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Protein Detection using Hydrogel-based Molecularly Imprinted Polymers Integrated with Dual Polarization Interferometry.

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23

24 Abstract

25 A polyacrylamide-based molecularly imprinted polymer (MIP) was prepared for bovine haemoglobin
26 (BHb). A 3 mg/ml solution of BHb was injected over a dual polarisation interferometer (DPI) sensor
27 to form a physisorbed layer typically of 3.5 ± 0.5 nm thickness. Onto the pre-adsorbed protein layer,
28 MIP and NIP (non-imprinted polymer) were separately injected to monitor the interaction of BHb
29 MIP or NIP particles under different loading conditions with the pre-adsorbed protein layer. In the
30 case of NIP flowing of the protein layer, there was negligible surface stripping of the pre-adsorbed
31 protein. When a protein-eluted sample of MIP particles was flowed over a pre-adsorbed protein
32 layer on the sensor chip, the sensor detected significant decreases in both layer thickness and mass,
33 suggestive that protein was being selectively bound to MIP after being stripped-off from the sensor
34 surface. We also integrated thin-film MIPS for BHb and BSA onto the DPI sensor surface and were
35 able to show that whereas BHb bound selectively and strongly to the BHb MIP thin film (resulting in
36 a sustained increase in thickness and mass), the BHb protein only demonstrated transient and
37 reversible binding on the BSA MIP. MIPS were also tested after biofouling with plasma or serum at
38 various dilutions. We found that serum at 1/100 dilution allowed the MIP to still function selectively.
39 This is the first demonstration of MIPS being integrated with DPI in the development of synthetic
40 receptor-based optical protein sensors.

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42

43 Keywords: Hydrogel; MIP; biomimicry; interferometry; protein; biosensor

44

45 1. Introduction

46 Molecularly imprinted polymers (MIPs) continue to receive much attention in research effort as they
47 promise (and in some cases are delivering) synthetic materials capable of mimicking the selective
48 binding function of antibodies and enzymes. The implications for such biomimicry are immense in
49 the field of biosensor development and novel drug delivery modes. Over the past decade, there has
50 been an exponential increase in research activity in developing hydrogel-based molecularly
51 imprinted polymers (HydroMIPs) for the imprinting of proteins [1]. Hydrogels are insoluble,
52 crosslinked polymer network structures composed of hydrophilic homo- or hetero-co-polymers,
53 which have the ability to absorb significant amounts of water [2]. Monomers that have commonly
54 been used for non-covalent molecular imprinted hydrogels are generally chosen on their ability to
55 form weak hydrogen bonds between the monomer and the template [2-4]. Polyacrylamide
56 hydrogels are known to be very inert, offer hydrogen bonding capabilities, and are biocompatible.
57 For these reasons, acrylamide has been commonly used for molecular imprinting [5-9].

58 The use of optical sensor platforms in conjunction with imprinted polymers have been recently
59 reported, primarily detailing the use of SPR [10] and quantum dots/array technologies [11-12]. Both
60 applications have been reviewed in depth by Al-Kindy et al. [13].

61 Interferometric sensors based on dual polarisation interferometry (DPI) can be used for biological
62 detection and the sensing is accomplished by directly monitoring a bioconjugate reaction occurring
63 within an evanescent field extending out from the interferometer's sensing channel [14]. There are
64 no subsequent steps, which are typical in many other sensing schemes, in which a second
65 bioconjugate is reacted with the first to produce a sandwich complex.

66 The optical analytical technique has been designed specifically for the study of thin films, which uses
67 electromagnetic evanescent wave probes to characterise the film above a planar waveguide surface.
68 By including an optical bridge in the form of a buried reference waveguide, the sensitivity of thin film

69 measurements is maximised with the resolutions determined by the interaction length between thin
70 film and the evanescent field [15].

71 The measurement principle involves the use of a HeNe laser light ($\lambda=632.8$ nm) source coupled to
72 the end facet of a silicon substrate and a ferroelectric liquid crystal halfwave plate switches the
73 plane of polarisation of the input beam between Transverse Electric (TE) and Transverse Magnetic
74 (TM) at frequencies of typically 50 Hz. At the output, interference fringes in the far-field form on a
75 digital camera screen, with the fringes being representative of the relative phase position of the
76 sensing and reference light paths at the output. Any thin film changes thereof on the sensor surface
77 of the waveguide will interact with its evanescent field and change its effective refractive index (RI).
78 Such changes will move the phase of the light exiting the sensing waveguide, and the position of the
79 fringes on the camera will move.

80
81 The phase positions refer to the two orthogonal polarisations (TE and TM) that are measured by the
82 instrument. The absolute effective index of a waveguide mode is found by solving Maxwell's
83 equations of electromagnetism for a system of uniform multiple dielectric layers in which the fields
84 in the semi-infinite bounding layers are exponentially decaying solutions. The parameters required
85 are the RI and thickness of each layer for each of two polarisations. Provided the input information
86 is complete, an effective index value is obtained which is representative of the distribution of optical
87 power amongst the layers. If a new layer is introduced to (or removed from) the system, it will alter
88 the effective index. For each of the two polarisations, the new effective index can satisfy a
89 continuous range of thickness and refractive index values. However, there will only be one unique
90 combination that satisfies the index of the two polarisations.

91 Using different evanescent field profiles, various characteristics of the thin-film can be resolved.
92 Different polarisations are used to resolve the optical density and the thin film thickness
93 simultaneously at resolutions of $<1\text{pg}/\text{mm}^2$ and $<10\text{pm}$ [16]. If a new layer is introduced to (or

94 removed from) the system, it will alter the effective index. For each of the two polarisations, the
95 new effective index can satisfy a continuous range of thickness and refractive index values.
96 However, there will only be one unique combination that satisfies the index of the two polarisations.

97 The technique allows the precise behaviour of layers to be determined in terms of both their density
98 (absolute RI) and thickness in real time and therefore, mass, surface coverage and concentration can
99 be calculated. Integral to this is the calibration of both the sensor chip and bulk refractive index,
100 which allows the accurate derivation of the data, and takes into account subtle changes film
101 parameters and variations in chip structure which may give rise to errors in sensitivity. As a result,
102 the DPI technology presents the opportunity to further provide a rapid method for the
103 characterisation and quantification of molecular binding events.

104 To date, work using dual polarisation interferometry has been focused on investigations into the
105 studying nucleic acid interactions [17], DNA immobilisation [18], antibody-antigen interactions [19],
106 protein characterisation [20-23], and polymer characterisation [24-26]. Herein is the first report of
107 DPI to characterise HydroMIPs and develop thin film HydroMIP-based protein sensors.

108

109 2. Materials & Methods

110 Acrylamide, glacial acetic acid (AcOH), ammonium persulphate (APS), N, N' –
111 methylenebisacrylamide; ethanol (EtOH), sodium dodecyl sulphate (SDS); N, N, N', N' –
112 tetramethylethyldiamine (TEMED), phosphate buffered saline (PBS), succinic acid, tris base, and tris
113 hydrochloric acid were purchased from Sigma-Aldrich. Bovine haemoglobin (BHb); and bovine serum
114 albumin (BSA) were purchased from Sigma-Aldrich. Unmodified silicon oxynitride sensor chips were
115 purchased from Farfield Sensors (Crewe, UK) and a programmable syringe pump (PHD 2000) was
116 purchased from Harvard Apparatus (Holliston, MA, USA). Plastic syringes (1ml & 5ml with Leur Lock
117 fittings) were purchased from Becton Dickinson UK Ltd (Oxford, UK). Pooled plasma and serum
118 samples were used in the biocompatibility studies.

119

120 2.1 Preparation of Solutions

121 A solution of 10% (w/v) AcOH:SDS was prepared for use in the wash stages before and after the
122 reloading stage of the rebinding studies. SDS (10g) was dissolved in 90ml of MilliQ water. 10ml of
123 AcOH was added and mixed thoroughly using a magnetic stirrer. A 0.3mg/ml stock solution of BHb in
124 RO water was prepared, as were SDS solutions to give final percentages of 10%, 5%, 2%, 0.5%, 0.1%
125 and 0.05% (w/v). A reverse osmosis (RO) water stock solution was degassed under vacuum (with
126 stirring) for 10mins, as was an 80% (w/w) solution of ethanol (EtOH) in degassed RO water. A stock
127 solution of bovine haemoglobin (BHb) template solution was prepared in MilliQ water.

128

129 2.2 HydroMIP production

130 Molecularly imprinted (MI) hydrogels were produced using our optimised methodology [5]. It was
131 shown that when imprinting BHb using bulk polyacrylamide hydrogels, a 10% crosslinked
132 polyacrylamide/ N, N'- methylenebisacrylamide hydrogel produced the optimal imprint for BHb in
133 terms of specificity and rebinding efficiency of the MIP compared to the non imprinted polymer

134 (NIP). Hawkins et al. also demonstrated that using a 10% AcOH:SDS during the elution stage of the
135 rebinding studies performed resulted in optimal protein recovery for BHB specific MIPs.

136 Therefore, MI hydrogels were produced as follows for 1ml of gel: 54mg of functional monomer
137 (acrylamide), 6mg of crosslinker (N, N'- methylenebisacrylamide), and 12mg of template protein
138 were all dissolved in PBS or MilliQ water and added together to create the MIP solution. 20 μ l/ml of a
139 10% (w/v) ammonium persulfate (APS) solution was added to the MIP solution, and the solution was
140 purged with nitrogen for 5 minutes. Once the solution was degassed, 20 μ l/ml of a 5% (v/v) N, N, N',
141 N'- tetramethylethyldiamine (TEMED) was added and the solution was then left to polymerise
142 overnight at room temperature. We looked at freshly prepared MIP following granulation and water
143 washing (hereafter, referred to as MIP1). A sample of MIP1 was washed with SDS and acetic acid
144 followed by a further water wash to produce sample MIP2. MIP3 was the sample produced after
145 MIP2 was reloaded with target protein (bovine haemoglobin).

146 For every BHB MIP created a NIP was also created using the same material concentration as the MIP
147 but without the protein template in order to serve as a control. BSA MIPs were also prepared using
148 12mg of BSA template instead of BHB. The BSA MIPs were used as control polymers for BHB binding
149 to be compared against BHB MIPs.

150

151 **2.3 Dual Polarisation Interferometry AnaLight®Bio200 Set-up**

152 All DPI experiments were performed on the Farfield AnaLight®Bio200 instrument, which had been
153 installed and internally calibrated by the manufacturer. The AnaLight®Bio200 instrument provides a
154 flexible platform that incorporates a modular fluidics arrangement to enable a wide range of
155 experimentation to be undertaken. Integral to all experimentation is the sensor chip used to exploit
156 the technology. The sensor is a multiplayer deposited waveguide structure on a silicon wafer, and is
157 manufactured to a high tolerance to enable accurate measurement to take place. Opening windows

158 in the top cladding of the waveguide define the two active areas on the chip, which also define the
159 active path length of the sensor and are lithographically produced to micron levels of precision. The
160 precise sensitivity is dependant on the waveguide thickness and refractive index. Many variations in
161 chip functionalisation and design are available and should be used following the careful
162 consideration of the method of immobilisation of molecules on the sensor surface. Hydroxylation of
163 the silicon oxynitride sensor is one of the simplest and most commonly employed surface
164 modifications and is typically used for the physisorption of proteins and the functionalisation of
165 “smart” polymers. Modifications in chip functionalisation can also include amination, biotinylation
166 and thiolation to name but a few.

167

168 The fluidic design of the AnaLight®Bio200 is based on a flow cell, which comprises two sample
169 chambers fitted with two independent feeds and drains, and makes up the core of the system. The
170 two channels can be configured in series or (more commonly) in parallel with a number of sample
171 introduction methods possible that should be chosen to optimise the experimental aim. When
172 performing an experiment, a pump supplies running buffer to the sensor chamber, with an injection
173 loop allowing the controlled introduction of the sample. The AnaLight®Bio200 instrument software
174 also allows the extensive post experimental analysis of results using an analysis of the interference
175 fringe position and operation of Maxwell’s equation. The technique allows the precise behaviour of
176 layers to be determined in terms of both their density (absolute RI) and thickness in real time and
177 therefore, mass, surface coverage and concentration can be calculated.

178

179 Each experiment was carried out on an unmodified silicon oxynitride sensor chip and analysed using
180 the AnaLight® Bio200 software (version 2.1.12/inject application version 1.0.1). Prior to each
181 experiment, RO water running buffer and 80% (w/w) EtOH was degassed. A 50ml syringe was filled
182 with the degassed running buffer, and fitted into the syringe pump. The Farfield fluidic system was
183 attached to the buffer syringe, the flow rate set at 200µl/min, and flow started (initially directed to

184 waste) to eliminate any air bubbles from the fluidic piping immediately associated to the buffer
185 reservoir. The flow rate was changed to 100 μ l/min (50 μ l/min per channel) to set the experimental
186 fringes. Degassed EtOH was loaded and injected across both channels in duplicate to wash the new
187 sensor chip before switching back to buffer and waiting for the fringes to stabilise (approximately
188 45seconds). The automated fringe selection function was employed to select the fringe positions,
189 with good amplitude and agreement being the key factors governing fringe choice. Prior to each
190 experiment, the sensor chip was calibrated by performing a routine EtOH injection. The injection
191 loop was filled with EtOH and injected across the experimental channels being used at 100 μ l/min
192 initially (3mins) before being changed to 15 μ l/min per channel (30 μ l/min if both channels were
193 used). The injection was allowed to run completely through to running buffer (rather than abruptly
194 stopping the injection) over a period of approximately 20mins.

195

196 **2.4 DPI - BHb Physisorption Studies**

197 The DPI system was set up and calibrated as described above. A 0.3mg/ml BHb solution was loaded
198 into the injection loop, and injected across both experimental channels at a flow rate of 50 μ l/min for
199 approximately six minutes. The flow was switched back to the buffer, and the sensor chip recycled
200 by injecting EtOH in duplicate followed by 2% SDS at 100 μ l/min. The DPI instrument phase responses
201 were recorded throughout the experiment.

202 Non-imprinted HydroNIP control gels were diluted 1/10 and 1/15 using RO water. The 1:15 dilution
203 of the HydroNIP suspension was loaded into the injection loop and injected across the sensor
204 surface at 50 μ l/min for approximately six minutes, followed by an EtOH injection each time at
205 100 μ l/min. Following the three 1/15 dilution HydroNIP injections, two 1/10 dilutions of HydroNIP
206 were injected in an identical fashion, followed once again by EtOH. Upon conclusion of the
207 experiment, the sensor chip was recycled by injecting 2% SDS at 100 μ l/min. The experiment was
208 then repeated with MIP1, MIP2 and MIP3.

209

210 **2.5 Interrogation of HydroMIP Imprinting Effect**

211 A 0.3mg/ml BHB solution was loaded and injected as before. Approximately five minutes after the
212 end of the BHB injection, a 1:15 dilution of the HydroNIP control was injected in duplicate as
213 previously described. Two EtOH injections and a 2% SDS injection were then performed at
214 100 μ l/min to recycle the sensor chip. This cycle (BHB; hydrogel sample x 2; EtOH x 2 and 2% SDS)
215 was repeated in an identical fashion for the remaining gel samples (MIP 1, MIP 2 & MIP 3).

216

217

218 **2.6 DPI – Data Analysis**

219 All experimental analyses were performed in the first instance using the AnaLight®Bio200 software.
220 In each experiment (where applicable), layer tables were constructed which in turn allowed the
221 quantification of specific binding and analysis events in terms of changes in thickness, refractive
222 index, layer density and mass of the deposited layers upon the sensor surface. All data were
223 exported into Microsoft Excel, which was employed to produce graphical representations of the raw
224 data obtained.

225

226 **2.7 Thin film MIP application to DPI chip**

227 Thin films were created by applying pressure to the hydrogel solutions over the optical waveguide
228 chips. This was done by injecting 50 μ l of free-radical initiated polymerising hydrogel solution over
229 the channels of the DPI chip and placing an 18mm² cover slip over the chip, thereby sandwiching the
230 hydrogel polymerising solution in between the cover slip and the DPI chip surface. Pressure (2kPa)
231 was then applied using bronze weights placed on top DPI chip for 10 minutes. After polymerisation
232 was completed, the weight and cover slip were removed the DPI chip was stored in a fridge at 4°C
233 overnight. Dual polarisation interferometry was used to characterise the real time binding effects
234 of BHB template molecules to BHB specific MI hydrogels. The selectivity and rebinding effect were
235 characterised using BHB specific MIPs as MIPs selective for the reloaded template, and BSA specific

236 MIPS as controls. DPI chips were first calibrated using blank chips. The materials used to calibrate
237 the chips were 80% ethanol and MilliQ water. The DPI chips were first exposed to running PBS buffer
238 for 20 min at a flow rate of 50 μ l/min. 100 μ g/ml of BHB template was then injected over both sets of
239 MIPs for 10 minutes at 20 μ l/min flow rate. This was followed by an injection of 100 μ g/ml of BSA
240 which was left to flow over the MIPs using the same association time and flow rate.

241 Stopping the flow of running buffer after BHB and BSA injections was also investigated to increase
242 the association time of the proteins to the MIPs, however this did not have a major effect on the
243 rebinding of BHB or BSA to the MIPs as results obtained were similar to those under constant flow
244 conditions.

245 **3 Results & Discussion**

246 **3.1 DPI Sensing of BHB Protein Adsorption**

247 Figure 1 shows the changes in thickness, mass and density observed due to BHB protein (0.3 mg/ml)
248 adsorption on a cleaned DPI chip surface. As expected, both the thickness and mass of the
249 deposited protein layer increase rapidly at first as protein physisorbs to the sensor surface. Upon
250 switching the flow from BHB solution back to the running buffer, excess BHB is clearly removed from
251 the sensor surface. It is probable that this was caused by the formation of a bi-layer of protein as a
252 result of the relatively high concentration of protein deposited upon the sensor surface [21].

253 <INSERT FIGURE 1>

254 It can be seen that the density of the deposited layer reaches a plateau with the continual
255 accumulation of protein upon the surface. As the injection ends and the flow switches to buffer, the
256 density of the deposited layer dramatically increases, at the same time as protein is removed from
257 the surface (as indicated by the changes in thickness and mass).

258 When the BHb molecules are removed, the remaining protein can re-organise and associate more
259 strongly with the sensor surface allowing physisorption to occur to a greater degree [20]. The
260 increase in the spatial association that occurs between the protein molecules and the sensor
261 surface, could be resulting in a more densely bound protein layer due to the ability of the protein
262 molecule to form hydrogen bonds with the sensor surface.

263 **3.3 DPI sensing of selective stripping of pre-adsorbed protein layers by MIP.**

264 All gels were granulated to 75 μ m (the internal diameter of the tubing in which fluid flow passes in
265 the instrument, was 250 μ m). Because of the viscous nature of the hydrogels, dilution was required
266 in order for the gel particles to be dispersed enough to flow. Initial experiments showed that flow
267 would not be possible at dilutions of 1:2 through to 1:8 as the samples were congealed resulting in
268 potentially clogging the fluid channels. Dilutions of 1:10 and 1:15 were appropriate for the injection
269 of HydroMIP and HydroNIP samples across the sensor surface. The MIP samples were found to be
270 stable for at least over a 3 month period when stored at 4°C when not in use. Table 1 summarises
271 the change in thickness, mass and density for various injections of protein (BHb adsorption),
272 followed by double injections of NIP, MIP1, MIP2 or MIP3.

273 <INSERT TABLE 1>

274 **3.3.1 Effect of NIP on pre-adsorbed protein layer**

275 Table 1 shows the changes in thickness, mass and density of the deposited protein layer upon the
276 sensor surface during protein deposition and following the injection of HydroNIP control gels. A final
277 BHb layer with a thickness of 2.74nm and a mass of 1.99ng/mm² was adsorbed upon the sensor
278 surface following protein deposition. Following the injection of the first HydroNIP sample, there is
279 a small decrease in both thickness and mass of layer, which rises slightly following the second
280 HydroNIP injection. Overall, there is no significant protein stripping effect. This is expected, as other
281 than the random formation of cavities that demonstrate the appropriate architecture and selectivity

282 to accept the protein molecules, there is no reason why the control gel would strip protein from the
283 sensor surface.

284 **3.3.2 Effect of MIP1 on pre-adsorbed protein layer**

285 The injection of BHB across the sensor surface results in a layer 4.32nm thick with a mass of
286 2.39ng/mm² being deposited upon the sensor surface (see Table 1). The initial MIP 1 sample
287 injection contributes a small increase in both mass and thickness, with the second injection
288 contributing to a small decrease. The density of the protein layer fluctuates slightly, but overall, the
289 MIP 1 sample has little effect, as would be expected, in the removal of BHB from the sensor surface.
290 The imprinted cavities within the MIP 1 sample are still occupied with the original template protein,
291 with very few (if any) imprinted cavities exposed. As a result, association between the immobilised
292 protein and imprinted cavities could not occur explaining why the MIP 1 gels had no effect upon
293 removal of protein from the sensor surface.

294 **3.3.3 Effect of MIP2 on pre-adsorbed protein layer**

295 Figure 2 shows the changes in thickness, mass and density of the deposited protein layer upon the
296 sensor surface during protein deposition, followed by the injection of MIP 2 HydroMIP samples.
297 Following the physisorption of BHB upon the sensor surface, a protein layer that was 4.43nm thick
298 with a mass of 2.48ng/mm² remained. Following the first MIP 2 injection, this layer decreased
299 significantly in thickness to 3.38nm, and once again to 3.26nm following the second injection. The
300 mass responded in an identical manner with a decrease to 2.31ng/mm² and 2.26ng/mm²

301 <INSERT FIGURE 2>

302 respectively following the two MIP injections. The small change following the second injection in Fig
303 2 confirms that the majority of the stripping effect occurs as a result of the first injection. In contrast
304 to the NIP control and MIP 1 samples, the MIP 2 sample which possessed exposed imprinted
305 cavities, was able to strip away BHB from the sensor surface. The layer density values also support

306 this event, as an increase from 0.55g/cm^3 (BHb protein layer) to 0.68g/cm^3 (injection 1) and
307 0.70g/cm^3 (injection 2) was observed as protein was removed from the surface apparently allowing
308 the remaining protein molecules to associate themselves in a denser fashion upon the sensor
309 surface.

310 As previously observed, the deposition of BHb (in terms of thickness and mass) results in an
311 immediate and distinctive (peaked) binding curve, followed by a plateau that relates to the
312 remaining deposited protein layer.

313 Immediately following the first injection of HydroMIP, there is a rapid increase in the density of the
314 protein layer. This suggests that protein is being removed from the sensor surface, which allows the
315 remaining protein that has not been stripped to orientate itself upon the surface in a highly ordered
316 manner (in comparison to the relative disorder of the saturated protein layer). Upon returning to
317 running buffer, the density falls slightly but once again increases when the second HydroMIP
318 injection is performed. An identical effect occurs, resulting in a final baseline density value that is
319 higher than that obtained following the first injection and considerably higher than the value
320 obtained prior to both the MIP2 injections.

321 The suggestion that protein is removed from the sensor surface is supported by the values relating
322 to the decreased mass of the protein layer. Following the injection of both HydroMIP samples, the
323 mass upon the sensor surface rises sharply, before decreasing to give values lower than those
324 observed prior to the injections. This strongly suggests that protein is indeed being removed from
325 the sensor surface by MIP2.

326

327 ***3.3.4 Effect of MIP3 on pre-adsorbed protein layer***

328 In contrast to both NIP and MIP 1, the injection of the MIP 3 sample across the sensor surface results
329 in an increase in thickness and mass and a decrease in density of the protein layer (Table 1),

330 however not to the same degree as the MIP 2 sample comprising exposed (unoccupied) molecularly
331 imprinted cavities.

332 The elevation in the density of the protein layer as a result of the injection of the MIP3 sample
333 indicates that protein is being stripped from the sensor surface by the gel samples. It appears that
334 the MIP 3 HydroMIP is behaving in a similar manner to that of MIP 2, but to a much lesser degree.

335 The MIP 3 sample is similar in nature to MIP 2 sample, but has been subjected to the rebinding of
336 the target template molecule within the imprinted cavities that it possesses. The rebinding of the
337 template molecule within these imprinted sites can be highly efficient [5, 27], however it cannot be
338 assumed that it is a process that is 100% efficient and that every cavity becomes reoccupied
339 following rebinding. Some imprinted cavities that have not been reoccupied by the template protein
340 are available within the MIP3 sample. When the gel is removed by the buffer solution some surface-
341 adsorbed protein is stripped away with the gel. The above promising results demonstrate that we
342 can distinguish between different MIP loading states using DPI sensing by monitoring their effect on
343 a pre-adsorbed protein layer.

344 **3.4 Thin film HydroMIPs integrated to the DPI sensor and optimisation of biological conditions**

345 Having demonstrated the selective stripping-off of protein from the sensor surface by MIP2, we
346 investigated the development of a thin-film MIP integrated to the DPI sensor. With the MIP attached
347 to the sensor, we would effectively have the makings of biosensor for protein detection. Thin MIP
348 films were prepared as detailed in Section 2.7. Instead of using a NIP control, we used a MIP
349 prepared for BSA, a protein of similar molecular weight to haemoglobin. Thin-film MIPs for
350 haemoglobin and BSA were prepared separately (as shown in the methods) and tested for their
351 selectivity in rebinding haemoglobin. The sensing region of the DPI instrument used in this thesis
352 typically extends to 500nm from the waveguide surface of the DPI chips, therefore it was favourable
353 to produce films less or equal in thickness to this parameter. The maximum thickness obtained for

354 the gels was $381\text{nm} \pm 5\text{nm}$ and the minimum thickness obtained was $138\text{nm} \pm 9\text{nm}$ at 0.5 kPa and 2
355 kPa pressure applied during film formation respectively. Gels prepared at 2kPa pressure
356 demonstrated the highest degree of sensitivity in terms of changes in the mass and density of the
357 polymers on the surface of the chip and were therefore investigated further for their selective
358 nature. Prior to rebinding of BHb to either BHb MIP or BSA MIP, the MIP layer was washed with SDS
359 to remove surface imprinted protein, followed by DI water to remove any residual SDS. Both the
360 BHb and BSA MIPs were then exposed to BHb. Selective binding of cognate protein for the BHb MIP
361 gave a sustained increase in film thickness and mass (Fig 3), whereas when BHb was injected over
362 the BSA MIP although there was an initial increase in film thickness and mass, the response was
363 transient and returned to thickness and mass values before protein injection. This indicates that the
364 there is no net non-specific binding of BHb to the BSA MIP (Fig 3).

365 <INSERT FIG. 3>

366 The trace for BHb loading onto BHb MIP can be interpreted as a swelling after BHb is injected over
367 the BHb MIP. The gel then relaxes, and interestingly, the mass of the layer increases. This increase in
368 mass implies the reloaded BHb is adhering to the polymer as it flows over the BHb MIP. The
369 thickness of the material remains at steady state, and the density of the material increases. This
370 could indicate that the majority of the BHb rebinding is occurring in cavities below the surface of the
371 film. The BHb MIP sensor exhibited a linear response up to $200\ \mu\text{g}/\text{ml}$ with a limit of detection of
372 $2\ \mu\text{g}/\text{ml}$. Hb in urine is known as hemoglobinuria and can occur due to for example kidney cancer,
373 burns, and malaria. Brian et al. [28] when using surface plasmon resonance detection of hemoglobin
374 reported a very similar lower limit of detection of $1.3\ \mu\text{g}/\text{ml}$. The mechanism of detection did not
375 rely on a MIP layer but relied on rebinding of heme to surface immobilised apo-hemoglobin. Our
376 lower limit of protein detection suggests that with some improvement in, for example, film thickness
377 and MIP cavity density, the technique will be appropriate for the measurement of disease markers

378 such as the prostate cancer markers, prostate specific antigen (2-10 ng/mL) in blood and engrailed-2
379 protein (>42.5 ng/mL) in urine [29] and other such biomarkers in blood.

380 In order to assess their suitability in real biological samples, HydroMIPs were investigated for their
381 potential application for biological diagnostics using plasma and serum matrices as potential
382 interferences for template protein rebinding. We applied thin films of a BSA MIP and investigated the
383 effects of neat, 1/10 and 1/100 diluted plasma exposure of MIPs prior to template protein (BSA)
384 rebinding (Table 2).

385 <Insert Table 2>

386 As neat plasma is injected over a BSA MIP a very large thickness, mass, and density change occurs
387 which exceeds the limitations of the DPI instrument. This could be due to swelling of the gel and the
388 detection of highly concentrated plasma flowing over the channels by the DPI instrument. This event
389 is followed by a decrease in mass and thickness, and an increase in density. The loss in mass and
390 thickness of the layer is indicative of the majority of the reloaded plasma passing over the MIP, and
391 that the MIP is exhibiting limited non-specific binding of the plasma proteins. Upon BSA reloading, a
392 small contraction event occurs followed by re-swelling of the gel. A steady state response is then
393 subsequently observed. The loss in mass and thickness observed after BSA reloading was 0.07
394 ng/mm^2 and 0.15nm respectively, and an increase of density of 0.02 g/cm^3 . This response indicates
395 no specific rebinding of BSA to the BSA MIP which is likely due to protein fouling of the MIP from the
396 plasma injected. This suggests that whole plasma as a sample matrix is not suitable for MIP use.
397 When using a lowered concentration of plasma (1:10 dilution) the initial mass, thickness and density
398 changes of the gel are significantly lower when comparing with whole plasma reloading. As the
399 response of the layer reaches steady state, the mass and thickness of the gel demonstrate a lower
400 steady state response compared with whole plasma, giving a difference of 0.22ng/mm^2 and 0.33nm
401 respectively. As BSA is reloaded onto the BSA MIP, a small contraction event occurs. This is followed
402 by re-swelling of the gel and a steady state response. The response of the resolved mass, thickness

403 and density of the gel did not change significantly after BSA reloading, which could indicate that
404 even lowering the concentration of the plasma interferent by 1:10 dilution, a high degree of protein
405 fouling still occurs in the gel. Lowering the concentration of plasma further to a 1:100 dilution,
406 demonstrates an interesting effect for the BSA MIPs. As 1:100 diluted plasma is injected over the
407 BSA MIP, the thickness, mass and density of the gel initially increase. This is followed by a gradual
408 decrease in these parameters, which suggests that the injected plasma is flowing over the MIP and
409 showing a low degree of non-specific rebinding. As BSA is subsequently injected over the BSA MIP,
410 there is an initial decrease in mass and thickness of the gel, followed by an increase in these
411 parameters. This suggests that BSA is stripping away non-specifically bound plasma from the MIP
412 and replacing it with BSA. However, there is also a gradual decrease in thickness and mass after this
413 event occurs, which could imply that the rebound BSA is also gradually being lost from the surface of
414 the MIP. This was not ideal and so we investigated the use of serum at various dilutions to see the
415 effect on MIP binding (Table 2). Again, neat and 1/10 diluted serum samples demonstrate a high
416 degree of non-specific binding of proteins. Subsequent addition of template BSA leads to a decrease
417 in thickness but no apparent change in mass, suggesting possibly that BSA is adsorbing to the MIP
418 after some of the non-specifically bound serum proteins are removed. At 1:100 diluted serum, the
419 serum proteins have a small to negligible effect on thickness and mass. Subsequent BSA addition
420 results in selective uptake of these template molecules as evidenced by significant increases in
421 thickness and mass. Our results suggest that further optimisation of MIPs applied to blood sample
422 should be conducted using 1:100 diluted serum samples as this dilution demonstrates negligible
423 fouling of MIP.

424 **4. Conclusions**

425 We have shown that when a layer of protein was physically immobilised upon the sensor surface,
426 the cavity-containing HydroMIP gels clearly stripped protein from the sensor surface in contrast to
427 non-imprinted polymer and the HydroMIP samples that did not possess available imprinted sites

428 capable of accepting the template protein. This effect has been quantified in terms of layer
429 thickness, mass and density, and this is a firm indication of the presence of imprinted cavities within
430 the HydroMIP structure.

431 Thin film MIPs can be applied to the DPI sensor also. This section of work indicates that the
432 HydroMIP materials, may play a significant role as the selective recognition material of a thin film
433 MIP biosensor strategy. Possible applications of HydroMIPs generally extend to either diagnostics,
434 such as detection of drugs, viruses, pesticides, toxins, or bacteria, or therapeutics i.e. controlled
435 release systems. The HydroMIPs are able to retain their selectivity after exposure to real biological
436 samples only when used in 1:100 diluted serum.

437

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441

442 References

443 [1] K. Haupt, Imprinted polymers - Tailor-made mimics of antibodies and receptors, Chemical
444 Communications 34 (2003) 171-178.

445 [2] M.E. Byrne, K. Park, N.A. Peppas, Microfabrication of intelligent biomimetic networks for
446 recognition of D-glucose, Advanced Drug Delivery Reviews 54 (2006) 149-161.

447 [3] X. Liu, J.S. Dordick, Sugar acrylate-based polymers as chiral molecularly imprintable hydrogels,
448 Journal of Polymer Science Part A: Polymer Chemistry 37 (1999) 1665-1671.

449 [4] J.M. González-Sáiz, M.A. Fernández-Torroba, C. Pizarro, Application of weakly basic copolymer
450 polyacrylamide (acrylamide-co-N,N'-dimethylaminoethyl methacrylate) gels in the recovery of citric
451 acid, European Polymer Journal 33 (1997) 475-485.

- 452 [5] D.M. Hawkins, D. Stevenson, S.M. Reddy, Investigation of protein imprinting in hydrogel-based
453 molecularly imprinted polymers (HydroMIPs), *Analytica Chimica Acta* 542 (2005) 61-65.
- 454 [6] Y. Xia, T. Guo, M. Song, B. Zhang, B. Zhang, Hemoglobin recognition by imprinting in semi-
455 interpenetrating polymer network hydrogel based on polyacrylamide and chitosan,
456 *Biomacromolecules* 6 (2005) 2601-2606
- 457 [7] J.L. Liao, Y. Wang, S. Hjerten, Novel support with artificially created recognition for the selective
458 removal of proteins and for affinity chromatography, *Chromatographia* 42 (1996) 259-262.
- 459 [8] S.H. Ou, M.C. Wu, T.C. Chou, C.C. Liu, Polyacrylamide gels with electrostatic functional groups for
460 the molecular imprinting of lysozyme, *Analytica Chimica Acta* 504 (2004) 163-166.
- 461 [9] E. Saridakis, S. Khurshid, L. Govada Q. Phan, D. Hawkins, G.V. Crichlow, E. Lolis, S.M. Reddy N.E.
462 Chayen, Protein crystallization facilitated by molecularly imprinted polymers, *P. Natl. Acad. Sci. USA*
463 108 (2011) 18566-18566
- 464 [10] J.M. Costa-Fernandez, R. Pereiro, A. Sanz-Medel, The use of luminescent quantum dots for
465 optical sensing, *Trends in Analytical Chemistry*, 25 (2006) 207-218.
- 466 [11] C.I. Lin, A.K. Joseph, C.K. Chang, Y.D. Lee, Molecularly imprinted polymeric film on
467 semiconductor nanoparticles - Analyte detection by quantum dot photoluminescence, *Journal of*
468 *Chromatography A* 1027 (2004) 259-62.
- 469 [12] R. Levi, S. McNiven, S.A. Piletsky, S.H. Cheong, K. Yano, I. Karube, Optical detection of
470 chloramphenicol using molecularly imprinted polymers, *Analytical Chemistry* 69 (1997) 2017-2021.
- 471 [13] S. Al-Kindy, R. Badia, J-L. Suarez-Rodriguez, M-E. Diaz-Garcia, Room-temperature
472 phosphorescent complexes with macromolecular assemblies and their (bio)chemical applications,
473 *Critical Reviews in Analytical Chemistry* 30 (2000) 291-309.
- 474 [14] M. Zourob, S. Elwary, A. Turner, *Principles of Bacterial Detection: Biosensors, Recognition,*
475 *Receptors and Microsystems*, Springer ISBN: 978-0-387-75112-2, 2008
- 476 [15] G.H. Cross, A.A. Reeves, S. Brand, M.J. Swann, N.J. Freeman, J.R. Lu, The metrics of surface
477 adsorbed small molecules on the Young's fringe dual-slab waveguide interferometer, *Journal of*
478 *Physics D - Applied Physics*, 37 (2004) 74-80
- 479 [16] A. Kugimiya, T. Takeuchi, Surface plasmon resonance sensor using molecularly imprinted
480 polymer for detection of sialic acid, *Biosensors & Bioelectronics* 16 (2001) 1059-62.

- 481 [17] H. Berney, K. Oliver, Dual polarization interferometry size and density characterisation of DNA
482 immobilisation and hybridisation, *Biosensors and Bioelectronics* 21 (2005) 618-626.
- 483 [18] B. Lillis, M. Manning, H. Berney, E. Hurley, A. Mathewson, M.M. Sheehan, Dual polarisation
484 interferometry characterisation of DNA immobilisation and hybridisation detection on a silanised
485 support, *Biosensors and Bioelectronics* 21 (2006) 1459-1467.
- 486 [19] S. Lin, C.K. Lee, Y.H. Lin, S.Y. Lee, B.C. Sheu, J.C. Tsai, S.M. Hsu, Homopolyvalent antibody-
487 antigen interaction kinetic studies with use of a dual-polarization interferometric biosensor,
488 *Biosensors and Bioelectronics* 22 (2006) 715-721.
- 489 [20] S. Ricard-Blum, L.L. Peel, F. Ruggiero, N.J. Freeman, Dual polarization interferometry
490 characterization of carbohydrate-protein interactions, *Analytical Biochemistry* 352 (2006) 252-259.
- 491 [21] M.J. Swann, L.L. Peel, S. Carrington, N.J. Freeman, Dual-polarization interferometry: an
492 analytical technique to measure changes in protein structure in real time, to determine the
493 stoichiometry of binding events, and to differentiate between specific and nonspecific interactions,
494 *Analytical Biochemistry* 329 (2004) 190-198.
- 495 [22] J.R. Lu, M.J. Swann, L.L. Peel, N.J. Freeman, Lysozyme adsorption studies at the silica/water
496 interface using dual polarization interferometry, *Langmuir* 20 (2004) 1827-1832.
- 497 [23] E.A. Yates, C.J. Terry, C. Rees, T.R. Rudd, L. Duchesne, M.A. Skimore, R. Lévy, N.T.K. Thanh, R.J.
498 Nichols, D.T. Clarke, D.G. Fernig, Protein-GAG interactions: new surface-based techniques,
499 spectroscopies and nanotechnology probes, *Biochemical Society Transactions* 34 (2006) 427-443.
- 500 [24] Y. Tang, J.R. Lu, A.L. Lewis, T.A. Vick, P.W. Stratford, Structural effects on swelling of thin
501 phosphorylcholine polymer films, *Macromolecules* 35 (2002) 3955-3964.
- 502 [25] M.S. Lord, M.H. Stenzel, A. Simmons, B.K. Milthorpe, Lysozyme interaction with poly(HEMA)-
503 based hydrogel, *Biomaterials* 27 (2006) 1341-1345.
- 504 [26] C. Aulin, I. Varga, P.M. Claesson, L. Wågberg, T. Lindström, Buildup of polyelectrolyte multilayers
505 of polyethyleneimine and microfibrillated cellulose studied by in situ dual-polarization
506 interferometry and quartz crystal microbalance with dissipation, *Langmuir* 24 (2008) 2509-2518.
- 507 [27] S.M. Reddy, G. Sette, G. Q. Phan, Electrochemical probing of selective haemoglobin binding in
508 hydrogel-based molecularly imprinted polymers, *Electrochimica Acta* 56 (2011) 9203-9208.

509 [28] V.A. Briand, V. Thilakarathne, R.M. Kasi, C.V. Kumar, Novel surface plasmon resonance sensor
510 for the detection of heme at biological levels via highly selective recognition by apo-hemoglobin,
511 Talanta 99 (2012) 113-118.

512 [29] H. Pandha, K.D. Sorensen, T.F. Orntoft, S. Langley, S. Hoyer, M. Borre and R. Morgan, Urinary
513 engrailed-2 (EN2) levels predict tumour volume in men undergoing radical prostatectomy for
514 prostate cancer, BJU International 110(6) E287-E292.

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515 Captions for Figures/Tables:

516
 517 Figure 1 Changes in thickness (-x-), mass (-o-) and layer density (—) following the
 518 deposition of BHb upon the sensor surface.

519
 520 Figure 2 Changes in thickness (-x-), mass (-o-) and layer density (—) of deposited
 521 protein layer as a result of the injection of MIP 2 HydroMIP gel samples.
 522

523 Figure 3 DPI mass response for BSA MIP (bottom trace), and BHb MIP (top trace)
 524 when reloading 100µg/ml BHb.

525 Table 1 Changes in thickness, mass and density of a pre-deposited BHb layer and
 526 following the injection of NIP and MIP1 to MIP3 gel samples.

527 Table 2 Thickness, mass, density and refractive index changes after injecting either:
 528 whole; 1:10 diluted serum; or 1:100 diluted serum or plasma followed by
 529 100µg/ml BSA over BSA MIPs.

530

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568 produce industry, molecular epidemiology of enteric contamination within meat chains and
569 development of reagentless biosensors for biohazard detection.

Table 1 The quantified changes in thickness, mass and density of a pre-deposited BHb layer and following the injection of NIP and MIP 1 to MIP 3 gel samples.

Name	Th / nm ($\pm 0.10\text{nm}$)	Mass (ng/mm^2) ($\pm 0.20\text{ng}/\text{mm}^2$)	Density (g/cm^3) ($\pm 0.02\text{g}/\text{cm}^3$)
BHb	2.74	1.99	0.73
NIP Injection 1	2.67	1.96	0.73
NIP Injection 2	2.87	2.05	0.72
BHb	4.32	2.39	0.55
MIP 1 Injection 1	4.42	2.46	0.56
MIP 1 Injection 2	3.91	2.22	0.57
BHb	4.43	2.48	0.56
MIP 2 Injection 1	3.38	2.31	0.68
MIP 2 Injection 2	3.26	2.26	0.70
BHb	3.87	2.04	0.53
MIP 3 Injection 1	3.40	1.88	0.55
MIP 3 Injection 2	3.26	1.82	0.56

	Sample dilution	Thickness nm	Mass ng/mm ²	Density g/cm ³	Refractive index
BSA MIP					
Plasma reloading	0	1.28	0.75	0.58	1.44
	1 in 10	0.95	0.53	0.56	1.44
	1 in 100	0.52	0.20	0.38	1.41
After 100µg/ml BSA reloading	0	1.13	0.68	0.60	1.44
	1 in 10	0.94	0.51	0.54	1.43
	1 in 100	0.39	0.12	0.31	1.39
BSA MIP					
Serum reloading	0	0.97	0.68	0.70	1.46
	1 in 10	2.85	0.80	0.28	1.39
	1 in 100	0.15	0.09	0.57	1.44
After 100µg/ml BSA reloading	0	0.76	0.60	0.78	1.48
	1 in 10	2.02	0.63	0.31	1.39
	1 in 100	1.45	0.66	0.45	1.42

Table 2 Thickness, mass, density and refractive index changes after injecting either: whole; 1:10 diluted serum; or 1:100 diluted serum or plasma followed by 100µg/ml BSA over BSA MIPs.

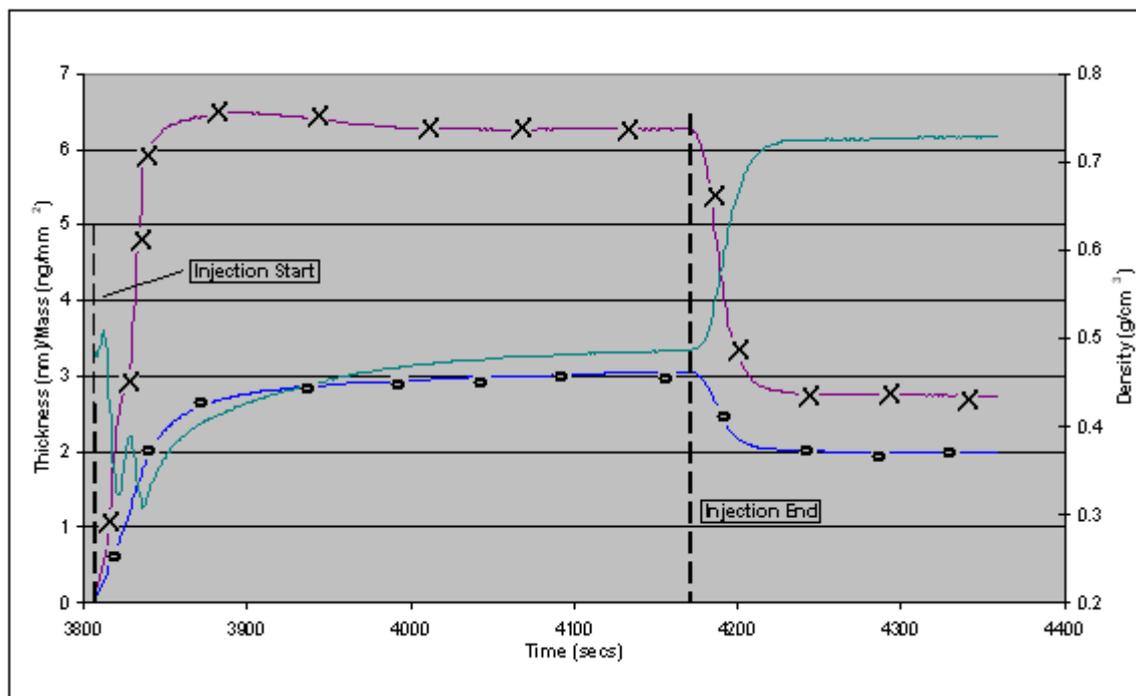


Figure 1 Changes in thickness (-x-), mass (-o-) and layer density (—) following the deposition of BHB upon the sensor surface.

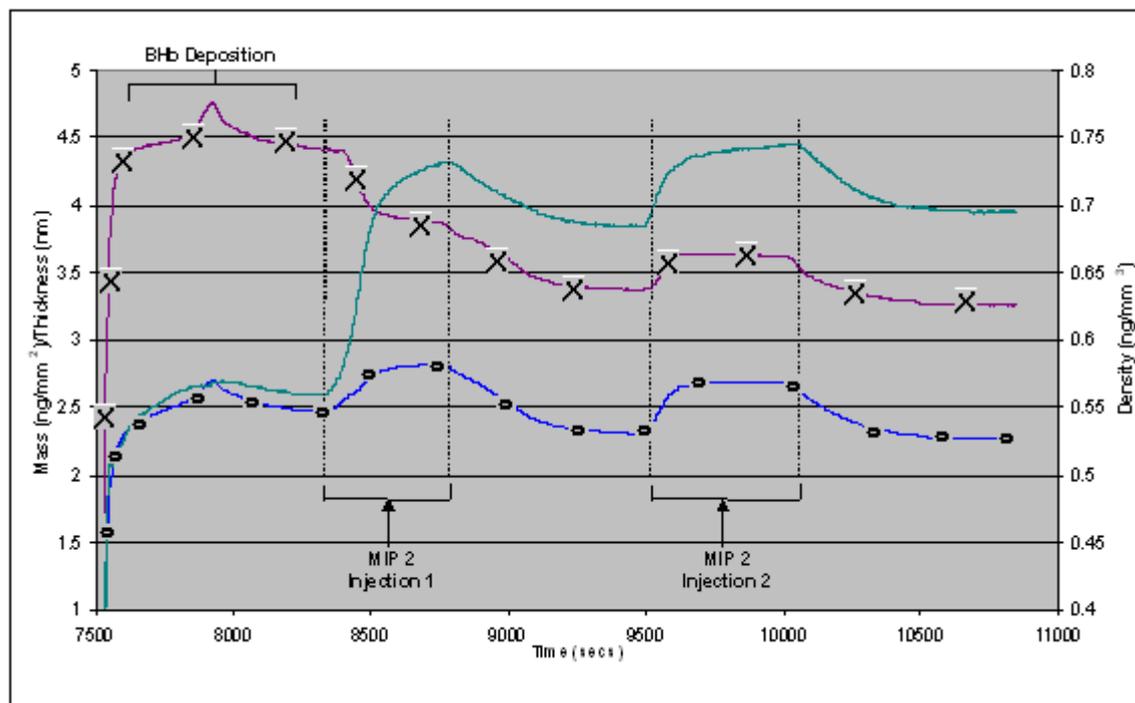


Figure 2 Changes in thickness (-x-), mass (-o-) and layer density (—) of deposited protein layer as a result of the injection of MIP 2 HydroMIP gel samples.

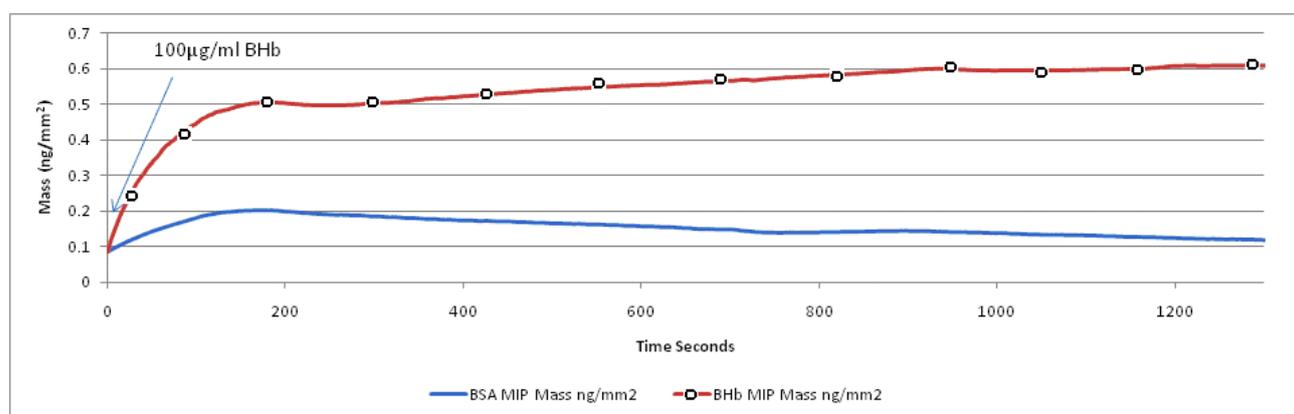


Figure 3: DPI mass response for BSA MIP (bottom trace), and BHb MIP (top trace) when reloading 100µg/ml BHb.