chromatograms. The developed HPLC-SEC method was optimised and validated according to ICH guidelines and applied successfully in conjunction with the produced MIPs to extract and quantify pepsin in saliva

samples. Finally, we can say that the combined use of the developed MIPs and the HPLC-SEC method could provide a highly promising and an effective diagnostic tool for patients with GERD through a very simple, non-invasive and rapid procedure.

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Conflict of interest

Authors declare no conflicts of interests.

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Figures

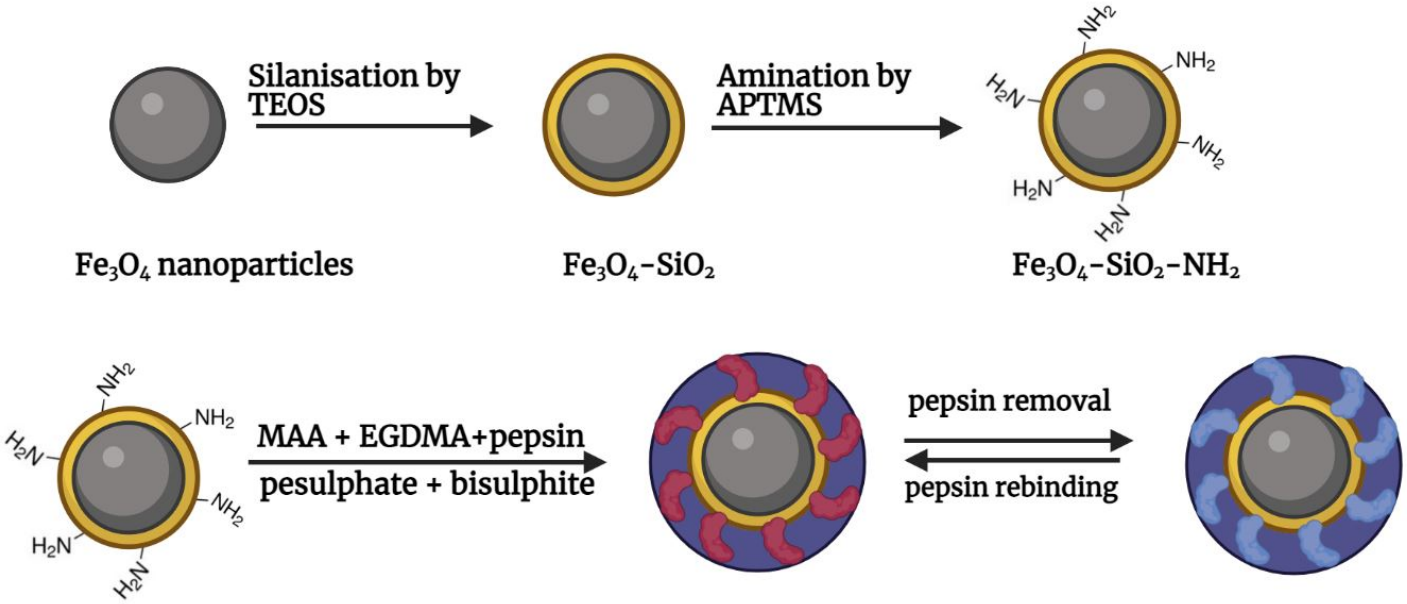

Figure 1. Functionalisation of magnetite and synthesis of magnetic molecularly imprinted polymers.

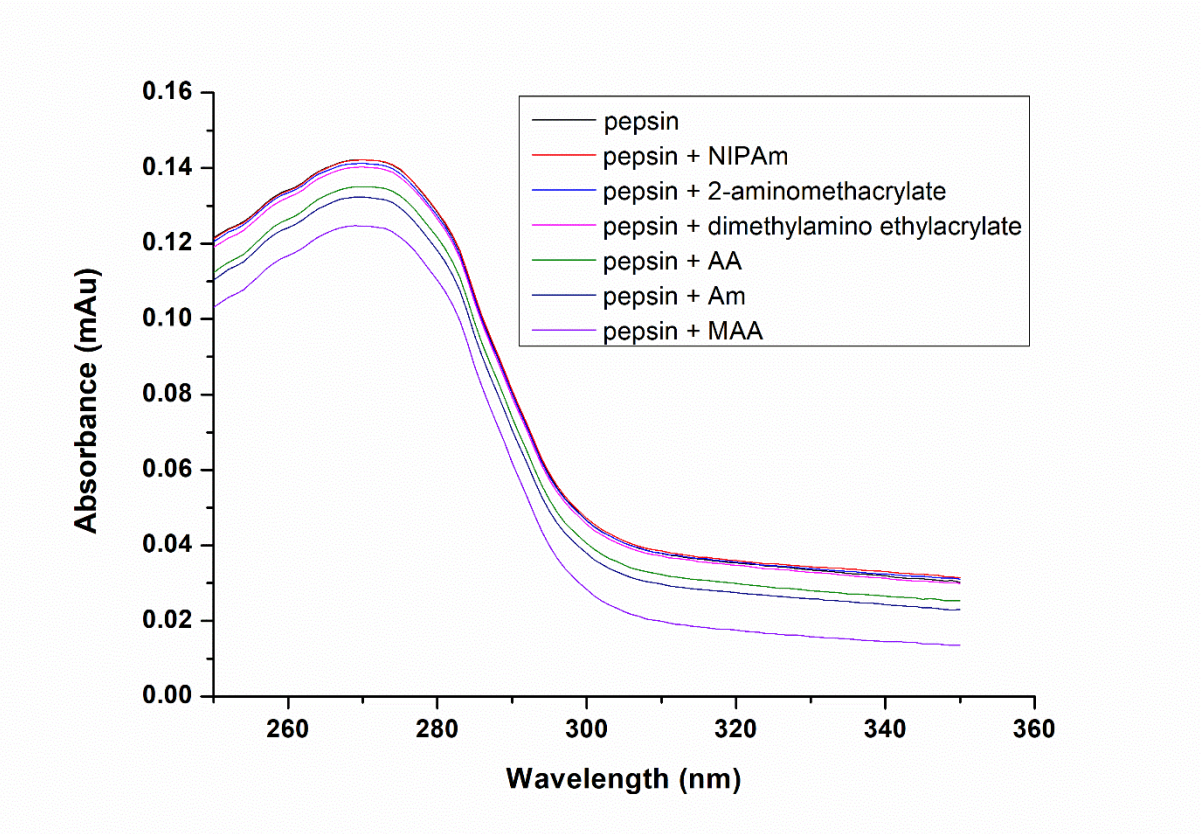

Figure 2. UV spectra of pepsin solution titrated with potential monomers.

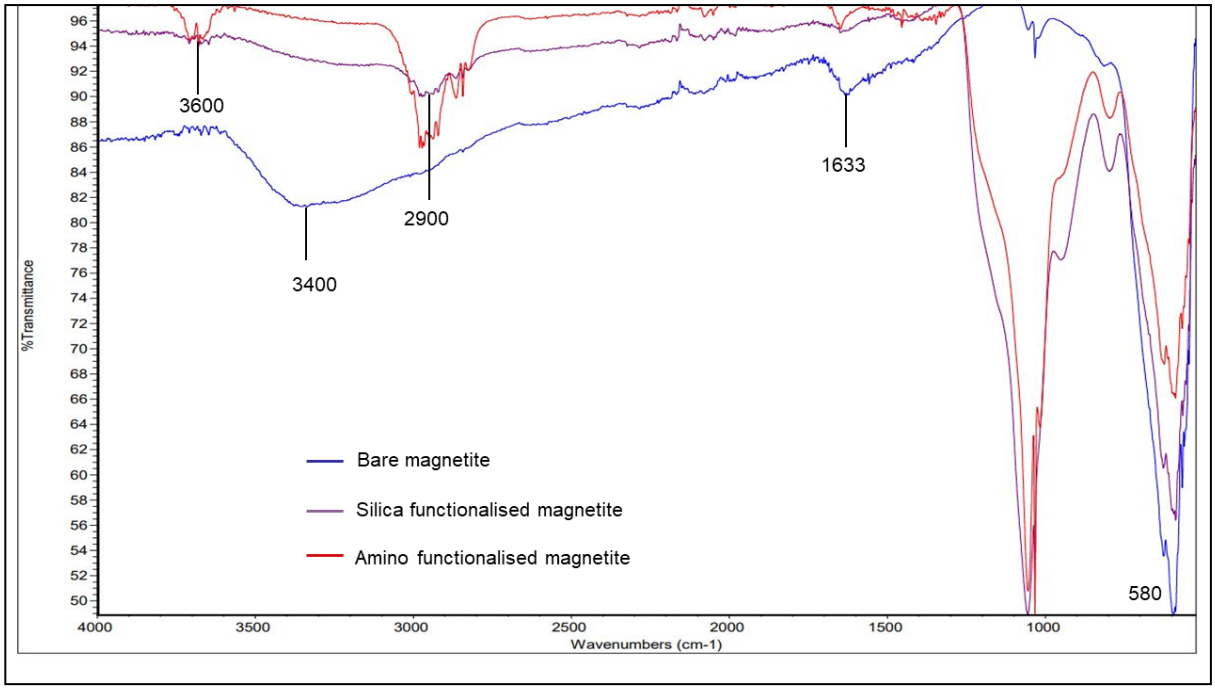

Figure 3. Infrared spectra of bare magnetite, silica functionalised magnetite (Fe_3O_4-SiO_2), and amino functionalised magnetite (Fe_3O_4- SiO_2-NH_2).

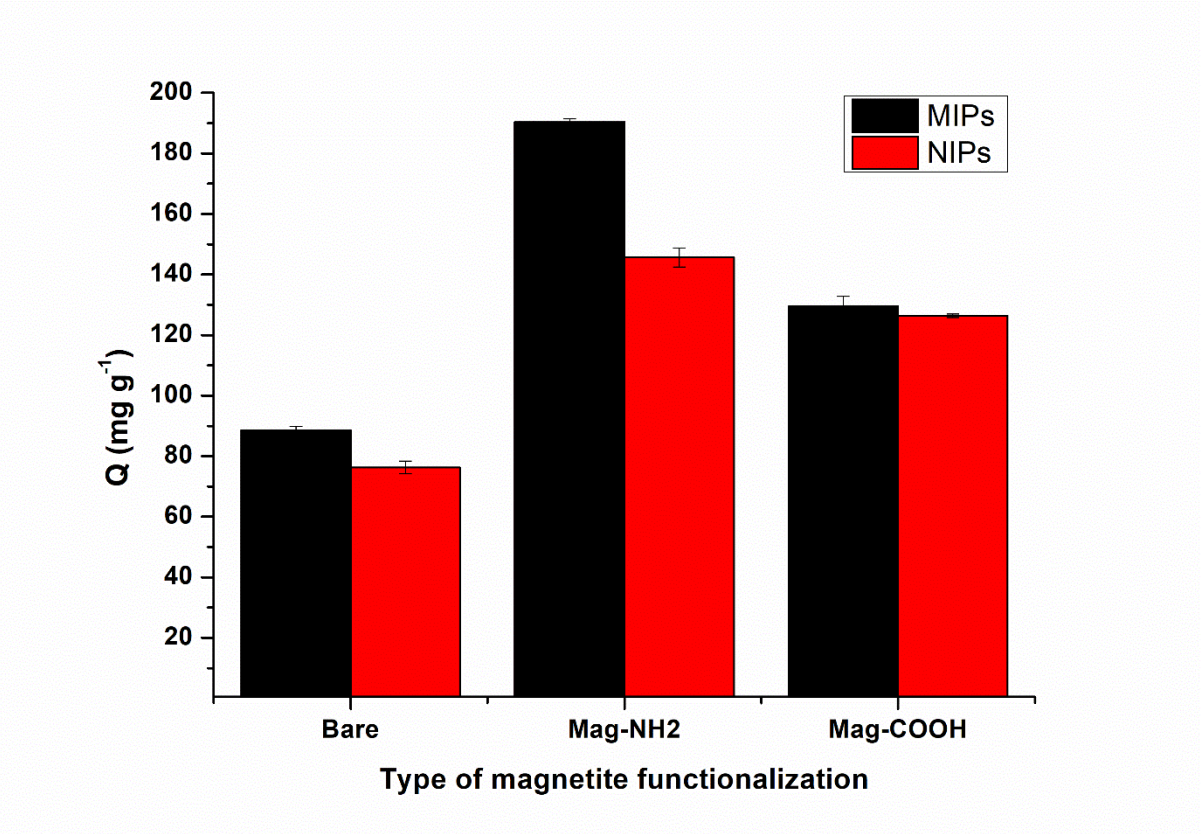

Figure 4. Optimisation of the type of magnetite functionalisation.

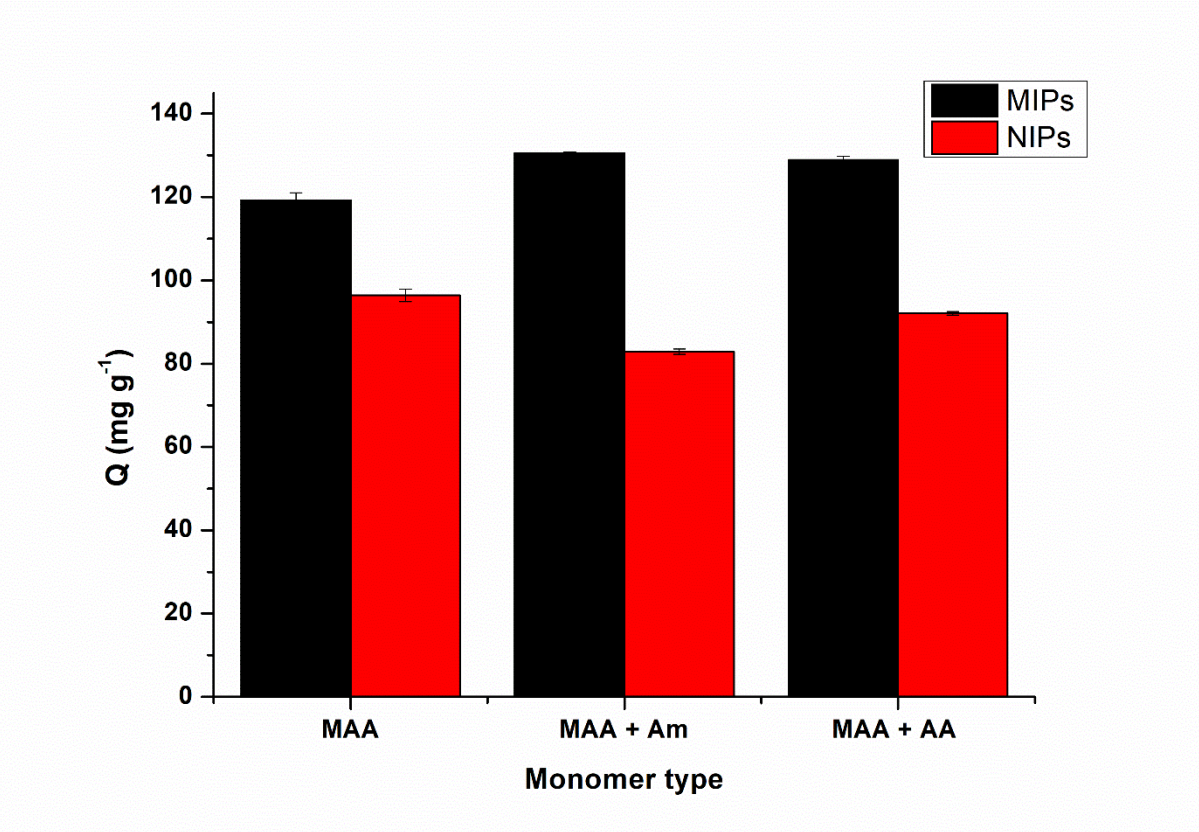

Figure 5. Optimisation of the type of monomer.

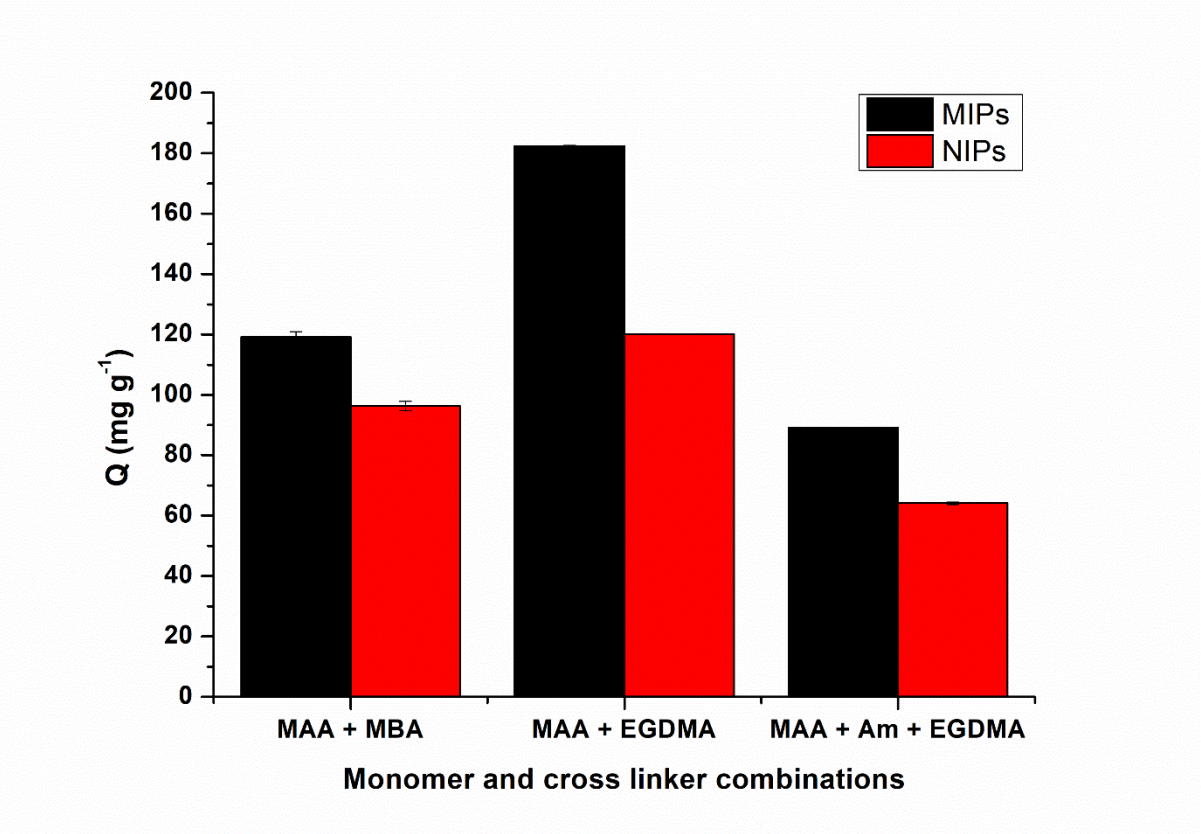

Figure 6. Optimisation of the type of crosslinker.

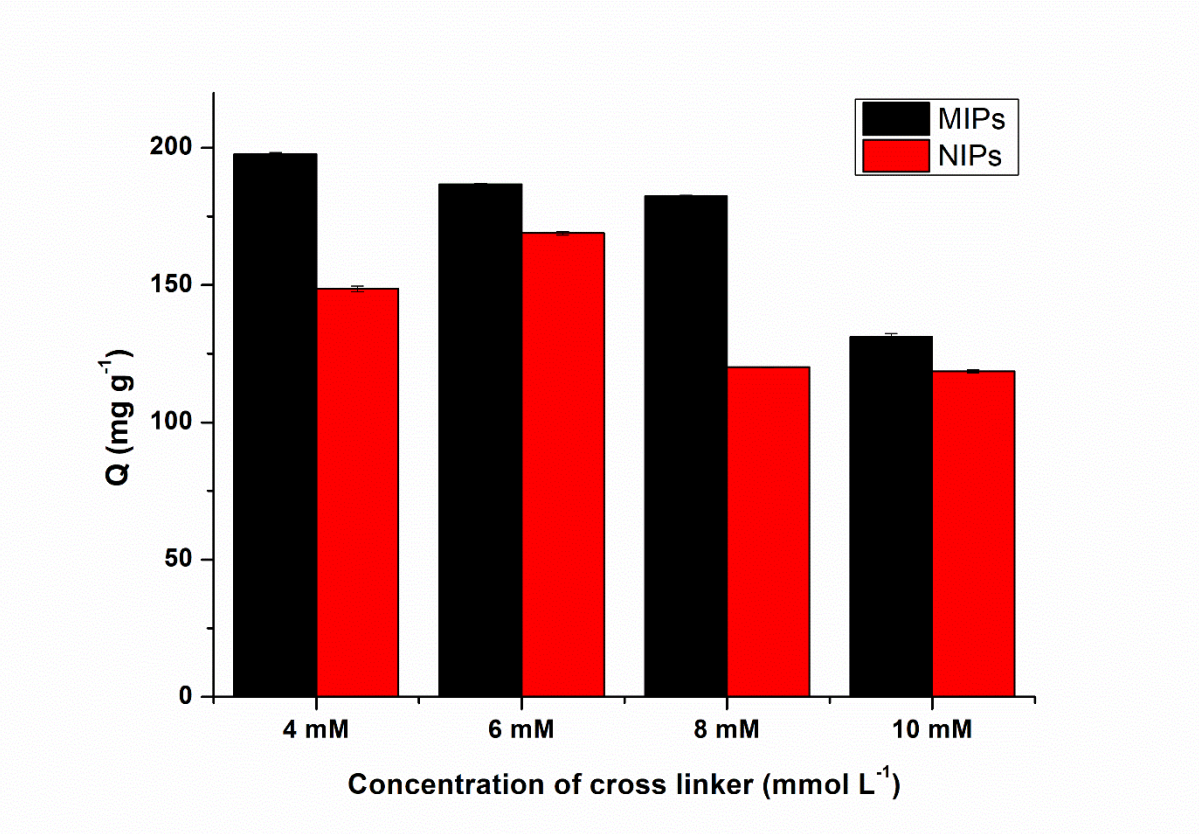

Figure 7. Optimisation of the concentration of crosslinker.

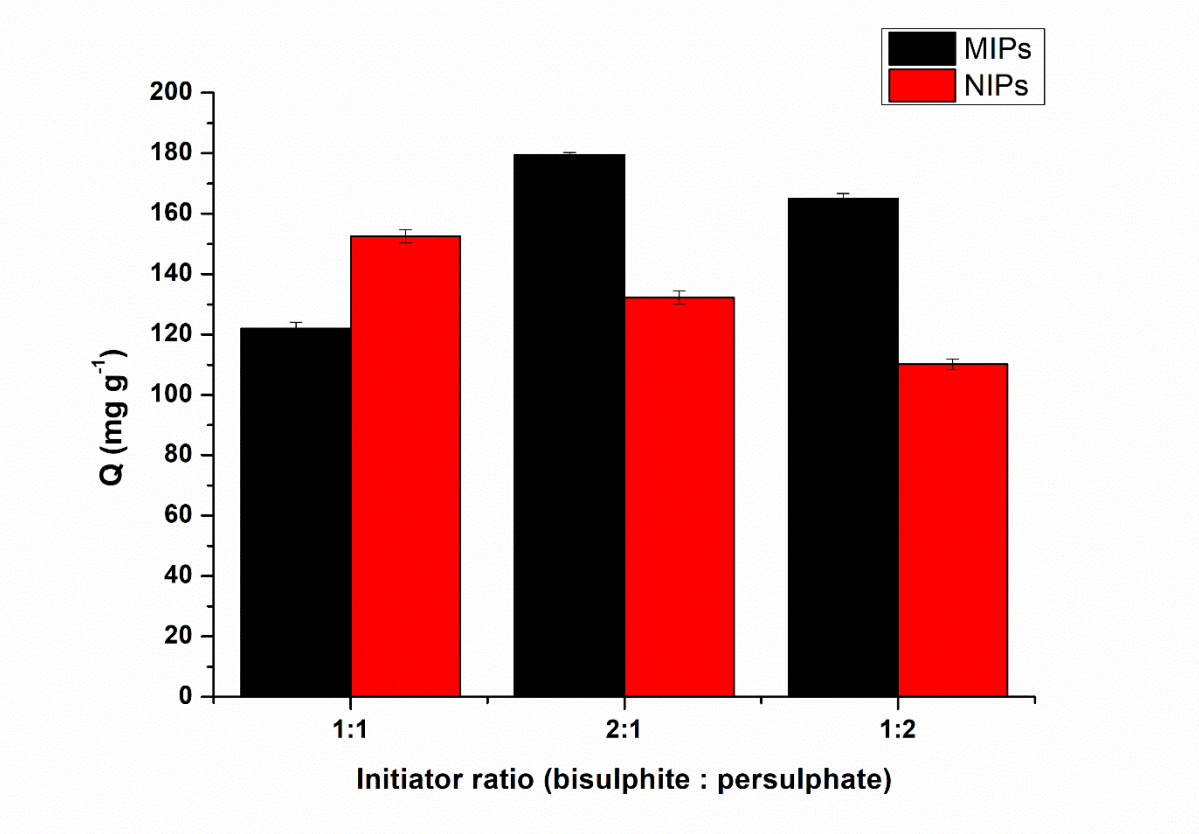

Figure 8. Optimisation of the ratio of initiators.

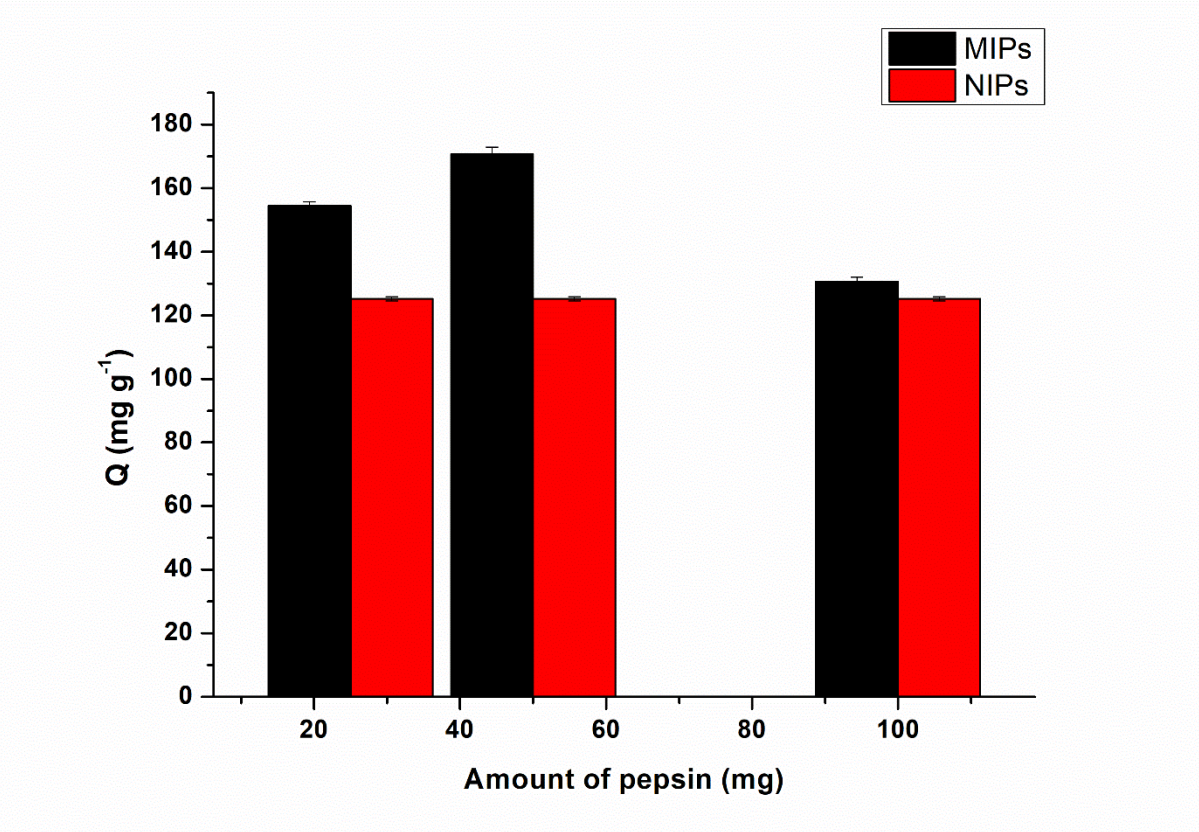

Figure 9. Optimisation of the amount of pepsin.

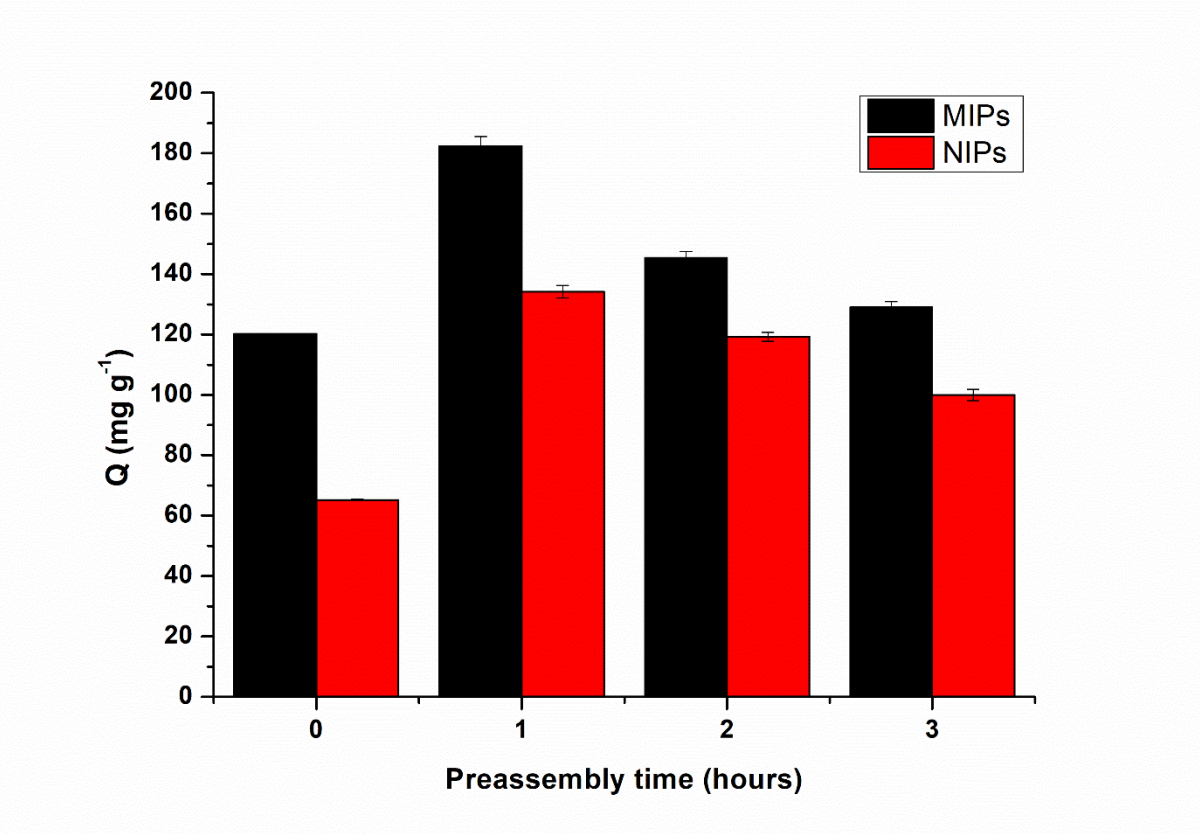

Figure 10. Optimisation of the preassembly time.

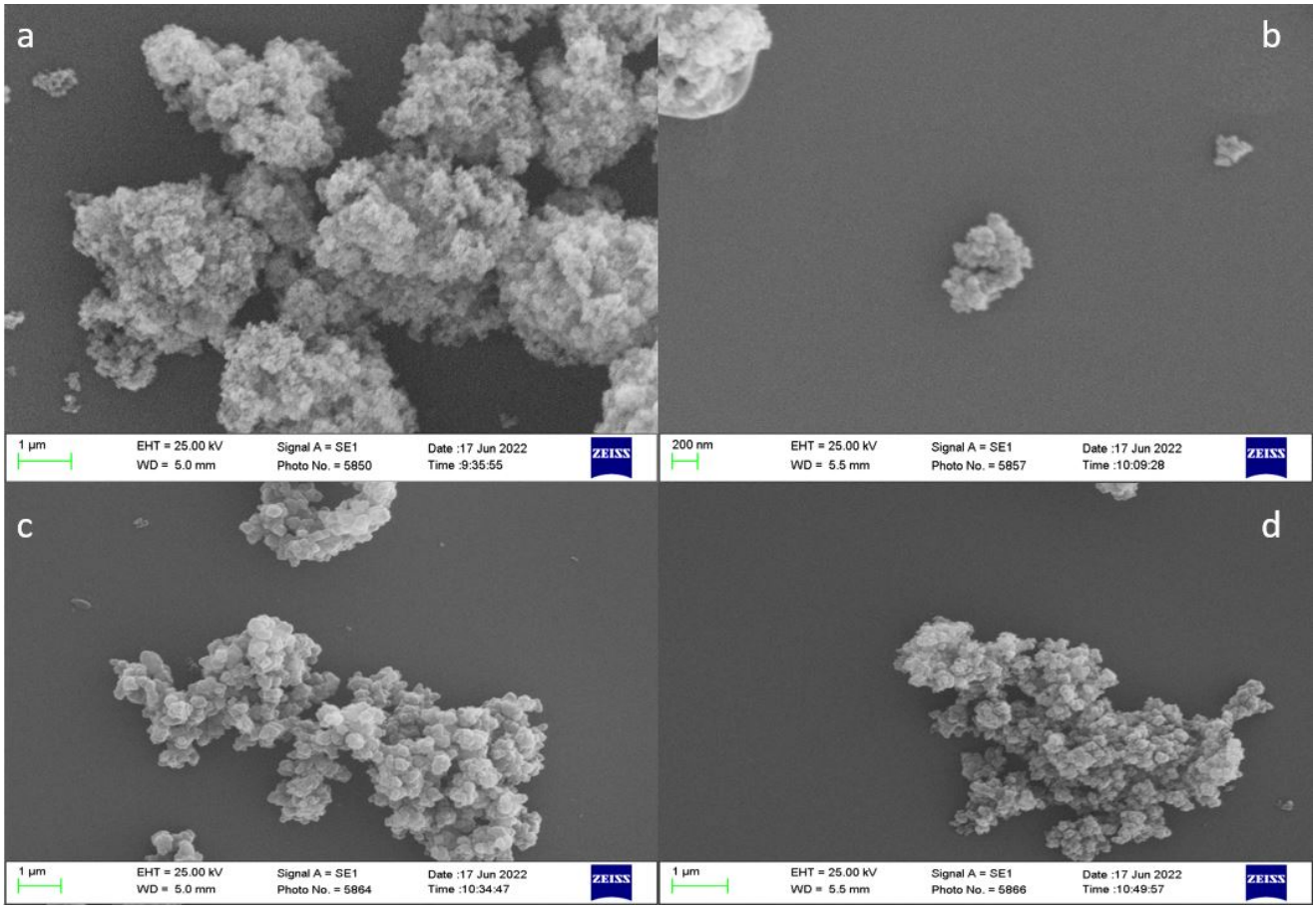

Figure 11. SEM pictures of (a) bare magnetite (Fe_3O_4), (b) amino functionalised magnetite (Fe_3O_4-SiO_2-NH_2), (c) magnetic MIPs, (d) magnetic NIPs.

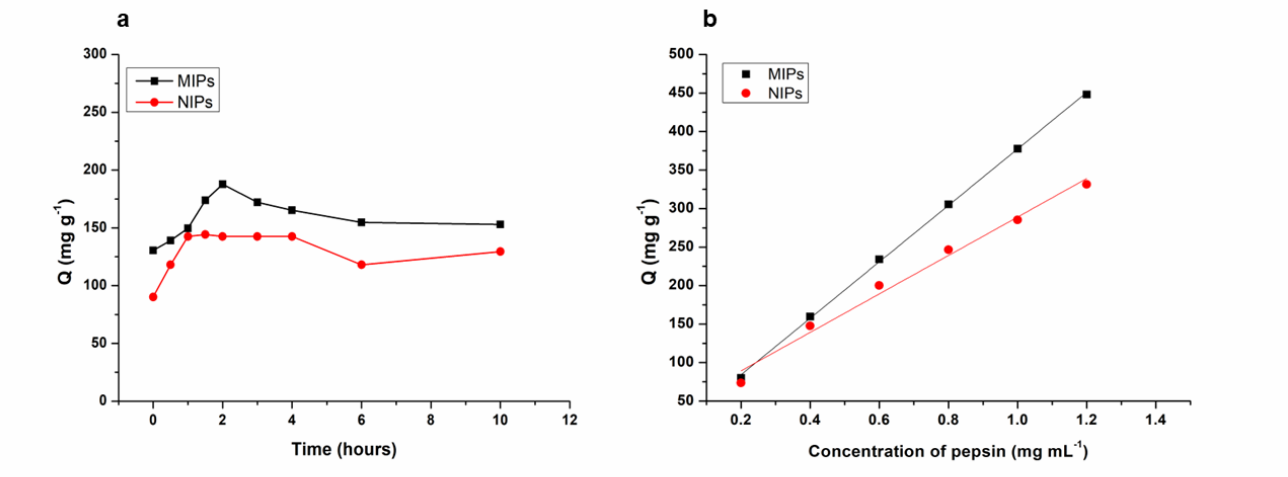

Figure 12. Functional characterisation (a) binding kinetics and (b) binding isotherm for both MIPs and NIPs.

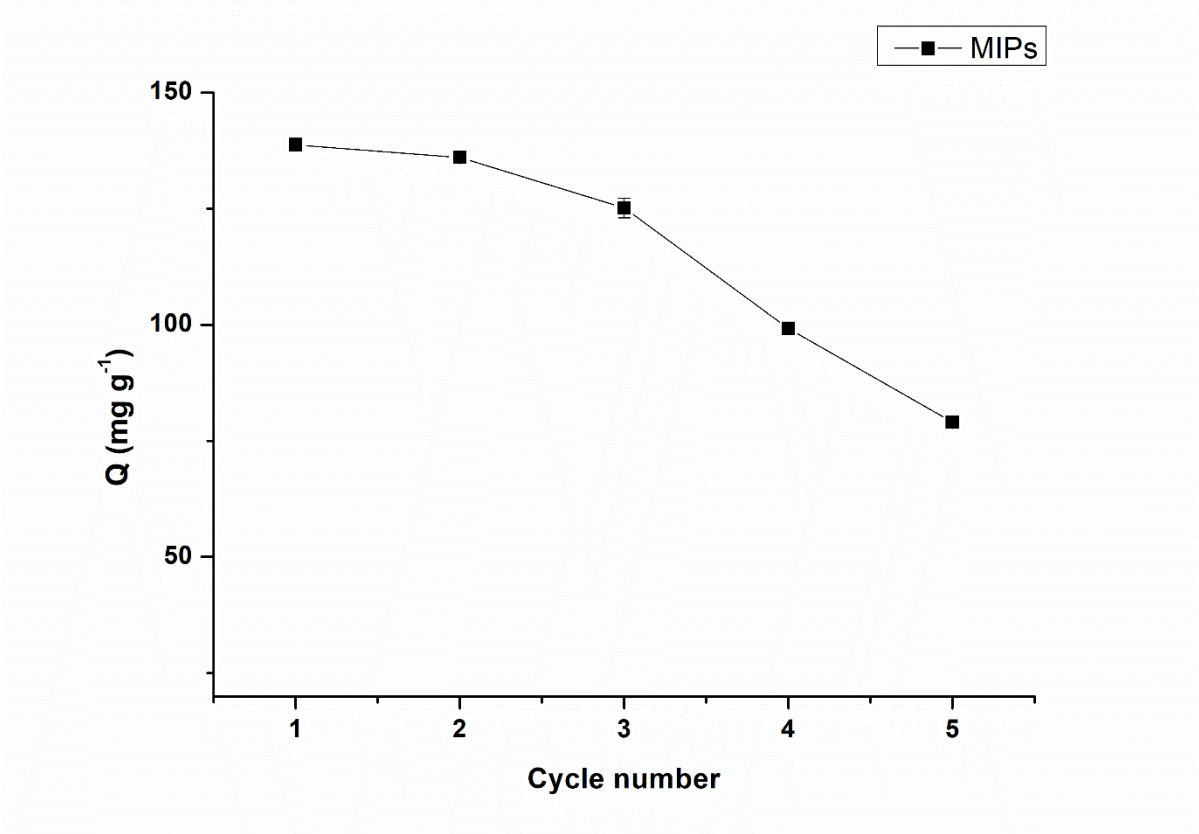

Figure 13. Reusability of the developed MIPs expressed as cycles of adsorption and desorption.

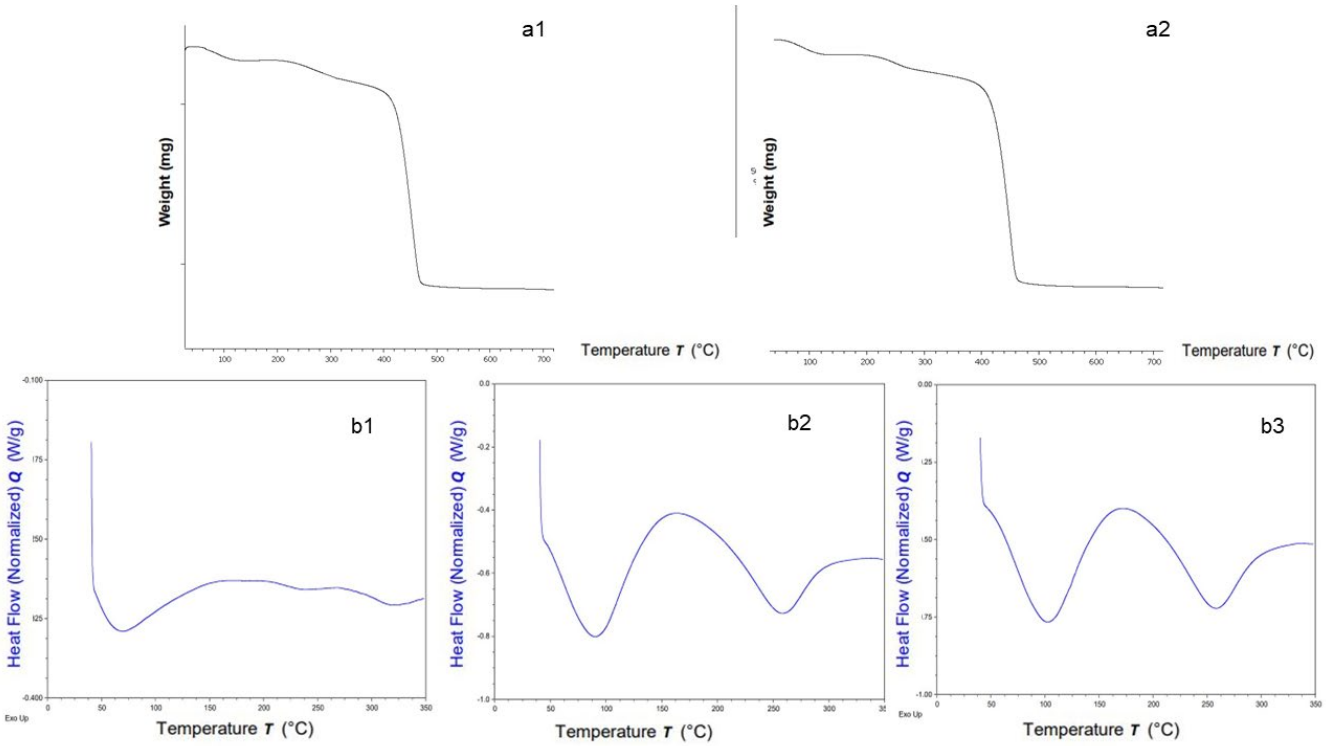

Figure 14. Thermal characterisation (a1) TGA thermograph of MIPs (a2) TGA thermograph of NIPs. (b1) DSC curve for (Fe_3O_4-SiO_2-NH_2), (b2) DSC curve for MIPs, (b3) DSC curve for NIPs.

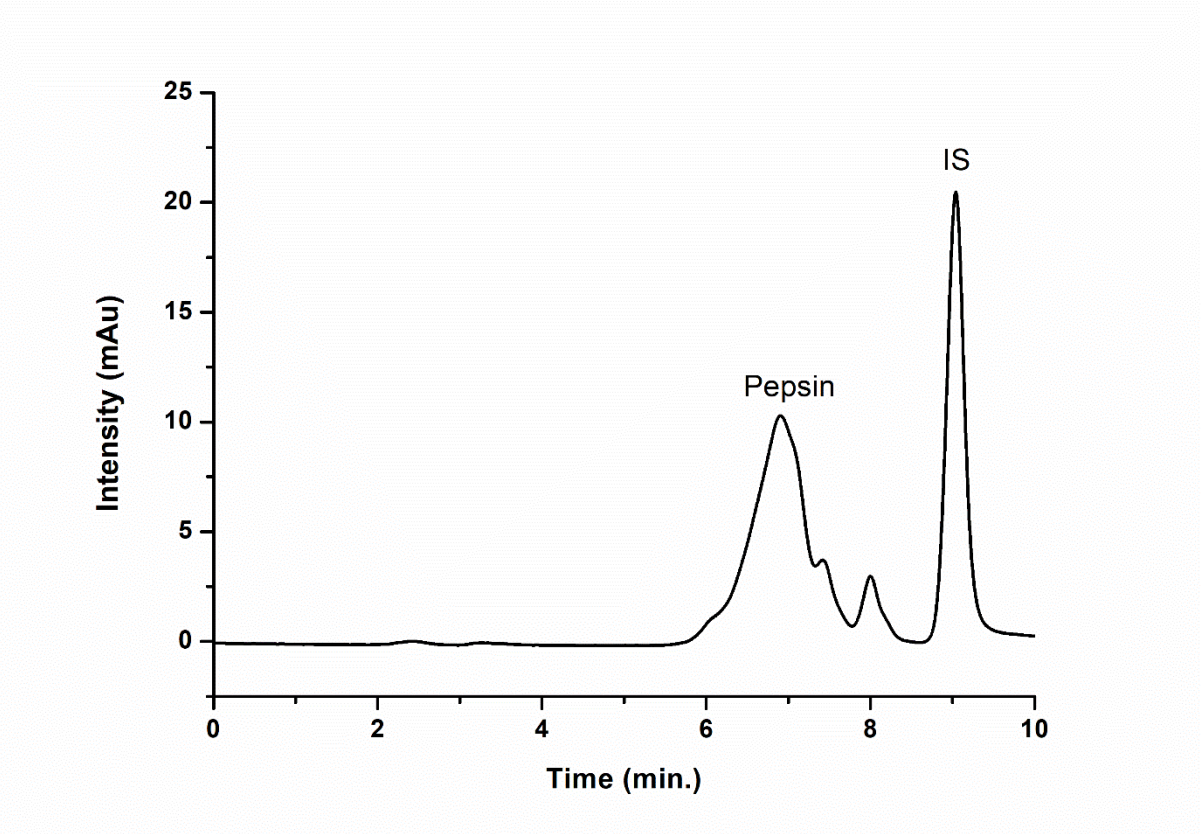

Figure 15. HPLC-SEC chromatogram of pepsin and internal standard in saliva at a concentration of 25 µg mL^{-1} and 2.5 µg mL^{-1} respectively.