

1 **MIP-based Electrochemical Protein Profiling**

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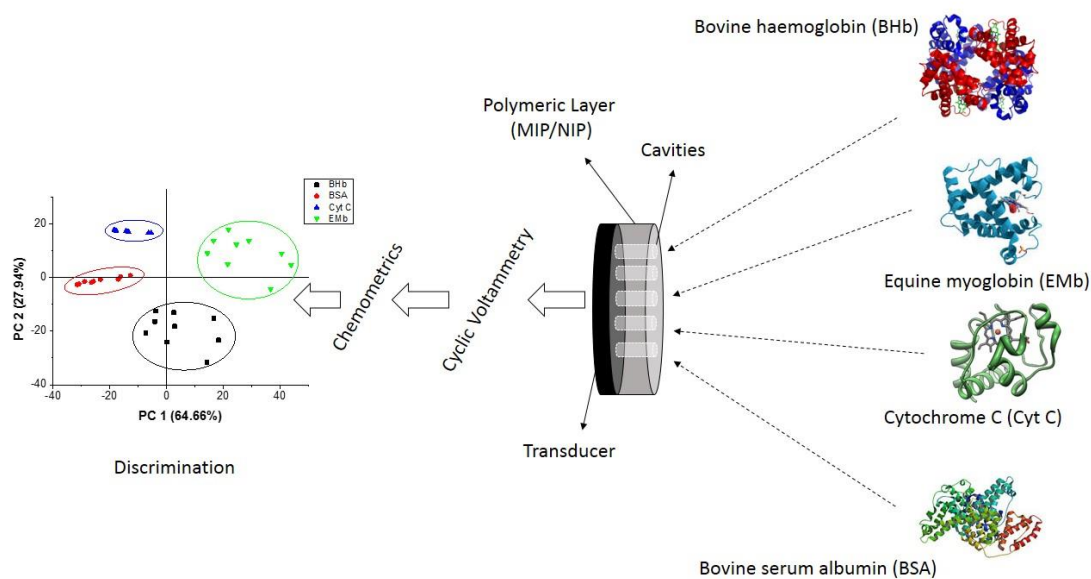
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15 Graphical Abstract



16

17 Highlights

- 18 • Electrochemical MIP-based biosensor fabricated for rapid protein detection.
- 19 • We report the coupling of electrochemical and pattern recognition techniques.
- 20 • Selective synthetic MIP recognition of a range of bio-significant proteins.
- 21 • Protein fingerprint profiling by principal component analysis
- 22 • Faster detection rates at lower concentrations.

23

24 **Abstract**

25 We present the development of an electrochemical biosensor based on modified
26 glassy carbon (GC) electrodes using hydrogel-based molecularly imprinted polymers
27 (MIPs) has been fabricated for protein detection. The coupling of pattern recognition
28 techniques via principal component analysis (PCA) has resulted in unique protein
29 fingerprints for corresponding protein templates, allowing for MIP-based protein
30 profiling. Polyacrylamide MIPs for memory imprinting of bovine haemoglobin
31 (BHb), equine myoglobin (EMb), cytochrome C (Cyt C), and bovine serum albumin
32 (BSA), alongside a non-imprinted polymer (NIP) control, were
33 spectrophotometrically, and electrochemically characterised using modified GC
34 electrodes. Rebinding capacities (Q) were revealed to be higher for larger proteins
35 (BHb and BSA, $Q \approx 4.5$) while (EMb and Cyt C, $Q \approx 2.5$). Electrochemical results
36 show that due to the selective nature of MIPs, protein arrival at the electrode via
37 diffusion is delayed, in comparison to a NIP, by attractive selective interactions with
38 exposed MIP cavities. However, at lower concentrations such discriminations are
39 difficult due to low levels of MIP rebinding. PCA loading plots revealed 5 variables
40 responsible for the separation of the proteins; E_p , I_p , $E_{1/2}$, $I_{at -0.8 V}$, $\Delta I_{decay \text{ peak current to } -0.8}$
41 v. Statistical symmetric measures of agreement using Cohen's kappa coefficient (κ)
42 were revealed to be 63% for bare GC, 96% for NIP and 100% for MIP. Therefore, our
43 results show that with the use of PCA such discriminations are achievable, also with
44 the advantage of faster detection rates. The possibilities for this MIP technology once
45 fully developed are vast, including uses in bio-sample clean-up or selective extraction,
46 replacement of biological antibodies in immunoassays, as well as biosensors for
47 medicine, food and the environment.

48

49 **Keywords**

50 Biosensors; Electrochemical protein detection; Molecularly imprinted polymers;
51 Pattern recognition; Modified electrodes; Electronic tongue.

52

53 **1. Introduction**

54 Molecularly imprinted polymers (MIPs) are rapidly becoming viable
55 alternatives to natural antibodies for sensor technology [1-4]. MIPs offer many
56 advantages in terms of shelf-life, stability, robustness, cost, and ease of preparation
57 [5]. While biological antibodies are routinely used in diagnostic tests and are able to
58 give precise results, they are notably unstable and require lengthy procedures to grow,
59 isolate, and treat before they can be used; ethical issues surrounding the use of animal-
60 based antibodies are also a common drawback [6].

61 Over the years molecular imprinting has become an effective method for
62 imprinting highly specific and selective recognition sites in synthetic polymers [7,8].
63 As such, MIPs have been regarded as ‘antibody mimics’ and have shown clear
64 advantages over actual antibodies for sensor technology as they are highly cross-
65 linked, intrinsically stable, robust, and have potential use in extreme environments [9].
66 However, in the imprinting community, bio-macromolecules such as proteins present
67 a variety of challenges and successful imprints are highly sought after. Proteins are
68 relatively labile and have changeable conformations that are sensitive to various
69 factors (e.g., solvent environments, pH and temperature) [7,10-12]. Moreover, a large
70 number of proteins are vital markers; for example, in the case of haemoglobin,
71 mutations in genes that encode for the protein’s subunits can result in hereditary
72 diseases such as *sickle cell anaemia*, *thalassaemia*, and *haemoglobinopathies* [1].
73 However, protein-detecting arrays remain under-developed due to the lack of highly

74 selective and specific binding agents that interact with protein surfaces through
75 complementary interactions [13]. It is therefore imperative to develop new
76 methodologies based on protein detection for applications in proteomics, medical
77 diagnostics, and even pathogen detection [13].

78 Differential receptor arrays, that in nature routinely conduct pattern-based
79 recognition, have already been artificially constructed using synthetic
80 receptors/transducers and could provide a possible solution. Such devices have been
81 labelled as electronic noses for smell recognition and electronic tongues for taste
82 recognition. These synthetic receptors/transducers or sensors have low selectivity and
83 consequently exhibit over-lapping signals for different species, providing a fingerprint
84 of a sample that could be used for qualitative discrimination [14]. The operation of
85 these electronic devices uses a concept of the human tongue and nose known as global
86 selectivity [15], in which the biological system does not identify a particular substance
87 but brings together all of the extracted information into patterns that the brain
88 decodes. An electronic sensor that works in a similar way is a chemometric tool e.g.,
89 principal component analysis (PCA). These tools decode complex information and
90 classify standards for recognition [16-18]. Takeuchi et al. previously applied the
91 electronic tongue strategy to the molecular recognition of proteins by using imprinted
92 acrylic acid (AA) and 2-dimethylaminoethyl methacrylate (DMA) polymers [19,20].
93 Three-dimensional PCA scores of the binding data described by Takeuchi et al.
94 revealed that a clear protein distinction was possible and that protein-imprinted
95 polymer arrays can be applied to protein profiling by pattern analysis of binding
96 activity for each polymer [19-21]. PCA has also been used in conjunction with
97 electrochemical methods such as cyclic voltammetry [16,17,22-25]. An attractive

98 approach for the development of biochemical sensors would be the integration of
99 smart materials (e.g., MIPs) with said electrochemical techniques

100 This paper demonstrates the use of pattern recognition techniques to uniquely
101 identify fingerprint profiles for four different proteins by coupling electrochemical
102 sensor strategies with hydrogel-based MIPs. The four proteins chosen were on the
103 basis of their different biological roles, sizes, and electrochemical activities. Bovine
104 hemoglobin (BHb, 64.5 kda) well known for its function in the vascular system as a
105 carrier of oxygen, also in aiding the transport of carbon dioxide and regulating blood
106 pH [26]. Both BHb and EMb (17.5 kda) exhibit well-known electrochemical
107 behaviour [1,24,27,28]. Cytochrome complex (Cyt C, 12.5 kda) is an essential
108 component of the electron transport chain but exhibits a lack of oxygen binding,
109 despite being an iron-containing metalloprotein that is capable of undergoing
110 oxidation and reduction. Bovine serum albumin (BSA, MW 66.0 kDa) is a non-
111 metalloprotein with similar molecular weight to BHb, and serves to test the selectivity
112 of the BHb-MIP to BSA compared to template BHb.

113 Our results demonstrate sensitivity and selectivity; if such devices can be
114 further optimised for MIP parameters, then perhaps these MIP-based strategies can
115 offer viable methods for the characterisation of proteins. With the aid of inexpensive
116 synthetic smart material hydrogel MIPs, new biosensor platforms for rapid screening,
117 diagnosis, and monitoring of a variety of disorders can be readily developed within
118 the years to come [8,29].

119

120 **2. Materials and methods**

121 **2.1 Materials**

122 Acrylamide (AA), N,N-methylenebisacrylamide (bis-AA), ammonium
123 persulphate (APS), N,N,N,N-tetramethylethyldiamine (TEMED), sodium dodecyl-
124 sulphate (SDS), glacial acetic acid (AcOH), phosphate buffered saline (PBS) tablets
125 ($137 \text{ mmol L}^{-1} \text{ NaCl}$; $27 \text{ mmol L}^{-1} \text{ KCl}$; $10 \text{ mmol L}^{-1} \text{ Na}_2\text{HPO}_4$; 1.76 mmol L^{-1}
126 KH_2PO_4), methyl viologen, bovine haemoglobin (BHb), bovine serum albumin
127 (BSA), cytochrome C (Cyt C), and equine heart myoglobin (EMb) were all purchased
128 from Sigma-Aldrich (Poole, UK). Sieves ($75\mu\text{m}$) were purchased from Inoxia Ltd.
129 (Guildford, UK). Polycarbonate membranes 25 mm in diameter, $0.8 \mu\text{m}$ pore size
130 were purchased from Osmonic Inc., Minnetonka, USA.

131

132 **2.2 Hydrogel production**

133 Hydrogel MIPs for BHb, EMb, Cyt C and BSA were synthesised by separately
134 dissolving AA (54 mg) and bis-AA as cross-linker (6 mg) along with template protein
135 (12 mg) in $960 \mu\text{L}$ of MilliQ water. The solutions were purged with nitrogen for 5
136 minutes, followed by an addition of $20 \mu\text{L}$ of a 10% (w/v) APS solution and $20 \mu\text{L}$ of
137 a 5% (v/v) TEMED solution. Polymerisation occurred at room temperature ($\sim 22 \text{ C}^0$),
138 giving final total gel densities (%T) of 6 %T, AA/bis-AA (w/v) and crosslinking
139 densities (%C) of 10 %C (9:1, w/w). For every MIP created, a control non-imprinted
140 polymer (NIP) was prepared in an identical manner but in the absence of protein.

141

142 **2.3 Hydrogel conditioning**

143 After polymerization, the gels were granulated separately using a $75 \mu\text{m}$ sieve.
144 Of the resulting gels, 500 mg were conditioned by washing with five 1 mL volumes of
145 150 mmol L^{-1} PBS buffer (pH 7.4). This was followed by five 1 mL volumes of a
146 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8). A further five 1 mL washes of 150 mmol

147 L⁻¹ PBS buffer (pH 7.4) were conducted to remove any residual SDS:AcOH eluent
148 and equilibrate the gels. Each conditioning step was followed by a centrifugation
149 using an Eppendorf mini-spin plus centrifuge (Fisher Scientific, Loughborough, UK)
150 for 3 minutes at 6000 rpm (RCF: 2419 x g). All supernatants were collected for
151 analysis by spectrophotometry to verify the extent of template removal. It should be
152 noted that the last water wash and SDS:AcOH eluent fractions were not observed to
153 contain any protein. Therefore we are confident that any remaining template protein
154 within the MIPs did not continue to leach out during future studies.

155

156 **2.4 Hydrogel characterization**

157 The rebinding efficiency of the MIPs and NIPs produced were characterized
158 using a UV mini-1240 CE spectrophotometer (Shimadzu Europa, Milton Keynes,
159 UK). After elution washing of the polymers MIP and NIP (500 mg) were treated with
160 3 mg/ml of protein in an eppendorf and polymer/protein solution mixed on a rotary
161 vortex mixer for 5 minutes followed by centrifugation. The supernatant was removed
162 and protein concentration measured spectrophotometrically (at 404 nm for BHb; 280
163 nm for BSA, 408 nm for Mb and 402 nm for Cyt C). Protein loaded MIPs and NIPs
164 were then washed with five sequential washes of water (1ml each) and the washes
165 combined. Again the absorbance of the washes was also taken. All protein
166 unaccounted for at this stage was deemed to be selectively bound to the MIP or NIP
167 and determined by subtraction of the protein levels in supernatants (after loading and
168 water washing) from the initial load. Optical microscope images of granulated and
169 washed MIPs and NIPs were also taken.

170

171 **2.5 Electrochemical analysis**

172 Glassy carbon (GC) working electrode surfaces were individually modified
173 with a 20 mg conditioned hydrogel layer of each: NIP, BHb MIP, Cyt C MIP, BSA
174 MIP, and EMb MIP. The layer was kept in place by a polycarbonate membrane (0.8
175 μm) placed over the modified electrode surface and held down with the aid of a
176 rubber ring. The polycarbonate membrane was chosen because its pores are small
177 enough to retain the gel (75 μm particle size) and, at the same time, large enough to
178 allow protein in solution to diffuse through. The potential range used in all
179 electrochemical measurements was 0.0 to -0.9 V with a scan rate of 100 mV s^{-1} ; a
180 Ag/AgCl reference electrode (saturated KCl) and platinum counter electrode
181 connected to an Autolab II potentiostat/galvanostat were used in this study (Utrecht,
182 Netherlands). The modified electrodes were first placed in a solution of PBS (pH 7.4)
183 and SDS 5% (w/v) and analysed after a 20 min period of equilibration. Subsequently,
184 15.4 $\mu\text{mol L}^{-1}$ protein solutions (BHb, BSA, EMb and Cyt C) dissolved in PBS buffer
185 (pH 7.4) and SDS 5% (w/v) were placed independently in the cell and
186 voltammograms were obtained at 10 min intervals for 60 min. It should be noted that
187 protein solutions were stirred between measurements for 3 minutes; GC electrodes
188 were cleaned, polished, and tested with methyl viologen between each new MIP/NIP
189 experiment. Cyclic voltammograms using bare GC electrodes were also recorded for
190 the PBS (pH 7.4) and SDS 5% (w/v) buffer solution and for the 15.4 $\mu\text{mol L}^{-1}$ protein
191 solutions (BHb, BSA, EMb and Cyt C) dissolved in PBS buffer (pH 7.4) and SDS 5%
192 (w/v).

193

194 **2.6 Principal component analysis**

195 Principal component analysis (PCA) and hierarchical cluster analysis (HCA)
196 were performed in Statistica 11.0 (StatSoft Inc., Tulsa, USA). The analysis was

197 carried out using voltammetric current density values without any previously pre-
198 processing and scaling from bare GC or modified GC electrodes as input. PCA was
199 used to reduce the large data sets to 2D plots, which can be easily used to discriminate
200 protein samples. All voltammetric curves were recorded three times for each sample
201 in a random order using a clean bare GC or a new modified GC electrode surface.

202

203 **3. Results and discussion**

204 **3.1 Characterisation**

205 Figure 1A and 1B show the optical microscope images of granulated and
206 washed BHb MIPs and NIPs. The MIPs appear denser than the NIPs due to the light
207 contrast apparent from protein which is still locked in the bulk of the MIP. It is also
208 evident that the MIP particles form larger agglomerates with each other compared
209 with the NIP. This is because there is still surface entrapped protein in the MIP
210 particles which is attracted to more surface entrapped protein within other MIP
211 particles. This is not observed with the NIP.

212 The molecular imprinting effect is characterised by the rebinding capacity (Q)
213 of protein to the gel polymer (mg/g) exhibited by the protein-specific MIP and the
214 control NIP, and is calculated using Eq. (1), where C_i and C_r are the initial protein and
215 the recovered protein concentrations (mg/ml) respectively (which specifies the
216 specific protein bound within the gel), V is the volume of the initial solution (ml), and
217 g is the mass of the gel polymers (g).

$$218 \quad Q = [C_i - C_r] V/g \quad (1)$$

219 Figure 1C shows the rebinding capacities for each protein studied. As
220 expected, the MIP exhibited superior selective binding of the target protein compared
221 with the NIP with a typical selectivity ratio of 10:1. Interestingly, the binding capacity

222 is highest for BHb-MIP_{polyAA} while both EMb-MIP_{polyAA} and BCat- MIP_{polyAA} exhibit
223 the lowest binding capacity. It has previously been observed that with smaller size
224 proteins a higher crosslinking density is necessary; the opposite is also true for larger
225 proteins [12,30]. Since the crosslinking density remained the same (10% by weight),
226 the low MIP affinities for BCat and EMb can be attributed to the fact that fewer
227 cavities were imprinted due too high, and too low of a crosslinking density
228 respectively.

229

230 **3.2 Electrochemical analysis**

231 *3.2 .1 Glassy carbon (GC) profiling*

232 Metallo-proteins are expected to produce an electrochemical signal because of
233 their metal-containing haem active centres in the protein molecules. However, the
234 extended three-dimensional structure of proteins results in the inaccessibility of the
235 electroactive iron centres. It can therefore be difficult for metallo-proteins to undergo
236 heterogeneous electron transfer; as a result, no detectable current appears at
237 conventional electrodes [1,4]. However, conformational changes due to partial or
238 complete protein denaturation, can allow haem groups to become accessible to a
239 subjacent electrode and be electrochemically reduced at GC electrodes via promotion
240 of electrocatalytic reduction of nascent oxygen [1]. For example, conformational or
241 structural changes in oxyhaemoglobin (Hb) complexes can be induced upon
242 denaturation in the presence of sodium dodecylsulphate (SDS) denaturant [1,11,31].

243 With this in mind, we attempted to evaluate the possibility to discriminate the
244 proteins using cyclic voltammetric information extracted from bare GC electrodes in
245 the presence of an SDS surfactant in solution. Cyclic voltammograms were recorded
246 in the presence of the four proteins that were studied at 15.4 $\mu\text{mol L}^{-1}$ (including one

247 non-metalloprotein as a control; BHb - 1 mg mL⁻¹, BSA - 0.98 mg mL⁻¹, EMb - 0.26
248 mg mL⁻¹, and Cyt C - 0.185 mg mL⁻¹) in a solution containing PBS buffer (pH 7.4)
249 and 5% SDS (w/v) (Figure 2A). In the presence of Cyt C and BSA, the cathodic
250 reduction signal of dissolved oxygen in solution could be seen (reduction peak at -0.6
251 V). The fact that the peak was due to dissolved oxygen was confirmed by bubbling
252 Ar in the latter protein solutions, which consequently led to depletion of the oxygen
253 reduction peak (results not shown). In the presence of BHb and EMb, a shift in the
254 peak reduction potential towards a less negative potential was observed, indicating an
255 iron centre-dependent electrocatalytic process for the oxygen reduction reaction at the
256 surface of the electrode. This effect was not observed in the absence of SDS, and is
257 therefore due to a probable SDS-induced change in the haemoglobin and myoglobin
258 structural conformation exposing the Fe(III) centre by partial denaturation. The
259 partial denaturation is induced only by SDS where at 5% (w/v) the CMC is reached.
260 Full denaturation however, requires a combination of SDS surfactant and an acid in
261 order to protonate the protein and hence be further attracted to and unravelled by
262 negatively charged SDS micelles [31]. With this modification, the reduction of the
263 oxygen does not directly happen at the electrode surface; the Fe(III) is reduced to
264 Fe(II) at the electrode surface and the oxygen reduction is subsequently
265 electrocatalysed by the oxidation of Fe(II) back to Fe(III).

266 Using hierarchical cluster analysis (HCA) the qualitative discrimination of the
267 proteins on the GC electrode was performed and data were analysed for their
268 discrimination and compiled as number of cluster recognition (Figure 2B). The results
269 reveal that a slight degree of separation between the proteins that, in solution, exhibit
270 and do not exhibit a shift in the peak reduction potential of the oxygen
271 electrochemical process, as clearly observed in the voltammetric profile. However,

272 further evaluation of the recognized sample similarities shows that the model using
273 data extract with a bare electrode was unable to clearly discriminate individual
274 proteins inside the groups of protein clusters.

275

276 3.2.2 Hydrogel profiling

277 That the MIP can detect partially denatured protein is of significance to the
278 exploitation of this electrochemical technique in protein discrimination. Indeed
279 Kryscico *et al.* recently demonstrated using CD spectroscopy that during the
280 imprinting process, some of the protein undergoes conformational changes and is
281 partially denatured [32,33]. The MIPs therefore are imprinted with both native as well
282 as partially denatured protein. The MIPs and NIPs were therefore analysed
283 electrochemically with SDS treated protein to give partially denatured protein.

284 Considering the selective nature of MIPs, protein arrival at the electrode
285 surface via diffusion should be delayed by the MIP due to attractive selective
286 interactions with exposed cavities [1]. With this in mind, GC electrode surfaces were
287 individually modified with a conditioned hydrogel layer (20 mg) of BHb MIP, EMb
288 MIP, Cyc C MIP, and BSA MIP.

289 To ensure the successful elution of protein from the MIP (and thus confirming
290 the presence of selective cavities through conditioning), BHb MIPs at different stages
291 were tested electrochemically on the electrode. Figure 3A characterizes the cyclic
292 voltammograms for freshly prepared MIP (with BHb still in the cavities; referred to as
293 MIP1), the same MIP washed to remove protein (referred to as MIP2) and also NIP.
294 The results clearly demonstrate that the MIP loaded with protein exhibits similar
295 electrochemistry to the BHb solution in Figure 2A. The reduction peak observed at
296 around -0.4V is the iron mediated reduction of oxygen. This suggests that the GC

297 electrode is able to detect the protein at the surface due to the ‘un-eluted’ MIP’s
298 presence and concurred with previously reported electrochemical MIP studies [1].
299 Conversely, when protein is not present in either the MIP or the NIP, the
300 electrochemistry (reduction peak at -0.6 V) reverts to direct electrochemical reduction
301 of dissolved oxygen.

302 Protein diffusion through MIP and NIP layers was initially studied at 154 and
303 15.4 $\mu\text{mol L}^{-1}$. Whereas the NIP response time remained constant at 10 min for all
304 protein concentrations, we found that the MIP response time decreases from our
305 previously reported 40 min [1] to 10 min at low protein concentrations. Figure 3B
306 illustrates the resulting voltammograms for 0 and 10 minutes of BHb exposure at 15.4
307 $\mu\text{mol L}^{-1}$ using a modified BHb-MIP layer (20 mg). It can be seen that a shift in the
308 peak reduction potential for the oxygen reduction was observed after only 10 min of
309 BHb exposure. Therefore, both MIP and NIP share the same reduced response time at
310 lower concentrations. This result suggests that the template protein exhibits little
311 interaction with the MIP cavities at the lower concentrations, which is associated with
312 a less tortuous path to the electrode. It could be that at low protein concentrations we
313 observe extensive protein denaturation in the presence of SDS and therefore there is
314 little or no interaction between denatured protein and the mixed population of MIP
315 cavities for native and partially denatured protein.

316 Another possibility is that the ‘template’ forms a mixed population of free and
317 clustered proteins when the template is imprinted at a very high concentration (12 mg
318 mL^{-1}). The resultant population of imprinted sites would therefore contain some
319 cavities that are associated with protein clusters. This phenomenon is supported by
320 our previous work [34,35], where force spectroscopy analysis of MIPs suggested that
321 the cavities accommodated an agglomeration of template protein molecules rather

322 than just a single molecule. It is therefore possible that the solution phase represents a
323 more dispersed protein population compared with the original imprinted template
324 population for rebinding protein at low concentrations. If the cavities only respond to
325 a critical number of protein molecules in a given arrangement, then this could explain
326 why the MIP does not appear to be selective at low protein concentrations.

327 However, although the presence of SDS in solution (5% (w/v)) allows for
328 protein detection at the electrode by iron exposure, it also implies that MIP
329 recognition within the specific cavities may technically not be able to rebind the
330 partially denatured and unfolded protein structures due to an altered size and shape. In
331 light of this, recent studies have shown that when imprinting a mixture of stable and
332 partially denatured proteins are present [9,32,33]. Therefore it is still possible that the
333 MIPs can function as a recognition element and rebind a small percentage of the
334 heterogeneous protein configurations.

335 In order to confirm these assumptions and elucidate the hypothesis that MIP
336 cavities undergo an electrochemical discrimination of their template proteins,
337 individually modified GC electrodes with all four hydrogel MIP layers were
338 separately tested across all four proteins. Cyclic voltammograms for all MIP were
339 recorded in a solution containing PBS (pH 7.4), SDS 5% (w/v), and $15.4 \mu\text{mol L}^{-1}$ of
340 the four proteins for different times of protein exposure (0-60 minutes). It was noted
341 that the current signal for both BHb and EMb at $15.4 \mu\text{mol L}^{-1}$ achieved steady state
342 behaviour after 10 minutes, indicating that this time could be used for all further
343 measurements. Therefore, using the voltammetric current density values PCA score
344 plots for each MIP and protein combination were plotted at 10 minutes of protein
345 exposure.

346 Figure 4A illustrates the average PCA score plot for the four MIPs as they all
347 shared the same cluster separation. A clear discrimination and separation (using 92.9
348 % of the original information) of the four proteins clusters at 10 min of protein
349 exposure can be seen. This indicated that MIP cavity interactions could play an
350 important role in the discrimination process. Of the four different clusters, Cyt C and
351 BSA clusters are far less scattered than BHb and EMb clusters. An explanation for
352 this behaviour could be ascribed to the fact that the BHb MIP was selective for both
353 BHb and EMb (which bear similarities in their structure), allowing for them to bind in
354 the MIP cavities and consequently making the diffusion rate less reproducible in the
355 MIP. The separation for Cyt C and BSA can be justified due to their adsorption at GC
356 electrode surfaces, subsequently changing the rate of the oxygen reduction. A change
357 in the peak current and in the current decay from peak current to -0.8 V ($\Delta I_{\text{decay peak}}$
358 $\text{current to } -0.8 \text{ V}$) for the oxygen reduction was observed for all the experiments with Cyt C
359 and BSA proteins when compared with a blank solution. These adsorption rates of
360 Cyt C and BSA can be related to previously published values [27,36]. It is plausible
361 that this adsorption effect and delayed diffusion due to MIP cavity interactions are
362 responsible for the discrimination process [37]. PCA loading plots revealed the
363 variables responsible for the separation of the proteins; 5 variables could be elected:
364 E_p , I_p , $E_{1/2}$, $I_{\text{at } -0.8 \text{ V}}$, $\Delta I_{\text{decay peak current to } -0.8 \text{ V}}$.

365 Thus, the effective diffusion rate of proteins through the composite
366 membranes could be a function of specific and non-specific cavities of the polymeric
367 MIP layer [37]. Therefore, the time of protein diffusion was considered an important
368 parameter for the discrimination process. This indicated that GC electrodes modified
369 with an acrylamide cavity-based MIP could be used as a sensor to discriminate
370 different kinds of proteins at 10 minutes of protein exposure. However, mechanical

371 obstruction of the polymeric layer using a control non-imprinted polymer (NIP) on the
372 GC electrode surface was conducted in order to validate the MIP-protein rebinding
373 profiles. This allows only for the non-specificity of the polymeric layer to be
374 evaluated due to the lack of selective cavities. All discrimination experiments were
375 executed identically as reported using the MIP layers; the only altered variable was
376 the modified NIP layer (20 mg). Unfortunately, PCA plots revealed NIP to have
377 similar protein discrimination (Figure 4B) to that of a MIP at 10 minutes of protein
378 exposure. Therefore only the protein diffusion rate through the polymeric layer could
379 be considered as a possible discriminating factor for the four proteins.

380 A closer look at the PCA data using interpreted HCA data compiled as number
381 of cluster recognition reveals that the four proteins are best profiled using both MIP
382 and NIP layers (Figure 5A and B, respectively) when compared with bare GC
383 electrode (Figure 2B). The symmetric measures between our protein discrimination
384 models, in terms of a percentage measurement of agreement using Cohen's kappa
385 coefficient (κ), are illustrated in Table 1. Since the approx. significance (p) = .000
386 (which actually means $p < .0005$), our κ coefficients are statistically significantly
387 different from zero (63% for bare GC, 96% for NIP and 100% for MIP). Therefore,
388 there is a clear comparison between the behavioural models for protein
389 discrimination.

390 Furthermore, clustering relationships for each of the four proteins are
391 apparent; this phenomenon is especially noticeable in the MIP and NIP PCA plots
392 (Figure 4). It should be noted that in different studies, involving bare GC electrodes,
393 MIP modified GC electrodes or NIP modified GC electrodes, all PCA protein clusters
394 fall into the same pattern recognition, thus providing an overall cohesive protein
395 profile. Each protein retains its own individual cluster within a single quadrant of the

396 PCA plot. Interestingly, our studies illustrate that proteins with a metal center behave
397 similarly; it can clearly be seen that both metalloproteins that exhibit a peak potential
398 shift (BHb and EMb) are on the right half of the vector, while BSA and Cyt C are on
399 the other. Moreover, the smaller sized proteins (EMB ~17.5kDa and Cyt C ~12.5kDa)
400 are on the top half of the plot. This recognition approach could be useful for future
401 protein speciation profiling.

402

403 **4. Conclusions**

404 The proposed electrochemical and PCA coupled method proved to be efficient
405 for discriminating four proteins (BHb, Mb, BSA and Cyt C), indicating that glassy
406 carbon (GC) electrodes modified with either a MIP or NIP layer could be used as a
407 fast sensor to discriminate between different kinds of proteins. At high concentrations,
408 the selective nature and integrity of MIPs delays the protein response and leads to an
409 obvious difference between MIP and NIP performance. At lower concentrations, such
410 discriminations are difficult due to an apparent lack of critical protein agglomeration
411 and/or complete denaturation of protein molecules impeding optimum protein binding
412 within cavities. With the use of PCA, protein discrimination has been achievable at
413 faster detection rates. Our results suggest that PCA could be used to interrogate and
414 discriminate between proteins when hydrogels are integrated to electrochemical
415 sensors.

416

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428

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534

535

536 **Table captions**

537

538 Table 1 - Symmetric Measures; Cohen's kappa coefficient (κ) as a percentage
539 measurement of agreement, asymptotic std. error not assuming the null hypothesis^a,
540 approximate T as the ratio of κ to the asymptotic standard error assuming the null
541 hypothesis^b, and the approximate statistical significance (p).

542

543

544 **Figure captions**

545

546 Figure 1 - Microscope imaging of 75 μ m hydrogel particles: (A) non-imprinted control
547 (NIP); (B) bovine haemoglobin (BHb) imprinted MIP_{polyAA}. (C) Rebinding capacities
548 and imprinting effects of MIP_{polyAA} and NIP_{polyAA} for several biological molecules
549 (bovine haemoglobin (BHb), bovine serum albumin (BSA), myoglobin (Mb),
550 Cytochrom C (Cyt C)). Data represents mean \pm S.E.M., $n = 3$.

551

552 Figure 2 – (A) Cyclic voltammograms recorded in PBS (pH 7.4), SDS 5% (w/v), and
553 in the presence of protein in solution (15.4 μ mol L⁻¹) (cytochrome C (a), bovine serum
554 albumin (b), equine heart myoglobin (c) and bovine haemoglobin (d)). Scan rate: 100
555 mV s⁻¹. Electrode: bare glassy carbon (GC) electrode. (B) Cluster analysis percentage
556 prediction scores for the four proteins using GC electrodes.

557

558 Figure 3 - Cyclic voltammograms recorded in PBS (pH 7.4), SDS 5% (w/v), and in
559 the presence of BHb in solution (15.4 μ mol L⁻¹) at scan rate of 100 mV s⁻¹: (A) Glassy
560 carbon (GC) electrode modified with hydrogel layers of NIP (a), unconditioned BHb-
561 MIP1 (b), conditioned BHb-MIP2 (c) after 0 minutes of protein exposure. (B) Glassy
562 carbon (GC) electrode modified with hydrogel layer of BHb MIP. Measurement made
563 after 0 (a) and 10 (b) minutes of protein exposure.

564

565

566 Figure 4 - PCA score plots: (A) glassy carbon (GC) electrode modified with hydrogel
567 MIP layer, results show the average response of all four different MIPs; (B) glassy
568 carbon (GC) electrode modified with a non-imprinted hydrogel layer. Voltammetric

569 data recorded in PBS (pH 7.4), SDS 5% (w/v), and in the presence of each protein
570 ($15.4 \mu\text{mol L}^{-1}$). Potential programme employed to record the voltammetric curves
571 used as input to perform PCA: $E_i=0.0 \text{ V}$, $E_{v1}=-0.9 \text{ V}$, $E_f= 0.0 \text{ V}$, and scan rate = 100
572 mV s^{-1} . Measurement made after 10 minutes of protein exposure.

573

574 Figure 5 - Cluster analysis percentage prediction scores for the four proteins; (A) MIP
575 modified GC electrodes, (B) NIP modified GC electrodes.

576

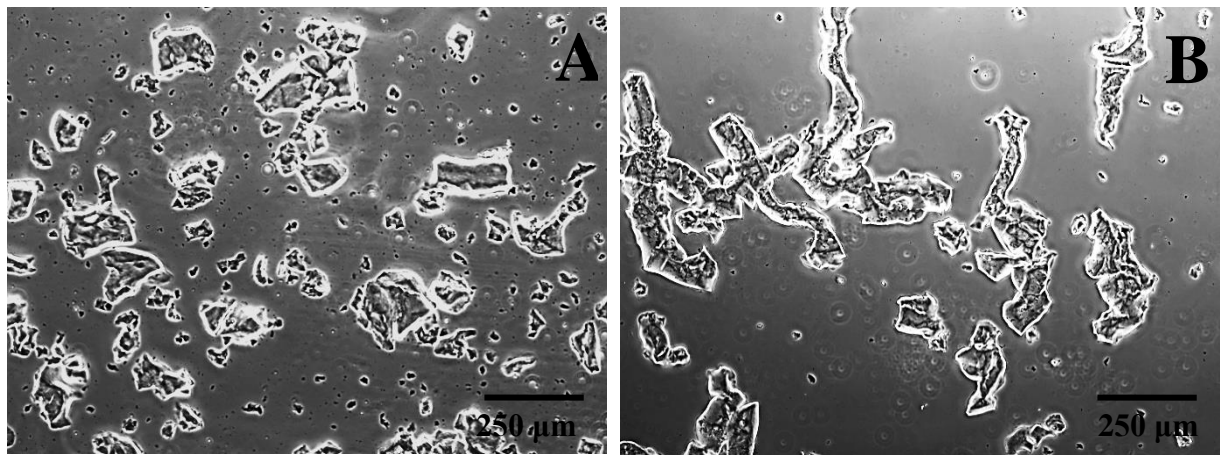
577 Table 1

Model	κ (%)	Asymp. Std. Error^a	Approx. T^b	Approx. Sig. (ρ)
Bare GCE	63%	0.1	6.543	0.00
NIP	96%	0.036	10.018	0.00
Mb MIP	100%	0	10.392	0.00
Cyt C MIP	100%	0	10.392	0.00
BSA MIP	100%	0	10.392	0.00
BHb MIP	96%	0.04	9.414	0.00

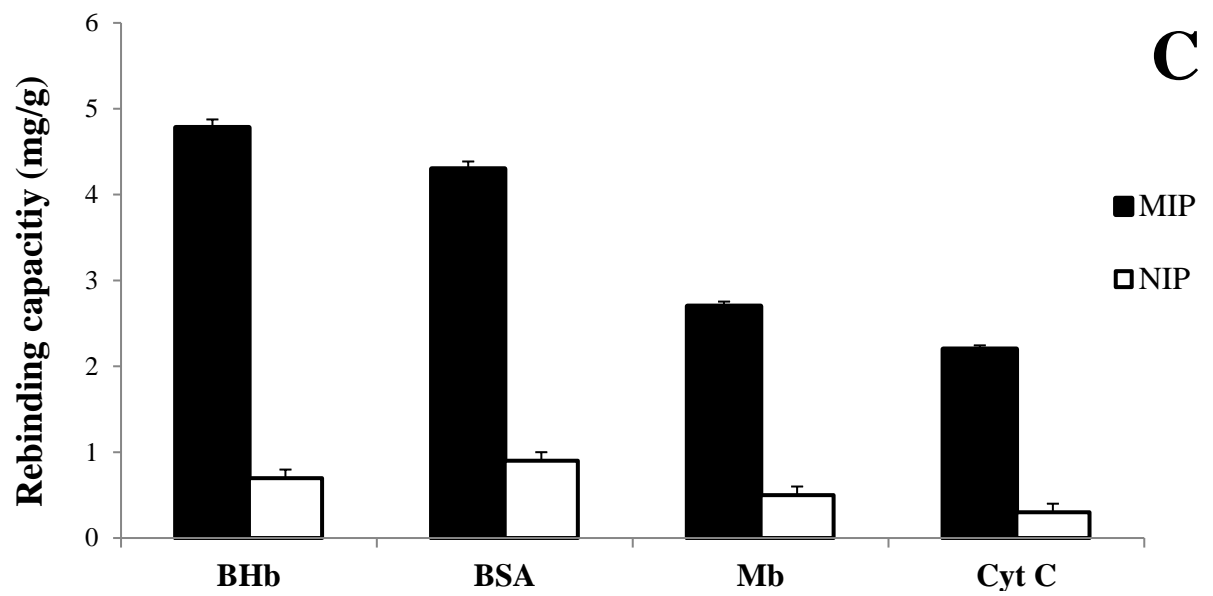
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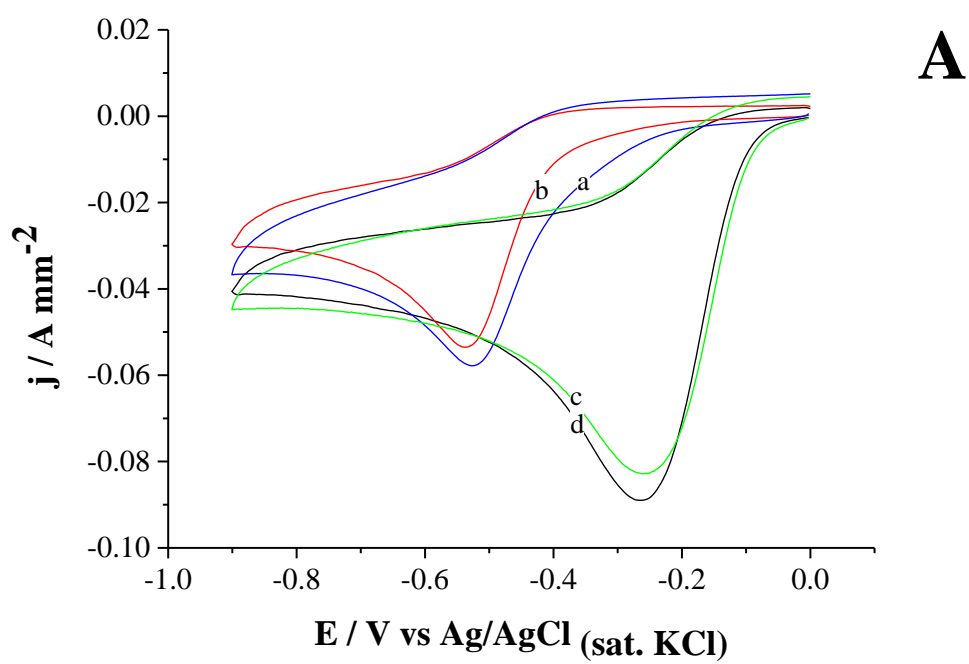


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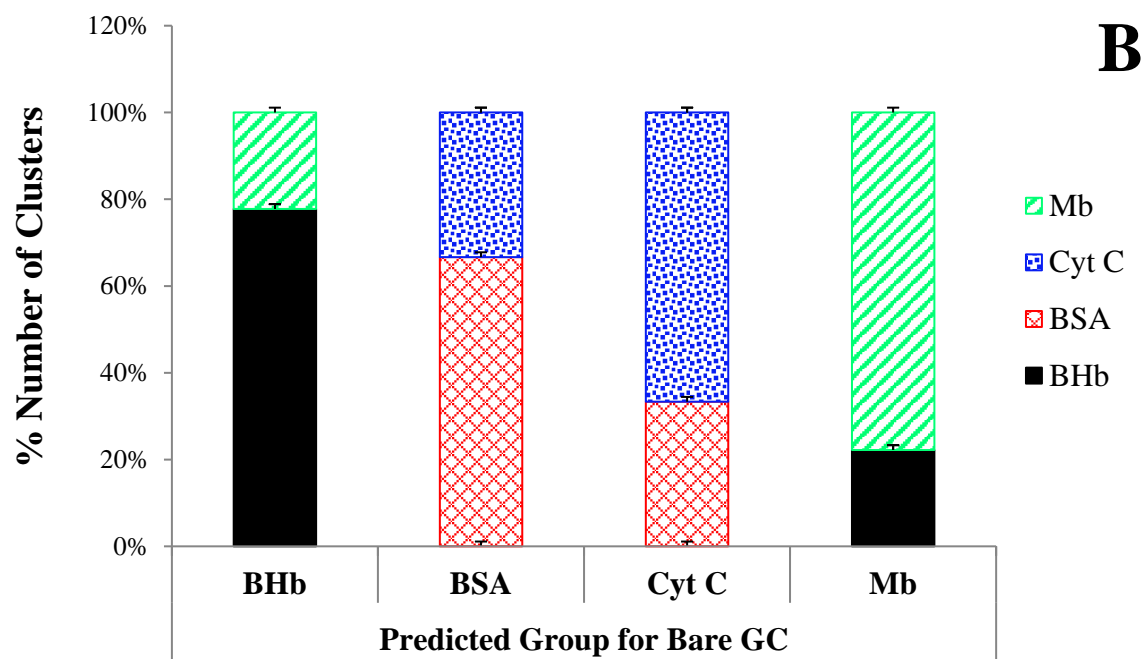


582 Figure 1

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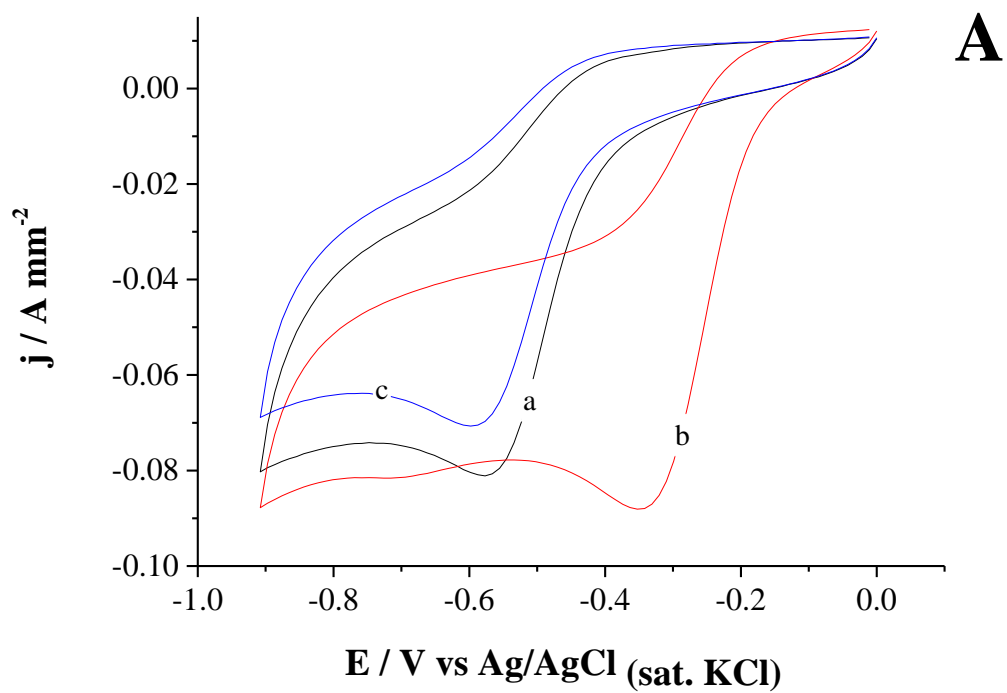


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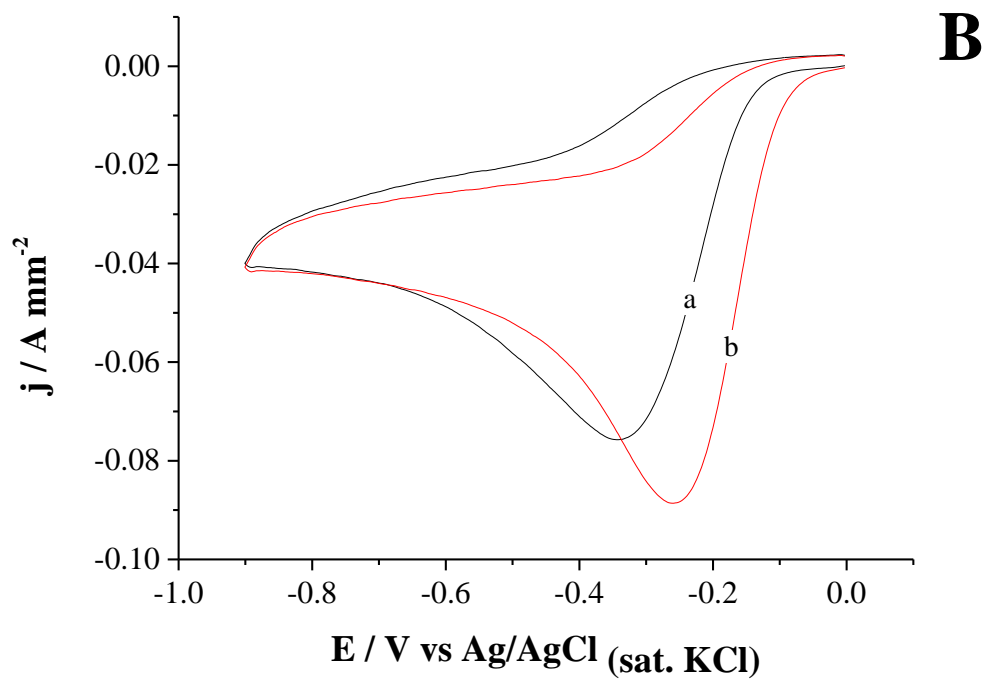


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586 Figure 2

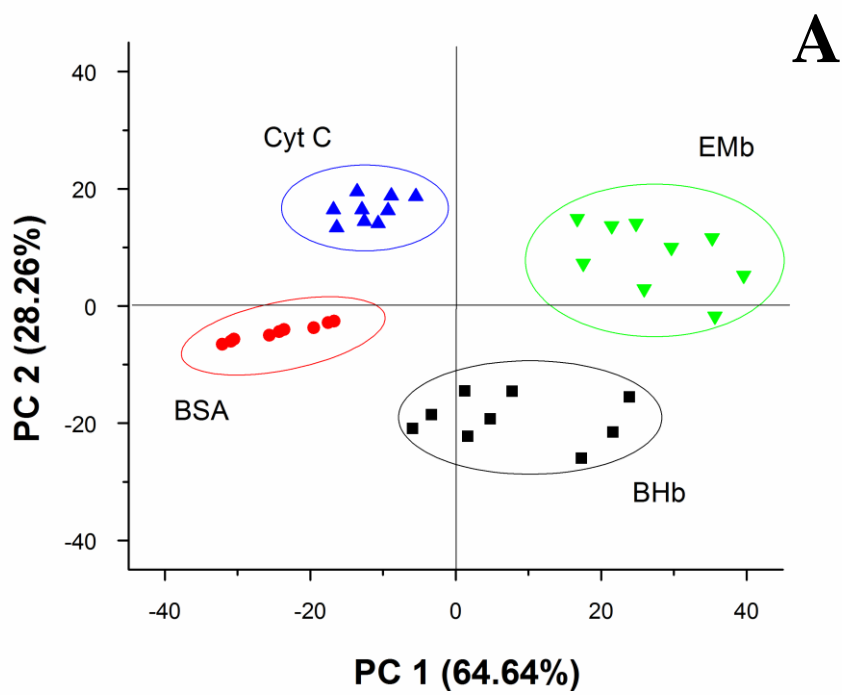


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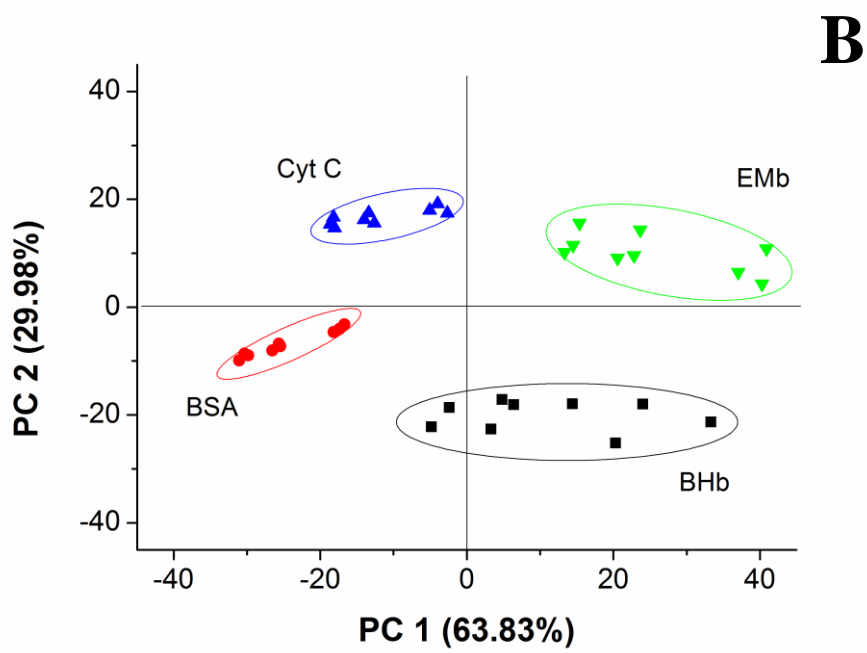


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589 Figure 3



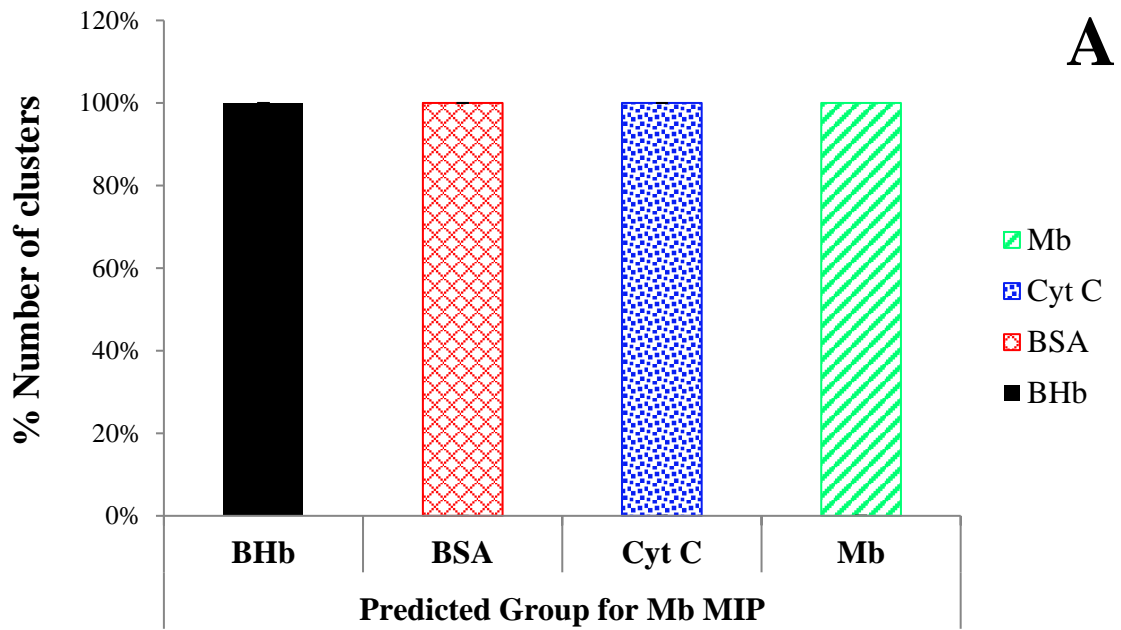
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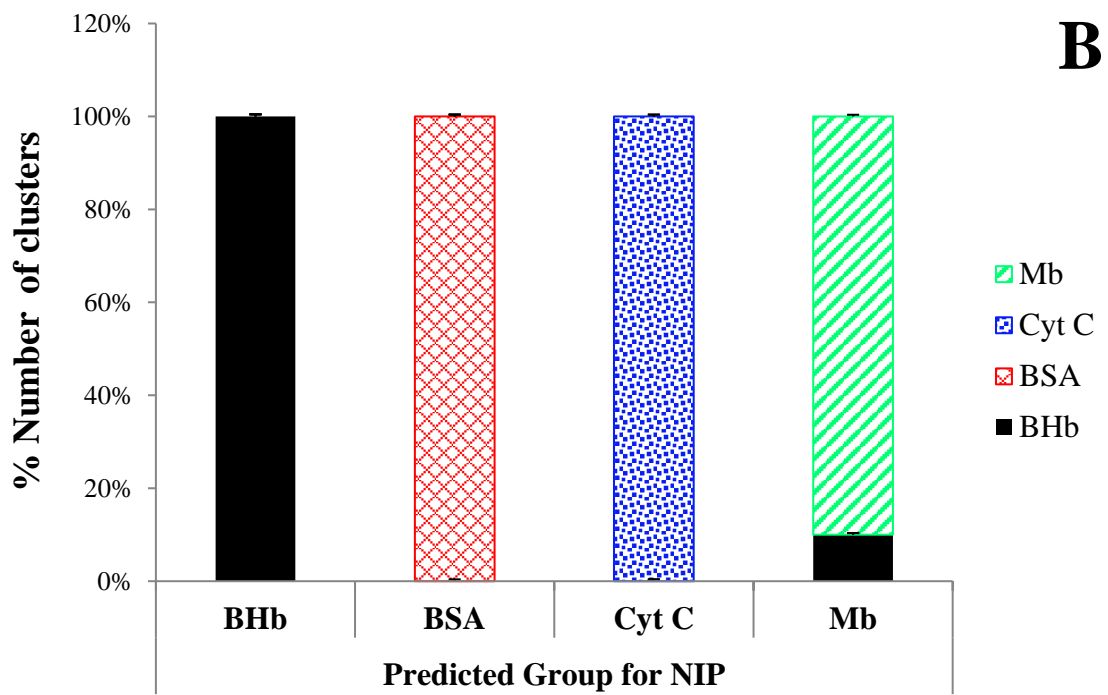
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592 Figure 4

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597 Figure 5

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