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MIP-based Protein Profiling: A Method for Interspecies Discrimination

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Abstract

Due to recent public concern and interest in the authenticity and origin of meat, for example, the 2013 “horsemeat scandal” in the human food chain, novel sensor strategies for the discrimination between protein species are highly sought after. In this work, molecularly imprinted polymers (MIPs) are utilised for protein discrimination using electrochemical sensor and spectrophotometric techniques. MIP selectivity between two proteins of similar molecular weight (haemoglobin and serum albumin) were compared across three different species, namely pork, beef and human. Bulk MIPs resulted in K_d and B_{max} values of 184±23 µM, and 582 µmol g^{-1} for BHb, 246.3±26 µM, and 673 µmol g^{-1} for HHb; 276±31 µM, and 467 µmol g^{-1} for PHb. With the aid of chemometrics, i.e. multivariate analysis and pattern recognition, distinctive protein profiles have been achieved for species discrimination in both spectrophotometric and electrochemical analysis experiments. MIP suitability and selectivity within complex matrices was also assessed using urine, human plasma and human serum. Pattern recognition MIP-based protein profiling demonstrated positive outputs yielding either a ‘bovine’ or ‘not-bovine’ outcome (p = 0.0005) for biological samples spiked with/without bovine using respective bovine haemoglobin MIPs.

Key Words
Molecularly imprinted polymers (MIPs); electrochemical biosensor; protein profiling; interspecies discrimination
Introduction

Proteins are essential parts of organisms and participate in virtually every process within cells. A large number of proteins are vital markers of disease. For example, mutations in genes that encode for the protein's subunits result in hereditary diseases such as sickle cell anaemia, thalassaemia, and haemoglobinopathies. The development of biosensor strategies for the detection of proteins is therefore imperative for applications in proteomics, medical diagnostics, and pathogen detection.

In the past decade, molecularly imprinted polymers (MIPs) have been developed for the imprinting of proteins, and are rapidly becoming viable alternatives to natural antibodies for sensor technology. MIPs offer many advantages in terms of shelf-life, stability, robustness, cost, and ease of preparation. However, the imprinting of large biomacromolecules, such as proteins, presents a variety of challenges. Proteins are relatively labile and have changeable conformations that are sensitive to various factors (e.g., solvent environments, pH, salt, and temperature). Due to the large size of proteins (~6000 Da to several million Da) it is essential to control the size and number of pores that are generated (in the bulk and on the surface) during MIP synthesis, together with the density of MIP network.

Takeuchi et al. previously demonstrated the use of a chemometric strategy via principle component analysis (PCA) for molecular recognition and classification of five proteins using plural imprinted acrylic acid and 2-dimethylaminoethyl methacrylate polymers. Six different protein-imprinted polymers were synthesized using three template proteins, cytochrome C (Cyt), ribonuclease A (Rib) and α-lactalbumin (Lac), and acidic or basic functional monomers of acrylic acid and 2-dimethylaminoethyl methacrylate (DMA) respectively. The resulting MIPs produced unique fingerprints when rebound with both corresponding and non-template (albumin and myoglobin) proteins. Three-dimensional PCA scores of the binding assay MIP data revealed that a clear protein distinction was possible, and that protein-imprinted polymer arrays can be applied to protein profiling by pattern
analysis of binding activity for each polymer. In our previous work, Bueno et al. also demonstrated the use of pattern recognition techniques to uniquely identify protein profiles by coupling electrochemical sensor strategies with hydrogel-based MIPs. They also used PCA techniques to discriminate between electrochemically and non-electrochemically active proteins by diffusion through MIP slurries immobilised at the surface of glassy carbon electrodes (GCE). In a bid to move away from bulk imprinting and the laborious need to form granular particles, Wu et al. successfully demonstrated the feasibility of fabricating a haemoglobin MIP sensor based on the electopolymerization of thin film PAM at GCE surfaces using an electrochemical probe ‘potassium ferricyanide’ for signal transduction. This technique demonstrated a more appropriate integration of electrochemical devices and MIPs, while also demonstrating good sensitivity and selectivity, features attractive for the development of biochemical sensor arrays.

There has been recent public concern and interest in the authenticity and origin of meat in the human food chain. For example, the 2013 ‘horsemeat scandal’ were the Food Safety Authority of Ireland (FSAI) announced the discovery of horse DNA in supposedly 100% beef burgers sold in British and Irish supermarkets. In light of this, novel sensor strategies for the discrimination between protein species are highly sought out. Recent developments using 60 MHz $^1$H NMR as a screening tool for distinguishing beef from horse meat has been demonstrated. While this represents a feasible high-throughput approach for screening raw meat, the method is inherently not portable and so cannot be used in-field. In this work, we look to discriminate between key proteins in 3 species using cheap, portable and synthetic smart material MIPs. MIP selectivity for two proteins of similar molecular weight (haemoglobin and serum albumin) are compared across three different species, namely Porcine (pig), Bovine (cow) and human using the combined latter mentioned techniques. Haemoglobin (Hb) is a well-known allosteric protein for its carbon dioxide and oxygen transport in the blood, as well as regulating blood pH. Hb is approximately 64.5 kDa in size (~5 nm) and has an iso-electric point (pI) of 6.8. Compared to smaller proteins, Hb will possess more anchor points with functional monomers and hence more flexible
conformational transitions in the imprinting process \(^{21}\). This results in more difficulties for Hb to form imprinted sites. Serum albumin (SA) with a molecular weight of 66.4 kDa and a pI of 4.7, is the main monomeric globular protein of plasma, and has a good binding capacity for water, \(\text{Ca}^{2+}, \text{Na}^+, \text{K}^+\), fatty acids, hormones, bilirubin and drugs. SA, particularly from bovine (BSA), is commonly used to determine the quantity of other proteins by comparing an unknown quantity of protein to known amounts of bovine serum albumin (BSA). Due to BSA having high stability, low cost, and a lack of effect in many biochemical reactions, it has served many uses as a carrier protein, as a stabilizing agent in enzymatic reactions, and in gel shift assays. These attributes serve as an excellent cross-selective template study for Hb.

The aim of this paper is to optimise synthetic hydrogel-based MIPs to specifically recognise and discriminate between species of proteins for future electrochemical diagnostic devices. The application of protein-specific MIPs along with multivariate analysis offers the potential for rapid in-field testing of meat samples based on analysing (the more abundant and readily accessible) protein levels and profiles with minimal sample preparation.

**Experimental Section**

**Reagents**

Acrylamide (AAm), \(N,N'\)-methylenebisacrylamide (MBAm), ammonium persulphate (APS), \(N,N,N',N'\)-tetramethylethyldiamine (TEMED), sodium dodecyl sulphate (SDS), glacial acetic acid (AcOH), phosphate buffered saline (PBS) tablets pH 7.2 (137 mmol L\(^{-1}\) NaCl; 27 mmol L\(^{-1}\) KCl; 10 mmol L\(^{-1}\) Na\(_2\)HPO\(_4\); 1.76 mmol L\(^{-1}\) KH\(_2\)PO\(_4\)), tris(hydroxymethyl)-amine (Tris-base), hydrochloric acid (HCl), potassium ferricyanide (K\(_3\)[Fe(CN)\(_6\)]), potassium chloride (KCl), sodium nitrate, potassium peroxydisulfate, acetone, nitric acid, bovine haemoglobin (BHb), bovine serum albumin (BSA), human haemoglobin (HHb), human serum albumin (HSA), porcine haemoglobin (PHb), porcine serum albumin (PSA), Negative Urine Control (Surine™) were all purchased from Sigma-Aldrich (Poole, UK). Sieves (75 µm) were
purchased from Inoxia Ltd. (Guildford, UK). Pooled plasma and serum samples from human
volunteers were used as complex biological matrices in the interspecies discrimination study.

**Bulk MIP Fabrication**

Individual bulk hydrogel-based MIPs (HydroMIPs) of polyacrylamide (PAM) for BHb, BSA,
HHb, HSA, PHb, and PSA were synthesised using 0.76 M of AAm monomer (54 mg) along
with 38.92 mM (6 mg) of MBAm as cross-linker for each hydrogel. Template protein (Hb
[64.5 kDa], or SA [66 kDa]; 12 mg, 186 µM and 181.8 µM respectively) was also added
followed by initiator (20 µL of a 10% (w/v) APS solution, 8.77 mM) and catalyst (20 µL of a
5% (v/v) TEMED solution, 8.61 mM) along with 50 mM Tris buffer pH 7.4 to give final
volumes of 1 mL. Solutions were purged with nitrogen for 5 minutes and polymerisation
occurred overnight at room temperature (~22 °C), giving final total gel densities (%T) of 6
%T, AAm/MBAm (w/v) and final crosslinking densities (%C) of 10 %C (9:1, w/w) for all
hydrogels. Molar ratios of monomer to template and cross-linker to template protein were
around 4180:1 and 214:1, respectively. For every MIP hydrogel created a non-imprinted
control polymer (NIP) was prepared in an identical manner but in the absence of template
protein. Both HydroMIPs and NIPs are semi-translucent and have a gel-like appearance and
texture that vary based on functional monomer/co-monomer, and %T gel composition.

After polymerization, the gels were granulated separately using a 75 µm sieve. Of the
resulting gels, 500 mg were washed with five 1 mL volumes of 50 mM Tris buffer pH 7.4
followed by five 1 mL volumes of 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8) and another five
1 mL volume washes of MilliQ water to remove any residual 10% (w/v):10% (v/v) SDS:AcOH
eluent followed by a further wash of 50 mM Tris buffer pH 7.4 to equilibrated the gels. Each
wash step was followed by a centrifugation, whereby the gels were vortexed then
centrifuged using an eppendorf mini-spin plus centrifuge for 3 minutes at 6000 rpm (RCF:
2419 x g). All supernatants were collected for spectrophotometric analysis to verify the
extent of template removal. It should be noted that the last water wash and SDS:AcOH
eluent fractions were not observed to contain any protein. Therefore, we are confident that
any remaining template protein within the MIPs did not continue to leach out during future studies.

Bulk MIP Characterisation

The subsequent rebinding effect of the conditioned and equilibrated MIPs and NIPs were characterized using a UV mini-1240 CE spectrophotometer (Shimadzu Europa, Milton Keynes, UK). Hydrogels (500 mg) were then treated (each) with 1 mL of a 3 mg mL\(^{-1}\) template protein solution of BSA, BHb, HSA, HHb, PSA, and PHb, polymer/protein solutions were then mixed on a rotary vortex mixer and then allowed to associate at room temperature (~22°C) for 20 minutes followed by centrifugation. The hydrogels were then washed four times with 1 mL MilliQ water. Each reload and wash step for the hydrogels was followed by centrifugation at 6000 rpm (RCF: 2419 x g) for 3 minutes. All supernatants were collected for analysis by spectrophotometry (at 404 nm for haemoglobins and 280 nm for serum albumins).

Bulk MIP Binding Affinity Studies

Tris buffer gels (BHb-MIP and NIP) were equilibrated, then 1 mL volumes of reload protein (BHb, HHb and PHb) solutions of known concentrations (3 mg mL\(^{-1}\) – 48 mg mL\(^{-1}\)) were allowed to associate at room temperature with the respective imprinted gels for 20 minutes. Each reload and wash step for all MIPs and NIP controls was followed by centrifugation at 6000 rpm (RCF: 2419 x g) for 3 minutes. All supernatants were collected for analysis by spectrophotometry. Curve fitting was carried out by non-linear regression using saturation binding - one site specific binding with Hill Slope equation in GraphPad Prism 6.

Electrochemical MIP Fabrication

Hydrogel-based MIP thin-film membranes for bovine haemoglobin (BHb) were fabricated by electrochemical polymerization of acrylamide solutions onto polished glassy carbon electrode (GCE) surfaces using 10 mL PBS (pH 7.2) containing 7.75 µM (5 mg mL\(^{-1}\)) BHb
protein template, 0.76 M (54 mg mL\(^{-1}\)) AAm as the functional monomer, 38.92 mM (6 mg mL\(^{-1}\)) MBAm as the cross-linker, 0.29 M (250 mg mL\(^{-1}\)) sodium nitrate, and 48.15 mM (130 mg mL\(^{-1}\)) potassium peroxydisulfate. The potential was cycled between −0.2 V and −1.4 V at 20 mV s\(^{-1}\) for five cycles. Prior to electropolymerization, the solution was deoxygenated by bubbling nitrogen gas for 10 minutes. Final total gel densities (%T) were 6 %T, AAm/MBAm (w/v) and final crosslinking densities (%C) were 10 %C (9:1, w/w) for all hydrogels. Molar ratios of monomer to template and cross-linker to template protein were around 98064:1 and 5022:1 respectively for each MIP. For every hydrogel MIP membrane created, a non-imprinted control polymer (NIP) was prepared in an identical manner but in the absence of template protein. All electrochemical measurements were performed using a standard three-electrode single-compartment cell comprising the GCE (3 mm in diameter), a Ag/AgCl reference electrode (saturated KCl) and a platinum counter electrode all connected to an Autolab II potentiostat/galvanostat (Utrecht, Netherlands). The GCE was polished before each experiment with α-alumina powder followed by sonication in 1:1 nitric acid, acetone and MilliQ water successively.

**Electrochemical MIP Characterisation**

Cyclic voltammetry (CV) was performed in 5 mM potassium ferricyanide solution containing 0.5 M KCl as supporting electrolyte to characterise the four different GCE phases (bare GCE, polymer modified GCE, eluted polymer modified GCE, and protein analysis [MIP and NIP reload]). Once electropolymerized, the modified GCE (MIP and NIP) was immersed firstly in a 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8) solution for 1.5 hr followed by a solution of 0.5 M H\(_2\)SO\(_4\) for 1 hr and then analysed to access the removal/elution of template protein. Both MIP and NIP the GCE was then immersed in PBS for 30 minutes to equilibrate the membranes. For protein selectivity studies, the modified GCE (MIP and NIP) was first incubated in BHb protein solution (100 µg mL\(^{-1}\)) for 30 minutes, washed with PBS to remove non-specifically bound protein, and then transferred into potassium ferricyanide solution for CV analysis. This was then followed by immersion in 10% (w/v):10% (v/v) SDS:AcOH (pH
2.8) then a solution of 0.5 M H₂SO₄ for an optimised time to elute the protein, equilibration in PBS (30 minutes), and then re-submersion in either HHb or PHb in series to assess selectivity again using potassium ferricyanide as the redox tracer.

**Interspecies Discrimination in Biological Matrices**

In order to assess MIP suitability in biological samples, both MIP and NIP membranes were investigated for their potential application for biological diagnostics using Surine™ along with human plasma and serum matrices to assess for potential interferents that could affect template protein rebinding. Reload samples of Surine™, diluted plasma and serum (1/10) were tested by incubating the modified GCE (MIP and NIP) for 30 minutes, and then washed with PBS to remove non-specifically bound protein. Surine™, plasma and serum samples were also spiked with a mixture of either all three proteins (BHb, HHb, PHb; 100 µg mL⁻¹ each) or a mixture in the absence of the original BHb template (HHb, PHb; 100 µg mL⁻¹ each) and allowed to associate with the modified GCE (MIP and NIP) for 30 minutes, then washed with PBS to remove non-specifically bound protein and transferred into potassium ferricyanide solution for CV analysis. Between each measurement the modified-GCEs were immersed in 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8) then a solution of 0.5 M H₂SO₄ for an optimised time to elute the protein, equilibration in PBS (30 minutes) then followed before assessing in ferricyanide.

**Statistical Analysis**

Discriminant function analysis (DFA) and hierarchical cluster analysis (HCA) were calculated using IBM SPSS Statistics, Version 21. Discriminant function plots were carried out using voltammetric current density values without any previous pre-processing and scaling from the modified GCE as input. Dendrograms were calculated using nearest the neighbour cluster method (single linkage) and Euclidean distance.

**Results and Discussions**
**Bulk MIP Characterisation**

The molecular imprinting effect or imprinting efficiency is characterised by the rebinding capacity \( Q \) of template to the polymer gel (mg g\(^{-1}\)) exhibited by the template-specific MIP and the control NIP. This is calculated using equation 1, where \( C_i \) and \( C_f \) are the initial template and the recovered template concentrations (mg/mL) respectively (which identifies the specific bound template within the gel), \( V \) is the volume of the initial solution (mL), and \( g \) is the mass of the gel polymers (g).

\[
Q = \frac{[C_i - C_f] V}{g} \quad \text{Equation 1}
\]

Fig. 1 shows the rebinding capacities and imprinting effects of polyacrylamide (PAM) MIP and NIPs for the several different proteins using a 50 mM Tris buffer (pH 7.4) MIP system. It can be seen that despite the polymer being the same, there is a distinctive rebinding capacity for each imprinted template. This is probably due to the varying sizes and attributes of the individual templates. In each case, the maximum binding capacity is shown for the protein template and in each case the NIP shows minimal binding capacity. Comparative studies using a water-based MIP system and a MIP prepared in 50 mM Tris buffer (pH 7.4) were conducted to assess the stability of both hydrogel and protein. Conformational stability of proteins is known to increase if anionic buffers are used above the \( pI \) of the protein (and conversely, if cationic buffers are used below the \( pI \)) \(^{11}\). At their \( pI \), proteins contain carboxyl and amide groups existing as \(-\text{NH}_3^+\) and \(-\text{COO}^-\). Above their \( pI \) however, proteins become negatively charged and the groups exist as \(-\text{NH}_2\) and \(-\text{COO}^-\). This overall negative net charge induces more favourable and complementary hydrogen bonding interactions, resulting in increased specific binding, and hence a Tris buffer (pH 7.4) system is preferred. Interestingly, despite similar molecular weights (within species and proteins) and \( pI \)s (within species, not proteins) the specific response of the polymer to the species of Hb and SA (also within the proteins themselves, i.e. either Hb or SA) suggests that the imprinted cavities distinguish the differences in protein structure between the two proteins, presumably due to
specific hydrogen bonding orientations between the SA and Hb to the PAM MIP matrix \(^{13, 14, 21, 22}\).

To further illustrate MIP affinity, fingerprint pattern recognition profiles were generated from the raw binding data based on the percentage that each of the individual proteins bound to MIP and NIP polymers collectively. Each protein exhibits an individual unique binding pattern for the MIPs and NIPs, within the Tris buffer system. Fig. 2 shows the discriminant function plot of DF1 vs. DF2 for the multiple proteins and species using a cumulative variance of 90% at a 0.999 canonical correlation. The discrimination in the plot shows different separations based upon different characteristics and illustrates a clear cluster discrimination of all proteins as unique protein fingerprints for corresponding protein templates, allowing for MIP-based protein profiling. Using LDA, 94% of the original grouped cases and the cross-validated grouped cases were correctly classified within the predicted group membership for the Tris buffer MIP system, significance \((\rho) = < 0.0005\).

According to global alignment tools, the similarity between Hb and SA within the same species varies by 13% for bovine, 11% for humans, and 12% for porcine species. Overall, the six proteins together have a 6.7% similarity, grouping porcine and bovine together in SA, whereas in Hb Human and bovine share a higher homology. Individually, the homology of the pig, bovine and human in serum albumin (PSA, BSA and HSA, respectively) sequence is 69%, sharing 420 and 124 identical and similar positions respectively. While the homology of the pig, bovine and human in haemoglobin (PHb, BHb, and HHb, respectively) sequence is slightly higher at 78%, sharing 451 and 77 identical and similar positions respectively.

Using hierarchical cluster analysis (HCA), a dendrogram was constructed to demonstrate the interspecies homology using an optimised MIP system (Fig. 3). Considering the high similarities between the proteins, specific MIPs are able to successfully discriminate between them and provide a clear protein cluster for each species, with the exception that both human and porcine are grouped in the case of both proteins. The above results indicate the possibility of these PAM MIPs possessing the ability of distinguishing template proteins perhaps not just based on molecular weight or size
separation, but also on the synergistic effect of shape memory/complementarity, and multiple weak hydrogen bonding interactions. Therefore, the shape, conformation, and/or amino acid composition of proteins continues to be an essential assertion to the recognition selectivity of imprinted gel polymers 13, 14, 21, 22.

**Bulk MIP Binding Affinity Studies**

Figure 4 illustrates the degree of affinity a BHb PAM MIP holds towards HHb and PHb respectively using a saturation binding profile using one site specific binding with Hill Slope (h) Equation 2. If h equals 1.0 then binding with no cooperativity to one site is occurring; when it is greater than 1.0, then multiple binding sites with positive cooperativity is implied. The Hill slope is less than zero when there are multiple binding sites with different affinities for ligand or when there is negative cooperativity. Using the latter approach, concentrations of haemoglobin were varied to measure binding of each species and dissociation constant, the ligand concentration that binds to half the receptor sites at equilibrium, (K_d) values and B_{max}, the maximum number of binding sites, (mol g^{-1} polymer) were determined (BHb: K_d = 184±23 µM, B_{max} = 582 µmol g^{-1}; HHb: K_d = 246±26 µM, B_{max} = 673 µmol g^{-1}; PHb: K_d = 276±31 µM, B_{max} = 467 µmol g^{-1}). Hill coefficients (n_h) for all MIPs demonstrated positive cooperativity (n_h >1), implying heterogeneous binding characteristics. Positive cooperativity also implies that the first protein molecules bind to the MIP polymer with a lower affinity than do subsequent protein molecules. This is in agreement with previous postulations that MIP formation can generate heterogeneous template protein populations, i.e. free and clustered proteins, when imprinting at high concentrations, such as at 12 mg mL^{-1} herein 23.

**Electrochemical MIP Characterisation**
In the previous section bulk MIP preparation was achieved via free radical polymerisation (FRP) using an equimolar ratio of APS and TEMED. Herein this section, free radicals are electrochemically generated by an electron transfer from the substrate to a redox-active initiator, i.e. the reduction of peroxodisulfate at the GCE surface, hence forming a PAM thin film.

Figures 5 a and b illustrate typical cyclic voltammograms for the electrochemical polymerization of PAM in the presence of BHb to form a MIP (Fig. 5 a) and a non-imprint control (NIP) (Fig. 5 b). It can be seen that the currents of the cycles decrease rapidly with the number of cycles, which is attributed to the non-conducting (insulating) PAM membrane layer formed on the electrode surface. This is especially true for the MIP (Fig. 5 a) in which the dielectric properties and permeability of the polymer membrane is dictated by the presence of BHb template. Thickness of wet PAM MIP-layers using the same parameters have been reported to be around 100±10 nm.

The electrochemical ‘ferricyanide probe’ characterisation of GCE before (clean) and after polymer modification for both MIP and NIP can be seen in Figures 5 c and d respectively, (labelled as ‘Clean’, ‘Polymer’, ‘Elute’ and Load). It can be seen that once the modification has occurred, the diffusion of the ferricyanide ion ([Fe(CN)₆³⁻]) is no longer possible (no redox signal observed), corroborating a successful polymerisation for both MIP and NIP (Figures 5 c and d, ‘Polymer’). Once both the modified GCE (MIP and NIP) are immersed in 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8) and 0.5 M H₂SO₄ solutions and analysed to access the removal/elution of template protein, typical redox peaks of [Fe(CN)₆³⁻] were observed for MIP modified GCE (Fig. 5 c, ‘Elute’), whereas the control NIP-modified GCE produced no electrochemical signal and remained unchanged due to its uniformly non-conducting PAM membrane properties concealing it (Fig. 5 d, ‘Elute’). Typically, the extraction of target BHb from the MIP results in the formation of biomimetic sites or cavities that are subsequently allowed to associate with cognate template to give a synthetic receptor binding event. In this instance, they can now also act as channels or pores, allowing access for the diffusion of the
[Fe(CN)₆]³⁻ probe to be oxidized or reduced at the GCE surface producing an
electrochemical signal which can be indicative of binding events.

To confirm this, protein selectivity studies were conducted; modified GCE (MIP and NIP)
were first incubated in BHb protein solution (100 µg mL⁻¹) for 30 minutes, washed with PBS
to remove non-specifically adsorbed protein, and then transferred into potassium
ferricyanide solution for CV analysis. The ferricyanide peak for the MIP modified GCE begins
to deteriorate in response to the loading of 100 µg mL⁻¹ (Figures 5 c, ‘BHb Load’), while the
NIP-modified GCE again remains unchanged (Figures 5 d, ‘BHb Load’). HHb and PHb
proteins were also tested (again by incubation of modified GCEs in solutions of 100 µg mL⁻¹
for 30 minutes) and ferricyanide peaks remained unchanged from that of the Elute phase.

These results suggest that the BHb MIP modified GCE does in fact exhibit selectivity
towards its native BHb template at a concentration of 100 µg mL⁻¹, and not PHb or HHb, due
to the rebinding of BHb which is potentially filling the selective cavities and causing a shift in
the [Fe(CN)₆]³⁻ response. Moreover, while the ferricyanide peak remains constant illustrating
no response to various external stimuli exhibited by the NIP control, this in turn is suggestive
of the NIP’s lack of selectivity towards target proteins and the robustness of the polymer
membranes architecture. MIP-modified GCE sensors also demonstrated good reusability,
i.e., the MIP-modified GCE sensitivity remained >90% after 9 cycles of binding and elution.

**Interspecies Discrimination in Biological Matrices**

In order to assess MIP suitability and selectivity in complex matrices, along with their
previously predetermined ‘bulk’ speciation ability, PAM-BHb HydroMIPs and NIPs were
investigated for their potential application for biological diagnostics using Surine™, human
plasma and human serum matrices (diluted to 1:10). This allows for the assessment of
potential interferents that could affect template protein rebinding and provides a proof of
concept that MIP-based pattern recognition functions within biological matrices. Reload
samples of Surine™, plasma and serum samples were spiked with a mixture of either all
three proteins (BHb, HHb, PHb; 100 µg mL⁻¹ each) or a mixture in the absence of the original
BHb template (HHb, PHb; 100 µg mL⁻¹ each) and were allowed to associate with the modified GCEs (MIP and NIP) for 30 minutes and then transferred into potassium ferricyanide solution for CV analysis. Figures 6a and b illustrates the resulting MIP and NIP discriminant function plots of DF1 vs. DF2 using the current density voltammograms data from the electrochemically modified GCEs. Using just the first two PC dimensions, since these contain ~95% of the original information content, a clear discrimination of all proteins clusters as unique protein fingerprints along with the corresponding biological sample matrix can be seen in Fig. 6a, approx. significance (p) = 0.0005. The boundary for the template BHb spiked samples is represented by an ellipse. It is clear to see that while the control NIP system is unable to discriminate between samples (Fig. 6b), the BHb MIP system is successfully able to discriminate between its native BHb template spiked within a mixture of pig and human haemoglobins in biological samples (Fig. 6a). These results suggest that these MIP systems could be used for future biosensor development that relies on electrochemical redox processes.

This MIP strategy opens up interesting possibilities for the testing of meat adulteration for example. The origin and purity of meat is of interest to the retailer and consumers alike in the supply chain. There have been incidents, for example the 2013 meat adulteration scandal in Europe which potentially put some of the meat production and distribution industries into disrepute. In some cases, meat products labelled as beef had as much as 100% adulteration by horse meat. The end-user would wish to have confidence in what they are consuming whether it is beef, pork or horse. The current gold standard tests for meat authenticity are based on DNA analysis allowing the discrimination between different meats in a mixture. Such analytical techniques require stringent levels of sample clean up and subsequent DNA amplification. Application of protein-specific MIPs and multivariate analysis offers the potential for rapid in-field testing of meat samples based on analysing (the more abundant and readily accessible) protein levels and profiles with minimal sample preparation.
Conclusions

In summary, a haemoglobin sensor based on a MIP modified GCE electrode by electrochemically induced redox polymerization of acrylamide has been fabricated. MIP selectivity between two proteins of similar molecular weight (haemoglobin and serum albumin) are compared across three different species (pig, cow and human) with the aid of chemometrics, i.e. pattern recognition and multivariate analysis. MIPs, along with non-imprint controls (NIP), both in bulk and on GCE sensor applications were able to demonstrate protein profiling and speciation within the pattern recognition system. This alternative MIP-based synthetic approach offers potential for rapid in-field testing of interspecies discrimination by protein profiling. Thus, this could lead to a viable application for future authenticity diagnostics i.e. in meat samples for authenticity based on analysing protein levels and profiles with minimal sample preparation.

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Figure 1 - MIP and NIP binding capacities $Q$ (mg g$^{-1}$ polymer) using a 50 mM Tris buffer (pH 7.4) MIP system. Data represents mean ± S.D., $n = 3$. 
Figure 2 – Discriminant function plot showing a clear discrimination of all proteins as unique protein fingerprint clusters. Of the original grouped cases and the cross-validated grouped cases 94% were correctly classified within the predicted group membership for the Tris buffer MIP system, significance ($\rho$) = < 0.0005.
Figure 3 – A Hierarchical cluster analysis (HCA) dendogram demonstrating successful interspecies homology using an optimised MIP system. Calculated using the nearest neighbour cluster method (single linkage) and Euclidean distance.
Figure 4 – One site specific binding with Hill slope saturation profiles for BHb imprinted PAM-MIPs. Data represents mean S.E.M., n = 3. Curve fitting was carried out by non-linear regression using saturation binding - one site specific binding with Hill Slope equation in GraphPad Prism 6.
Figure 5 – Cyclic voltammograms plotted in Origin 9.1 illustrating: the Electropolymerisation of both BHb-MIP (a) and non-imprint control (NIP) (b) in PBS (pH 7.2) at a scan rate of 20 mV s$^{-1}$; the electrochemical ‘ferricyanide probe’ characterisations of clean GCE and modified GCE (after polymerisation, after elution, and after BHb, PHb and HHb protein loading) for both MIP (c) and non-imprint control (NIP) (d) using 5 mM potassium ferricyanide solution containing 0.5 M KCl at a scan rate of 50 mV s$^{-1}$.
Figure 6 – Discriminant function plots showing a clear discrimination of all proteins as unique protein clusters for both MIP modified GCE (a) and non-imprint control NIPs (b). Surine™ (U), plasma (P) and serum (S) samples spiked with a mixture of all three proteins (BHb, HHb, PHb; 100 µg mL⁻¹ each, are noted as U1, P1 and S1 respectively); Samples spiked with a mixture in the absence of the original BHb template (HHb, PHb; 100 µg mL⁻¹ each, are noted as U2, P2 and S2 respectively). For demonstrative purposes, Both MIP and NIP plots were kept at the same scale to illustrate the profiling pattern recognition effect.