



## Article

# Spectroscopic and quartz crystal microbalance (QCM) characterisation of protein-based MIPs

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*El-Sharif, H.F., Aizawa, H. and Reddy, Subrayal M ORCID: 0000-0002-7362-184X (2015) Spectroscopic and quartz crystal microbalance (QCM) characterisation of protein-based MIPs. Sensors and Actuators, B: Chemical, 206 . pp. 239-245. ISSN 0925-4005*

It is advisable to refer to the publisher's version if you intend to cite from the work.  
<http://dx.doi.org/10.1016/j.snb.2014.09.053>

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1 **Spectroscopic and Quartz Crystal Microbalance (QCM) Characterization of Protein-**  
2 **based MIPs**

3

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5

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13

14 **Abstract**

15 We have studied acrylamide-based polymers of varying hydrophobicity (acrylamide, AA; N-  
16 hydroxymethylacrylamide, NHMA; N-isopropylacrylamide, NiPAm) for their capability of  
17 imprinting protein. Rebinding capacities (Q) from spectroscopic studies were highest for  
18 bovine haemoglobin (BHb) MIPs based on AA,  $Q = 4.8 \pm 0.21 < NHMA, Q = 4.3 \pm 0.32 <$   
19 NiPAm,  $Q = 3.6 \pm 0.45$ , while also demonstrating low selectivities for non-template proteins  
20 ( $<30 \pm 5\%$ ), with the exception of bovine serum albumin (BSA,  $>76 \pm 0.5\%$ ). When applied  
21 to the QCM sensor as thin-film MIPs, NHMA MIPs were found to exhibit best discrimination  
22 between MIP and non-imprinted control polymer (NIP) in the order of NiPAm  $< AA <$

23 NHMA. The extent of template removal and rebinding, using both crystal impedance and  
24 frequency measurements, demonstrated that 10% (w/v):10% (v/v) sodium dodecyl  
25 sulphate:acetic acid (pH 2.8) was efficient at eluting template BHb (with  $80 \pm 10\%$  removal).  
26 Selectivity studies of NHMA BHb-MIPs revealed higher adsorption and selective recognition  
27 properties to BHb (64.5 kDa) when compared to non-cognate BSA (66 kDa), myoglobin  
28 (Mb, 17.5 kDa), lysozyme (Lyz, 14.7 kDa) thaumatin (Thau, 22 kDa) and trypsin (Tryp, 22.3  
29 kDa). The QCM gave frequency shifts of  $\sim 1500 \pm 50$  Hz for template BHb rebinding in both  
30 AA and NHMA MIPs, whereas AA-based MIPs exhibited an interference signal of  $\sim 2200 \pm$   
31  $50$  Hz for non-cognate BSA in comparison to a  $\sim 500 \pm 50$  Hz shift with NHMA MIPs. Our  
32 results show that NHMA-based hydrogel MIP are superior to AA and NIPAM..

33

34 **Keywords:** Molecular imprinted polymer (MIP); Hydrogel; Protein; Biosensor; QCM

## 35 **1. Introduction**

### 36 1. Introduction

37 In recent years, molecularly imprinted polymers (MIPs) have allowed selective extractions  
38 that rival immunoaffinity-based separations, and have shown clear advantages over real  
39 antibodies for sensor applications: they are easy to fabricate, intrinsically stable, robust, and  
40 are able to operate in extreme environments [1], [2] and [3]. MIPs could provide an  
41 alternative, inexpensive, fast, and efficient diagnostic method for highly sensitive analytical  
42 procedures within the pharmaceutical area [3].

43

44 When imprinting complex bio-macromolecules some of the most significant drawbacks in  
45 MIP technology are the unprecedented degree of influence that the variation in pH [4], ionic  
46 strength and local matrix effects all have on the gel properties [5], [6], [7] and [8]. This can  
47 affect the three dimensional shape and chemical characteristics of the template molecule  
48 during polymerisation. This is particularly true when imprinting large bio-macromolecules  
49 such as proteins. Proteins are relatively labile, and have variable conformations which are  
50 sensitive to solvent environments, pH and temperature, all of which present a variety of  
51 challenges [5]. It has been thought that low imprinting capacities associated with bio-  
52 macromolecules could be caused by the use of charged functional monomers causing non-  
53 specific electrostatic interactions [5]. Moreover, as with antibodies, MIPs have also shown a  
54 degree of cross-selectivity, in that they can bind molecules similar to the native template and  
55 cause non-specific binding. It is thought that this is due in part to an excess of functional  
56 monomer molecules being randomly distributed and frozen within the imprinted cavity  
57 during polymerisation that have an affinity for non-template molecules [3] and [9]. Thus,  
58 more sophisticated monomers capable of forming better, stronger and more stable

59 interactions that offer better positioning and complementary functionality are widely being  
60 sought. Once these parameters are optimised, application to biosensors and analysis of actual  
61 biological samples would be more realistic [6], [10], [11] and [12].

62

63 Biosensors for proteins are currently expensive to develop because they require the use of  
64 expensive antibodies [3] and [13]. However, as MIPs are becoming more and more promising  
65 as viable alternatives to natural receptors new MIP-based sensor strategies are being  
66 developed [3]. The main advantage of biosensors is the ability to sample outside the  
67 laboratory environment with minimal user input. One important part of bio-sensing is  
68 transducers, which monitor the reaction between bio-selector and analyte. Among various  
69 physical transducers (electrochemical, peizoelectric, etc.), mass sensitive devices such as  
70 surface acoustic wave (SAW), surface plasmon resonance (SPR) and quartz crystal  
71 microbalance (QCM) have become popular for sensing applications [14], [15], [16] and [17].

72

73 Following the thorough analysis of QCM systems for use in fluids over the past 2 decades,  
74 this has allowed for more esoteric applications including bio-sensing [16]. In most cases  
75 quartz resonators are integrated to oscillator circuits to form a QCM for micro weighing  
76 applications. Normally, an equivalent circuit model is fitted to the impedance curve, and the  
77 obtained parameters can be used for calculating the resonant frequency and dissipation (D) of  
78 the quartz crystal i.e. mass and viscoelastic properties of the deposited layers [15] and [16].  
79 Determining the impedance curve has many advantages; first and foremost it has expanded  
80 the range of measurable parameters from rigid thin films, to biologically relevant films of  
81 “soft” viscoelastic material. These QCM couplings have been widely used for biomaterials  
82 and biosensor studies [10], [12], [16], [18] and [19], where surface confined bio-molecular

83 interactions have provided an insight into dissolution of polymer coatings, DNA  
84 hybridisation, cell response to pharmacological substances, molecular interactions of drugs  
85 and their delivery. The QCM has also been utilised as an immunosensor, where analytes are  
86 recognised by antibodies, which are immobilised on a thin layer deposited on a crystal surface.  
87 Resulting mass changes are transformed into an electronically measurable quantity. The  
88 objective behind the majority of QCM research is to use sensor technology to develop a rapid  
89 method for the measurement of bio-molecular affinity reactions, and an in-depth analysis of  
90 electrochemical deposition, adsorption and reaction mechanisms of polymers coated on  
91 electrodes as ‘thin films’ [10], [12], [18], [19] and [20].

92

93 The focus of this paper is the tailoring of QCM electrode surface chemistry (i.e. specialised  
94 polymer coatings), with a view that these devices can discriminate proteins for bio-sensing  
95 and basic surface-molecular interaction studies. In this work, we demonstrate the application  
96 of the QCM technique to distinguish between the behaviour of MIPs and NIPs in the  
97 presence of cognate and non-cognate proteins. Bovine haemoglobin (BHb, 64.5 kDa) was  
98 chosen as a model protein for its well-known function in the vascular system as a carrier of  
99 oxygen, also in aiding the transport of carbon dioxide and regulating blood pH [3] and [13].  
100 Bovine serum albumin (BSA, 66 kDa), a non metalloprotein of similar molecular weight to  
101 BHb, served to test the selectivity of the BHb-MIP to BSA compared to template BHb, and  
102 was compared across a family of acrylamide-based polymer hydrogels.

103

## 104 2. Experimental

### 105 2.1. Materials

106

107 Acrylamide (AA), N-hydroxymethylacrylamide (NHMA), N-iso-propylacrylamide (NiPAm),  
108 N,N'-methylenebisacrylamide (bis-AA), ammonium persulphate (APS), N,N,N',N'-  
109 tetramethylethylenediamine (TEMED), sodium dodecyl-sulphate (SDS), glacial acetic acid  
110 (AcOH), bovine haemoglobin (BHb), bovine serum albumin (BSA), hen egg-white lysozyme  
111 (Lyz), thaumatin from *Thaumatococcus daniellii* (Thau), bovine pancreatic trypsin (Tryp) and  
112 equine heart myoglobin (Mb) were all purchased from Sigma–Aldrich, Poole, Dorset, UK.  
113 Sieves (75  $\mu\text{m}$ ) were purchased from Endecotts Ltd. and Innoxia Ltd., UK. AT-cut quartz  
114 crystal pieces (9 MHz fundamental resonance) with gold-on-chrome electrodes were supplied  
115 by Nihon Dempa Kogyo Company Ltd. (Tokyo, Japan).

116

## 117 2.2. HydroMIP preparations

118

119 Hydrogel MIPs were synthesised by separately dissolving AA (54 mg), NHMA (77 mg),  
120 NiPAm (85.6 mg) and bis-AA as cross-linker (6 mg), (8.5 mg) and (9.5 mg), respectively  
121 along with template protein (12 mg) in 960  $\mu\text{L}$  of MilliQ water. The solutions were purged  
122 with nitrogen for 5 min, followed by an addition of 20  $\mu\text{L}$  of a 10% (w/v) APS solution and  
123 20  $\mu\text{L}$  of a 5% (v/v) TEMED solution. Polymerisation occurred at room temperature (RT, 22  
124  $\pm 2$   $^{\circ}\text{C}$ ) giving total gel densities (%T) of 6%T, AA/bis-AA (w/v); 8.5%T, NHMA/bis-AA  
125 (w/v); 9.5%T, NiPAm/bis-AA (w/v), and final crosslinking densities (%C) of 10%C (9:1,  
126 w/w) for all hydrogels.

127

128 For every MIP created a non-imprinted control polymer (NIP) was prepared in an identical  
129 manner but in the absence of protein. After polymerisation, the gels were granulated  
130 separately using a 75  $\mu\text{m}$  sieve. Of the resulting gels, 500 mg were transferred into 1.5 mL  
131 centrifuge Eppendorf tubes and conditioned by washing with five 1 mL volumes of MilliQ  
132 water followed by five 1 mL volumes of 10% (w/v):10% (v/v) SDS:AcOH (pH 2.8) and  
133 another five 1 mL volume washes of MilliQ water to remove any residual 10% (w/v):10%  
134 (v/v) SDS:AcOH eluent and equilibrated the gels. Each wash step was followed by a  
135 centrifugation, whereby the gels were centrifuged using an Eppendorf mini-spin plus  
136 centrifuge for 3 min at 6000 rpm (RCF:  $2419 \times g$ ). All supernatants were collected for  
137 analysis by spectrophotometry to verify the extent of template removal. It should be noted  
138 that the last water wash and eluent fractions were not observed to contain any protein.  
139 Therefore we are confident that any remaining template protein within the MIPs did not  
140 continue to leach out during the rebinding studies.

141

### 142 2.3. Rebinding studies

143

144 Once the gels (500 mg) were equilibrated, a 1 mL template protein solution prepared in  
145 MilliQ water containing 3 mg of protein was added to the target MIPs and NIP controls and  
146 was allowed to associate at RT ( $22 \pm 2$  °C) for 20 min. Selectivity studies were also  
147 conducted to assess the relative imprinting factor of the original protein template. This was  
148 achieved by loading non-cognate proteins on a BHb imprinted gel. Gels were then washed  
149 with four 1 mL volumes of MilliQ water. Each reload and wash step for all MIPs and NIP  
150 controls was followed by centrifugation at 6000 rpm (RCF:  $2419 \times g$ ) for 3 min. All  
151 supernatants were collected for analysis by spectrophotometry.



152

#### 153 2.4. Spectrophotometric analysis

154

155 Calibration curves in MilliQ water and 10% (w/v):10% (v/v) SDS:AcOH were prepared for  
156 BHB, BSA, Lyz, Tryp and Mb. Spectral scans revealed peak wavelengths for BHB in MilliQ  
157 water and 10% (w/v):10% (v/v) SDS:AcOH to be 405 nm and 395 nm, respectively. Peak  
158 wavelengths for BSA in MilliQ water and 10% (w/v):10% (v/v) SDS:AcOH were found to be  
159 288 nm and 290 nm respectively. Peak wavelengths for Lyz in MilliQ water and 10%  
160 AcOH:SDS were found to be 291 nm and 296 nm respectively. Peak wavelengths for Tryp in  
161 MilliQ water and 10% (w/v):10% (v/v) SDS:AcOH were found to be 293 nm. Peak  
162 wavelengths for Mb in MilliQ water, 10% (w/v):10% (v/v) SDS:AcOH were found to be 410  
163 nm, and 396 nm respectively. Analysis and subsequent determination of protein  
164 concentration in appropriate media was performed at specific peak wavelengths using a UV  
165 mini-1240 CE spectrophotometer (Shimadzu Europa, Milton Keynes, UK).

166

#### 167 2.5. Quartz crystal microbalance (QCM) analysis of thin film MIPs

168

169 QCM crystals were sealed and air capped (single-sided) with PVC glue in-order to prevent  
170 short circuiting when the QCM was submerged in solution [10]. Poly AA, NHMA and  
171 NiPAm gels for BHB were synthesised using the hydrogel production procedures outlined in  
172 Section 2.2. Before polymerisation, MIPs and NIPs were deposited as thin films onto the  
173 capped QCM crystals. Thin-films were achieved by beading and compressing 10  $\mu$ L of the  
174 polymerising solutions directly onto the crystals. QCM frequency and impedance

175 measurements were taken using an Agilent 4194A Impedance Analyser. An in-house written  
176 QBasic programme was used to drive the analyser and collect series resonance frequency and  
177 impedance data in real time.

178

#### 179 2.5.1. Elution and rebinding studies

180

181 MIP and NIP polyAA thin-film capped crystals were firstly immersed in MilliQ water,  
182 followed by 10% (w/v):10% (v/v) SDS:AcOH in order to remove imprinted protein primarily  
183 from the surface of the polymer. This was followed by another submersion in MilliQ water to  
184 remove any residual surfactant and to re-condition the hydrogel. After subsequent  
185 stabilisation of the QCM response, template protein was reloaded by immersing the QCM in  
186 a 3 mg/mL BHb solution and the response trace was recorded at RT ( $22 \pm 2$  °C).

187

#### 188 2.5.2. Selectivity studies

189

190 Continuous real-time scans were conducted in-order to assess characteristic impedance  
191 changes of the gels during surface exposure to wash, elute and protein rebinding conditions.  
192 During a typical run, the MIP thin-film capped crystals were submerged sequentially in  
193 various solutions such as 10% (w/v):10% (v/v) SDS:AcOH, MilliQ water or 3 mg/mL protein  
194 solutions (cognate BHb and non-cognate BSA, Thau, Lyz and Tryp) for a set time of 5 min  
195 each, and crystal impedance and frequency responses were recorded at RT ( $22 \pm 2$  °C). The  
196 latter procedure was followed for AA, NiPAm and NHMA based MIP hydrogels for BHb.

197

### 198 3. Results and discussion

#### 199 3.1. MIP selectivity

200 The molecular imprinting effect is characterised by the rebinding capacity (Q) of protein to  
201 the gel polymer (mg/g) exhibited by the protein-specific MIP and the control NIP, and is  
202 calculated using Eq. (1), where  $C_i$  and  $C_r$  are the initial protein and the recovered protein  
203 concentrations (mg/mL) respectively (which specifies the specific protein bound within the  
204 gel),  $V$  is the volume of the initial solution (mL), and  $g$  is the mass of the gel polymers (g).

205

$$206 \quad Q = \frac{[C_i - C_r]V}{g} \quad (1)$$

207

208 Fig. 1A shows the rebinding capacities and imprinting factors of polyacrylamide (polyAA)  
209 MIP and NIPs for several different proteins. The internal measure of the imprinting factor  
210 between MIP and NIP serves to demonstrate that the MIP possesses selective cavities for the  
211 rebinding of template molecule compared to NIP controls. It can clearly be seen that there is  
212 a distinctive rebinding capacity variation for each imprinted protein template within a  
213 polyAA MIP. This has previously been attributed to protein size, cross-linking density, and  
214 the initial degree of association within the polymer matrix [4].

215 Gels based on N-hydroxymethylacrylamide (NHMA) exhibited similar rebinding trends,  
216 whereas poly-N-isopropylacrylamide gels (polyNiPAm) demonstrated lower rebinding  
217 capacities. Thus, bulk gel characterisation revealed the highest rebinding capacities for BHb  
218 MIPs based on polyAA ( $Q = 4.8 \pm 0.21$ ), followed by polyNHMA ( $Q = 4.3 \pm 0.32$ ),

219 polyNiPAm ( $Q = 3.6 \pm 0.45$ ). These gel imprinting trends are in agreement with those  
220 previously published [4], [9] and [10].

221

222 Selectivity studies were also conducted to confirm a BHb specific imprinting effect and to  
223 assess the relative imprinting factor of cross-selective binding profiles. The cross-reactivity of  
224 the BHb-imprinted MIPs for non-cognate proteins was quantified using relative imprinting  
225 factors ( $k$ ), Eq. (2), where  $IF_{BHb}$  is the imprinting factor for BHb, and is calculated by  $IF =$   
226  $[C_i - C_r]MIP/[C_i - C_r]NIP$ , and  $IF_x$  is the imprinting factor of the cross-reacting non-  
227 cognate proteins on a BHb MIP. For the template BHb  $k = 1$ , and for non-cognate proteins  
228 that are less-specific for the BHb MIP,  $k < 1$ .

229

$$230 \quad k = \frac{IF_{BHb}}{IF_x} \quad (2)$$

231 The data (Fig. 1B) suggests that both non-cognate trypsin (Tryp) and lysozyme (Lyz) proteins  
232 have relatively low affinities for the BHb-specific MIP,  $k \approx 0.2 \pm 0.05$ . However, bovine  
233 serum albumin (BSA), which is a similar size to BHb, exhibited a high degree of interference  
234 binding (cross selectivity) resulting in high  $k$  values of  $0.65 \pm 0.05$ . Myoglobin (Mb) also  
235 exhibited some degree of cross-selectivity; this can be attributed to the size of Mb, which is a  
236 quarter that of BHb (17.5 kDa), and its similarity to a single BHb sub-unit [4]. Interestingly  
237 though, when reversed, a polyAA BSA-MIP exposed to non-target BHb protein had  
238 relatively low affinity. It would appear that BSA has a high ability to bind non-specifically to  
239 a BHb MIP, whereas BHb does not exhibit the same ability within a BSA MIP.

240

241 Competitive binding studies using a 50:50 mixture of BHb:BSA (3 mg/mL total) on a MIP-  
242 BHb were also conducted (Fig. 1B). The addition of BSA caused an obvious capacity  
243 decrease of BHb binding on the BHb-MIP, suggesting that the rebinding of BHb was  
244 displaced by the competing BSA or by protein-protein interactions [21]. As the size,  
245 structure, and specificity of the imprinted cavities should be in favour of the BHb template, it  
246 is rational that the addition of BSA as a competing protein would not bind to the BHb-  
247 specific imprinted cavities. Gai et al. previously demonstrated that BSA does not bind  
248 specifically to a BHb MIP, but rather displaces the non-specific recognition sites of cavities  
249 and the nonspecific binding of BHb to BHb-MIP [21]. Moreover, although BSA and BHb  
250 share similar sizes (66 kDa and 64.5 kDa, respectively), it should be noted that BSA has a pI  
251 of 4.6 and BHb a pI of (6.8–7.0). Since competitive binding was performed under MilliQ  
252 water (pH 5.4), conditions are in favour of BSA [21] and [22]. Above its pI, BSA becomes  
253 negatively charged and the groups exist as single bondNH<sub>2</sub> and single bondCOO<sup>-</sup>, this  
254 overall negative net charge induces more favourable and complementary hydrogen bonding  
255 interactions, resulting in increased specific as well as non-specific binding [4].

256

### 257 3.2. QCM sensor application of MIPs

258 Thin film BHb MIPs were prepared on the surface of a QCM chip and the sensor was  
259 exposed sequentially to MilliQ water, 10% (w/v):10% (v/v) SDS:AcOH and 3 mg/mL protein  
260 solutions at RT ( $22 \pm 2$  °C). We have previously published on the thickness of the thin films  
261 on sensor chips with an average thickness of  $138 \pm 9$  nm [6]. Given that for a 9 MHz crystal  
262 the shear wave decay length is 250 nm at RT [23], we are within the sensing region of the  
263 QCM to measure both bulk and surface effects within the MIP film.

264

265 Fig. 2A and B shows the QCM impedance and frequency responses following immersion in a  
266 solution of 10% (w/v):10% (v/v) SDS:AcOH in order to remove imprinted protein from the  
267 surface of the polymer. Previous investigations have shown that optimum conditions for  
268 protein removal of up to 80% have been achieved using 10% (w/v):10% (v/v) SDS:AcOH  
269 [9]. Using this acid/surfactant combination the positively charged protein attaches to the  
270 negatively charged surface of SDS micelles and disrupts the hydrophobic bonds. Since there  
271 is a significant shift in both resonance frequency and impedance it can be assumed that some  
272 of the BHb imprinted template has been successfully removed from the MIP.

273 It is worth noting the two distinct differences in the impedance response when compared with  
274 the frequency response. Firstly, the impedance response has much reduced noise in the signal  
275 in contrast to the frequency response. Secondly, there are significant additional transitions  
276 (e.g. at 350 and 650 s) in the signal which are being observed in the Z response, but not in the  
277 frequency response. It has been suggested that whereas the frequency response predominately  
278 demonstrates the QCM mass response only within an adlayer, the electrical impedance gives  
279 a combination response of the mass effect as well as subsequent changes in the viscoelasticity  
280 of the adlayer possibly due to molecular relaxations within the adsorbed layer over a longer  
281 timescale following initial immersion [23], [24] and [25].

282

283 After subsequent stabilisation of the QCM response, the template BHb was then reloaded by  
284 immersing the QCM in a 3 mg/mL BHb solution and the response trace recorded. Fig. 2C and  
285 D compares the final QCM impedance and frequency responses to template BHb exposure of  
286 each MIP and its corresponding NIP. It can be seen that upon addition of a 3 mg/mL BHb  
287 solution to the BHb MIP caused significant QCM responses compared with NIP thin-film  
288 hydrogels. This suggests that MIP thin-film gels are affected by specific binding of target

289 BHb. This distinct difference between responses exhibited by MIP and the NIP control  
290 strongly supports that binding and elution of target protein gave rise to distinct impedance  
291 transitions. The  $200 \pm 50$  Hz frequency shift observable by both MIP and NIP during the  
292 initial loading step (Fig. 2D) is suggestive of a solution viscosity effect.

293

294 Real time impedance response following sequential immersion in solutions of BHb, 10%  
295 (w/v):10% (v/v) SDS:AcOH and BSA were also measured. Three distinct types of responses  
296 were observed depending on the acrylamide-based monomer used. The key difference  
297 between the polymers is their hydrophilicity dictated by the hydrophilic hydroxyl group in  
298 NHMA and the hydrophobic isopropyl group in NiPAm. AA sits between the two in degree  
299 of hydrophilicity (polyNHMA > polyAA > polyNiPAm), which agrees with the order of best  
300 performance of the polymers as BHb MIPs in previous QCM studies [10]. Fig. 3 compares  
301 the final QCM response to cognate and non-cognate protein exposure of each MIP with its  
302 corresponding NIP. Interestingly, the NiPAm MIP and NIP both show a near zero frequency  
303 response to template BHb and non-cognate BSA, indicating that NiPAm is equally  
304 unselective for both proteins as is the control non-imprinted polymer. The non-response of  
305 NiPAm to either BHb or BSA suggests that there is a resistance to either protein to bind to  
306 the polymer. The striking difference in selectivities between cognate and non-cognate  
307 proteins for NHMA and AA suggests that the hydroxyl group in NHMA plays a significant  
308 role in the selective binding of BHb and the lack of binding of BSA.

309 Moreover, variations of the series resonance frequency demonstrated to be highly dependent  
310 on the test solution used (Fig. 4A). The impedance data is presented here because in  
311 comparison to the frequency response, there is much reduced noise in the signal following  
312 each solution phase immersion. It can be seen that MIP thin-films exposed to a 10%:10%

313 (v/v) SDS:AcOH solution exhibited an immediate significant decrease in impedance ( $500 \pm$   
314  $100 \Omega$ ); this is possibly due to the increase in the viscosity of the solution caused by the  
315 presence of SDS micelles in the solution. Moreover, it can clearly be seen that the  
316 introduction of non-template BSA also exhibits an impedance response within the poly AA  
317 MIP, suggesting some non-specific binding within the BHb-HydroMIP. Thus, there is a high  
318 degree of cross-selectivity present for our AA-based MIPs ( $>70\%$ ), and this interference is  
319 absent when NHMA-based MIPs are used ( $<20\%$ ) as seen in Fig. 3.

320 To further test the BHb-MIP selectivity, we investigated the rebinding of template BHb after  
321 exposing the MIP with non-target BSA. The resulting quantified imprinting effect of BHb for  
322 polyAA MIP thin-film gels can be seen in the impedance responses (Fig. 4B). The  
323 comparison demonstrates that both HydroMIP and HydroNIP films act differently under  
324 water wash, elution and load (solution immersion) treatments following BSA loading. It can  
325 be seen that when non-target BSA is loaded first, the QCM impedance response is now  
326 negligible. Interestingly, impedance responses are also almost negligible when BHb is  
327 introduced after prior exposure to BSA. Although the loaded BSA did not associate  
328 specifically with the BHb-MIP thin-film surface, an interesting and lasting effect inhibits  
329 BHb from easily binding to recognition sites. Indeed, BSA is similar to the template BHb in  
330 size, but the spatial arrangement of the effective groups on its exterior are different from  
331 BHb, and the recognition sites in the BHb-MIP cavities are not complementary in shape to  
332 BSA [22]. Therefore, little to no selectivity of BSA on BHb-MIPs should be expected.

333 Therefore, in the case of AA-based MIPs, the inhibition effect is most likely due to the ability  
334 of BSA to exhibit protein binding on the MIP surface but not within cavities. Formation of a  
335 BSA biolayer above (but not within) the cavities would block subsequent cavity-selective  
336 MIP binding for its cognate protein [22]. This is further indication that BHb-MIPs distinguish  
337 proteins not only based on molecular size, but also on the synergistic effect of shape memory,



338 and multiple weak hydrogen bonding interactions specific to template protein in  
339 macromolecular recognition [21], [22] and [26].

340

341 Moreover, further studies to interrogate the recognition capabilities of MIPs were carried out  
342 using a range of non-metalloproteins chosen for their different sizes and functionalities  
343 compared to BHb, BSA and Mb. Of these proteins: lysozyme (Lyz), a glycoside hydrolase  
344 enzyme (14.7 kDa) that is part of the innate immune system, and exists as a natural form of  
345 protection from pathogens like Salmonella, E. coli, and Pseudomonas [9], [10] and [27].  
346 Thaumatin (Thau), a sweetener or flavour modifier (22 kDa) often used in crystallisation  
347 studies due to its ease of use and crystal formation [27]. Trypsin (Tryp), a serine protease  
348 enzyme or proteinase 'digestive enzyme' (23.8 kDa) commonly imprinted within MIPs [9],  
349 [10] and [27]. Fig. 5 shows that the BHb-MIPs based on all three polymers (AA, NHMA, and  
350 NiPAm) are essentially non-responsive to the addition of the three smaller proteins Thau, Lyz  
351 and Tryp respectively. An average NIP response was calculated based on all three polymers  
352 and used as an illustration to demonstrate the negligible responses exhibited by the MIP  
353 properties. The negligible responses exhibited by the QCM sensor concur with the qualitative  
354 data and confirm that these small proteins exhibit no selective specific/non-specific binding  
355 characteristics to a BHb-imprinted MIP.

356

#### 357 4. Conclusions

358 A family of acrylamide-based MIPs have been characterised for their imprint efficiency using  
359 spectrophotometric and QCM sensor techniques for biosensor development. Varied rebinding  
360 capacities and relative imprinting factors have been achieved using bulk characterisation. We

361 have demonstrated that MIP selectivity is a function of the hydrophilicity of the acrylamide  
362 monomer used to form the MIP. Three distinct types of QCM responses were observed  
363 depending on the acrylamide used (polyNHMA > polyAA > polyNiPAm), which agrees with  
364 the order of best performance of the polymers in previously published QCM studies. The  
365 selectivity of BHb-MIP for BHb and BSA was also compared via QCM, along with several  
366 other proteins. Results demonstrated BHb-MIP to have better selective adsorption and  
367 recognition properties to BHb than BSA when using the hydrophilic NHMA as a MIP  
368 polymer matrix. Therefore, the QCM sensor was able to indicate MIP surface activity and  
369 provide physical interpretation in terms of hydrophilicity of the polymer matrix that forms the  
370 MIP and protein selectivity. Our QCM sensor also has the ability to assess the extent of  
371 specific protein binding by sensing surface-specific bound cognate protein to MIPs compared  
372 to non-imprint NIP controls. We expect, once fully developed, that the benefits of sensitivity,  
373 specificity and stability of MIPs coupled with discriminatory techniques, such as QCM, will  
374 be crucial to the future impact of portable diagnostics for personal healthcare and use by  
375 health professionals. The technology also presents major potential benefits to environmental  
376 and food monitoring.

377

### 378 Acknowledgements

379 The authors wish to thank the UK Engineering and Physical Sciences Research Council  
380 (EPSRC) Grants (EP/G014299/1) and NERC/ACTF (RSC) for supporting this work.

381

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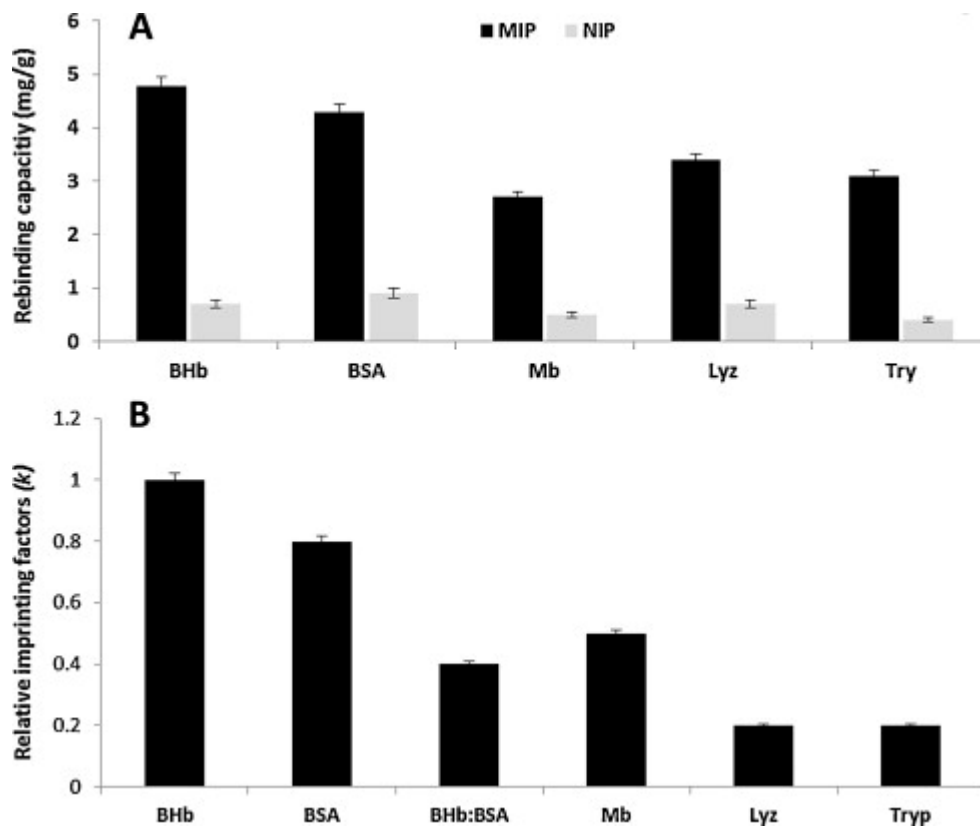
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465 Fig. 1.

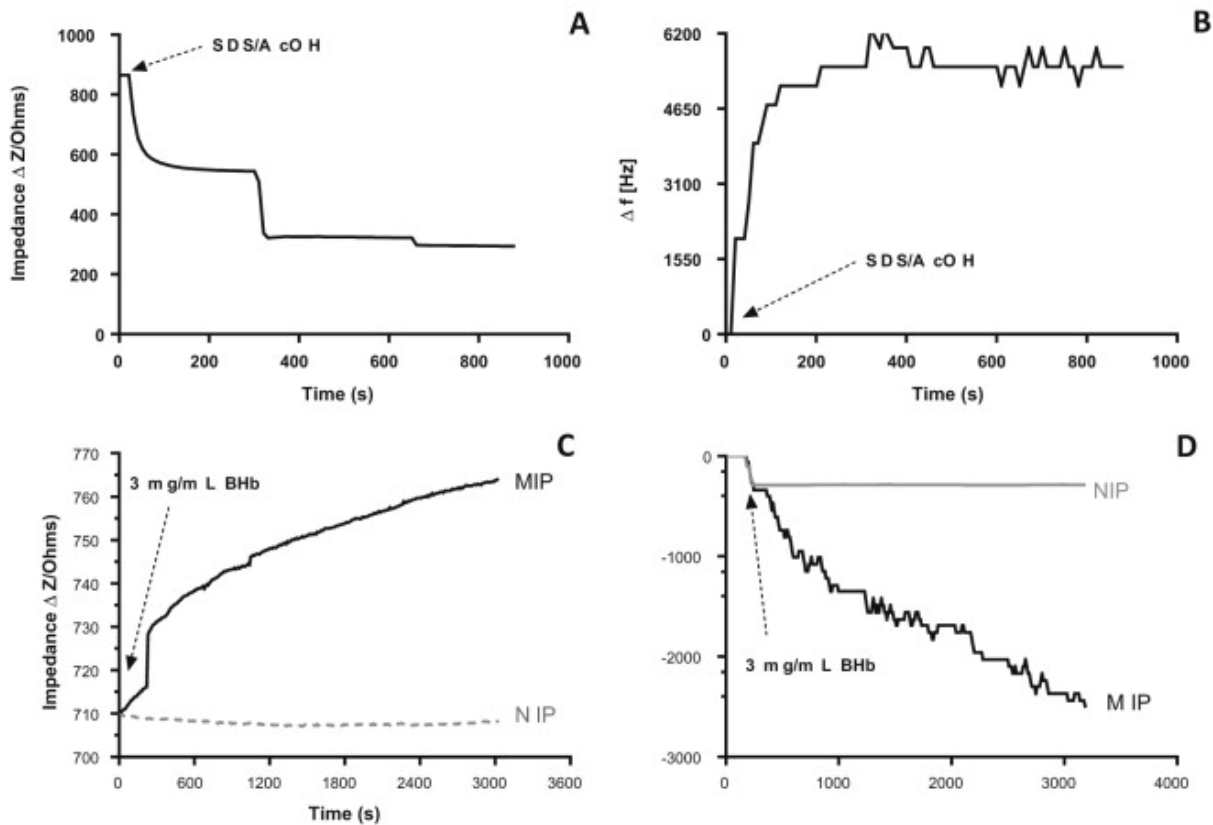
466 (A) Rebinding capacities ( $Q$ ) and imprinting factors of MIP<sub>polyAA</sub> and NIP<sub>polyAA</sub> hydrogels for several  
467 proteins in MilliQ water media: bovine haemoglobin (BHb), bovine serum albumin (BSA), myoglobin  
468 (Mb), lysozyme (Lyz), trypsin (Tryp); (B) relative imprinting factors ( $k$ ) for a range of BHb-  
469 MIP<sub>polyAA</sub> cross-reactants in MilliQ water media. Results illustrate higher MIP selectivities for cognate  
470 BHb and the degree of cross-selectivity for other non-template analytes. Data represents  
471 mean  $\pm$  S.E.M.,  $n = 3$ .  
472



473

474 Fig. 2.

475 QCM response to the immersion of polyAA-BHb hydrogel thin-film MIP in 10% (w/v):10% (v/v)  
476 SDS:AcOH in order to follow protein elution (arrow indicates time of MIP immersion): (A) impedance  
477 ( $\Delta Z$ ), (B) frequency ( $\Delta f$ ); QCM responses to BHb (3 mg/mL) loading onto a BHb imprinted polyAA  
478 hydrogel thin-film (arrow indicates time of immersion in protein solution): (C) impedance ( $\Delta Z$ ) and (D)  
479 frequency ( $\Delta f$ ).  
480



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482

483 Fig. 3.

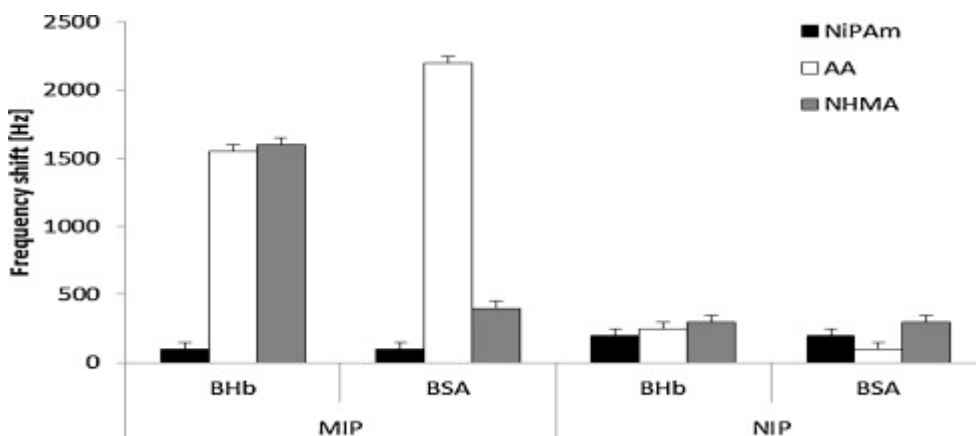
484 QCM frequency shift responses of NiPAm, AA and NHMA polymer MIPs and NIPs to cognate BHB

485 and non-cognate BSA loading (3 mg/mL) after 5 min of exposure. Data represents

486 mean  $\pm$  S.E.M.,  $n = 3$ .

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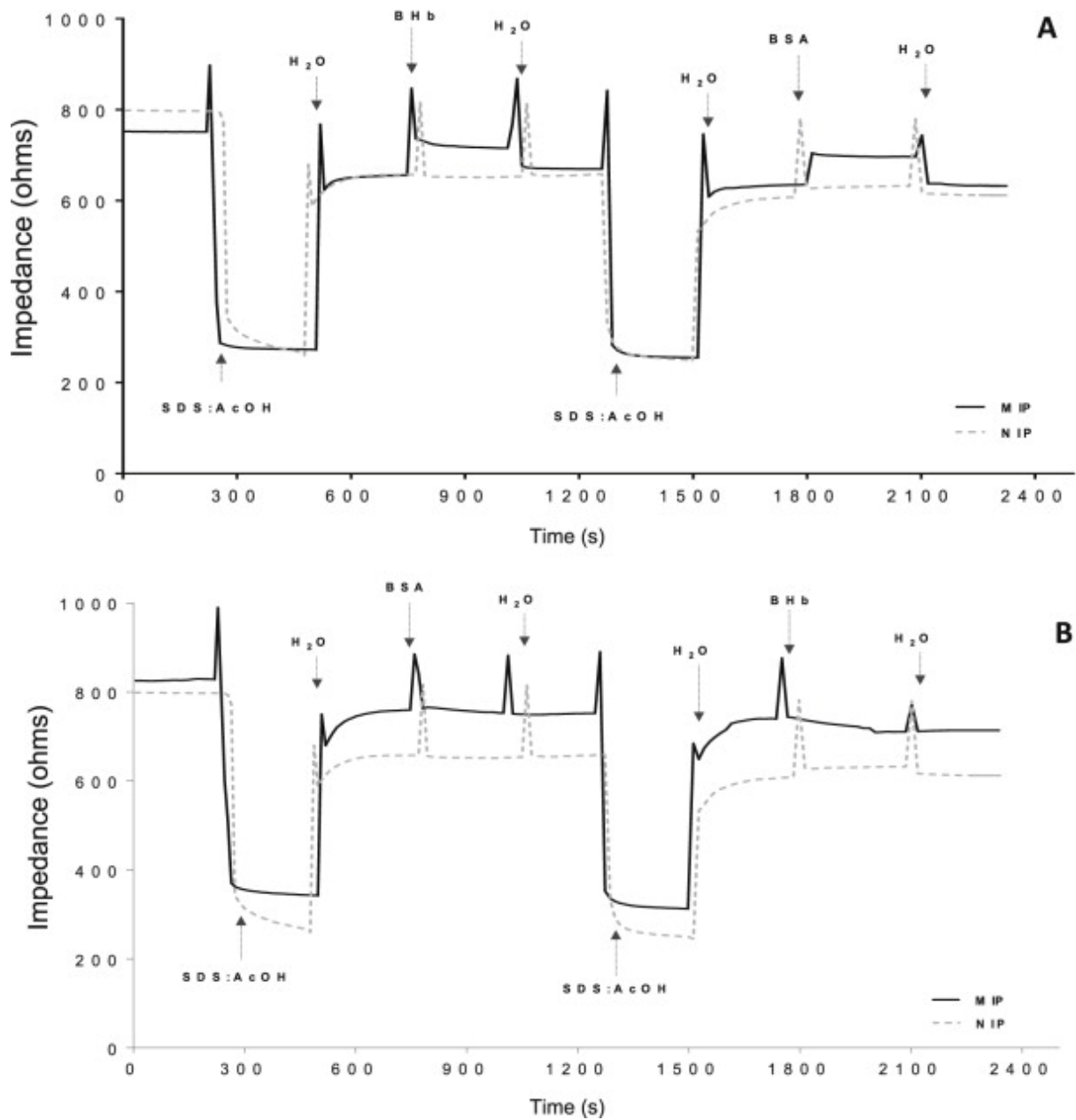
490 Fig. 4.

491 Real time QCM impedance responses: (A) direct BHB rebinding and BSA cross-selectivity on a BHB-

492 MIP<sub>polyAA</sub>; (B) BSA cross-selectivity on a BHB-MIP<sub>polyAA</sub> followed by BHB rebinding.

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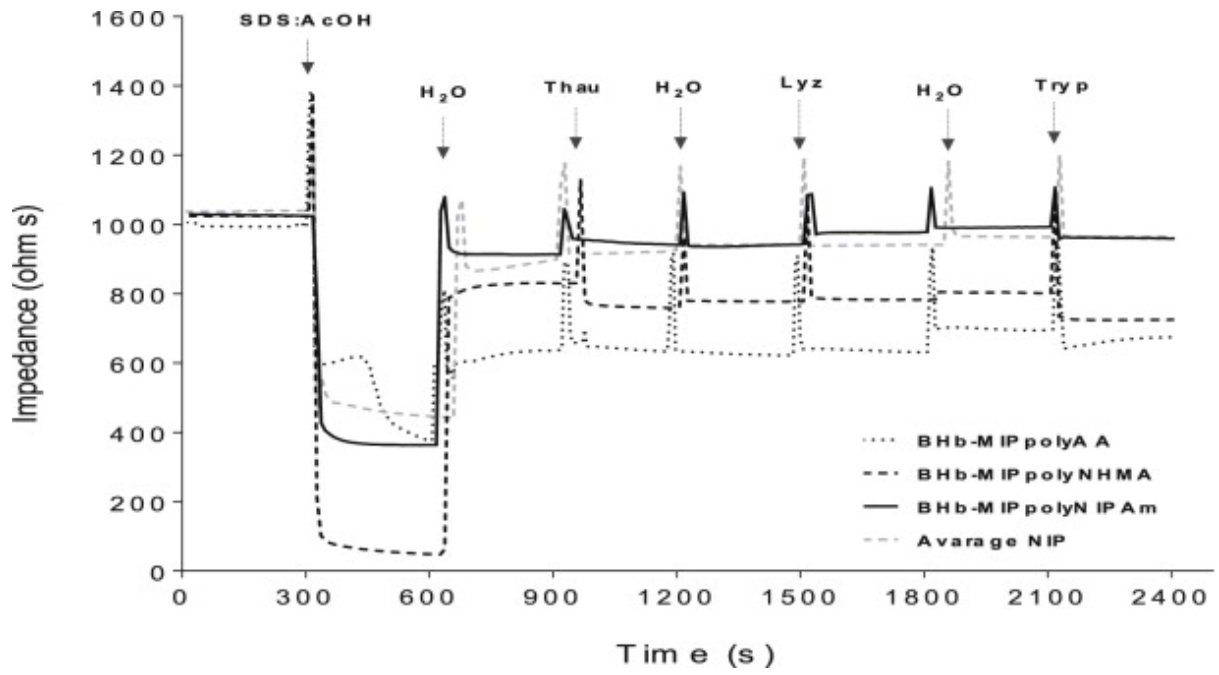
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496 Fig. 5.

497 QCM response of functionalised acrylamide BHB MIPs to non-cognate proteins thaumatin (Thau),  
 498 lysozyme (Lyz), and trypsin (Tryp) after H<sub>2</sub>O washes and an SDS:AcOH elute.

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