

1 **Enhanced selectivity of hydrogel-based molecularly imprinted polymers (HydroMIPs)**
2 **following buffer conditioning.**

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10

11 **Abstract**

12 We have investigated the effect of buffer solution composition and pH during the preparation,
13 washing and re-loading phases within a family of acrylamide-based molecularly imprinted
14 polymers (MIPs) for bovine haemoglobin (BHb), equine myoglobin (EMb) and bovine
15 catalase (BCat). We investigated water, phosphate buffer saline (PBS),
16 tris(hydroxymethyl)aminomethane (Tris) buffer and succinate buffer. Throughout the study
17 MIP selectivity was highest for acrylamide, followed by N-hydroxymethylacrylamide, and
18 then N-iso-propylacrylamide MIPs. The selectivity of the MIPs when compared with the
19 NIPs decreased depending on the buffer conditions and pH in the order of Tris > PBS >
20 succinate. The Tris buffer provided optimum imprinting conditions at 50 mM and pH 7.4,
21 and MIP selectivities for the imprinting of BHb in polyacrylamide increased from an initial
22 8:1 to a 128:1 ratio. It was noted that the buffer conditions for the re-loading stage was
23 important for determining MIP selectivity and the buffer conditions for the preparation stage
24 was found to be less critical. We demonstrated that once MIPs are conditioned using Tris or
25 PBS buffers (pH7.4) protein reloading in water should be avoided as negative effects on the

26 MIP's imprinting capability results in low selectivities of 0.8:1. Furthermore, acidifying the
27 pH of the buffer solution below pH 5.9 also has a negative impact on MIP selectivity
28 especially for proteins with high isoelectric points. These buffer conditioning effects have
29 also been successfully demonstrated in terms of MIP efficiency in real biological samples,
30 namely plasma and serum.

31

32 **Keywords:** Molecular imprinting; Protein binding; Selectivity; Biomimetic material; pH;
33 Biocompatibility

34

35 **1. Introduction**

36 Molecularly imprinted polymers (MIPs) are polymers which have been synthesized to have
37 tailor-made selectivity for a template molecule. Hydrogels are insoluble, crosslinked polymer
38 network structures composed of hydrophilic homo- or hetero-co-polymers, which have the
39 ability to absorb significant amounts of water [1]. Molecular imprinting using hydrogels
40 (HydroMIPs) have been well documented, with a vast array of monomers currently being
41 used for molecular imprinting [2]. Monomers that have commonly been used for non-
42 covalent molecular imprinted hydrogels are generally chosen on their ability to form weak
43 hydrogen bonds between the monomer and the template [1, 3, 4]. Acrylamide-based
44 hydrogels are known to be very inert, offer hydrogen bonding capabilities, and are
45 biocompatible [5]. For these reasons, acrylamide has been commonly used for molecular
46 imprinting [6-10].

47
48 Historically, molecular imprinting has been used for low molecular weight non-biological
49 molecules such as drugs and pesticides [11, 12]. This has been due in part to the fact that
50 MIPs have been hard to adapt to aqueous conditions due to the specific polar interactions
51 between good imprinted sites and the analyte which become weakened in the presence of a
52 polar solvent, and to the non-specific (hydrophobic) interactions between other small
53 molecules and the gel which become strengthened [6]. However, popularity for imprinting for
54 large macromolecule templates such as proteins has increased in the past decade, with a view
55 to developing sensors and new diagnostics for disease markers [13]. Organic solvents
56 traditionally employed in creating MIPs are not well suited to imprinting biological
57 molecules such as proteins, as the non-polar side chains are more soluble the hydrophobic
58 interactions that maintain the highly folded quaternary structure are weakened considerably.
59 This can result in a loss of structure and function leading to precipitation and denaturation [1,

60 6]. Also, for bioanalytical applications, it is desirable for the resultant MIPs to be efficiently
61 used under aqueous conditions [11]. Work carried out by Andersson [11, 12] suggested that
62 upon changing from organic solvent to aqueous based incubation, the selectivity of the MIPs
63 can be changed. It was suggested that in organic solvents the imprints recognise subtle
64 differences in polar functionalities of the template molecule, and in aqueous media the
65 hydrophobic parts of the template molecule are more efficiently recognised [11].

66

67 One of the holy grails for the molecular imprinting community is to achieve binding affinities
68 for MIPs that can be comparable to the high selectivity offered for instance antibody-antigen
69 binding [13]. Another example, the biotin-avidin interaction, is renowned for having a large
70 binding constant of 10^{15} M^{-1} [13]. This interaction is purely non-covalent, but the strength of
71 interaction comes from 15 amino acid residues on the avidin being in optimum positions to
72 specifically interact with the vitamin, biotin; approximately half through hydrogen bonding
73 interactions and the other half of the residues through hydrophobic interactions [13]. The high
74 specificity is compounded by the flexibility of the protein to subtly change its conformation
75 in order to lock into place upon biotin binding. This is quite a complex series of events which
76 is made to look easy by such natural systems. MIPs are typically highly cross-linked systems
77 and by virtue of their rigid structure are therefore unable to offer many degrees of freedom to
78 allow similar capture and locking to take place. However, hydrogel-based MIPs are able to
79 swell and contract depending on solvent, ionic strength and the presence of other dissolved
80 components in solution [1,3,11,14]. If these parameters can be optimised to improve selective
81 binding within MIPs compared to non-imprinted polymer controls, we could go some way in
82 improving the reputation of MIPs as biomimetic and antibody-like materials [14].

83

84 When protein imprinting, one often overlooked parameter is protein stability [10-12].
85 Conventionally, protein stability is achieved using lyophilisation, freezing and
86 homogenization techniques [15]. However in a MIP system, buffers offer a more attractive
87 choice for sustaining biological molecules in their native state, and thus have been
88 extensively used in a range of chemical and biochemical assays. In some cases, the assays
89 make use of a solid support upon which a biorecognition molecule (such as an enzyme or
90 antibody) is immobilised. There have been recent studies that have focused on the effect of
91 surface modifications and protein modifications on biomolecule stability [15,16]. For
92 example, Wei [15] demonstrated that buffer type as well as buffer concentration can have
93 significant effects on protein adsorption onto surfaces. It was suggested that at pH 7.4,
94 protein adsorption increased monotonically with a Tris buffer, while a PBS buffer induced
95 negative adsorption effects. This was attributed to the possibility of various phosphate ions
96 competing to adsorb with protein molecules [15].

97
98 This paper aims to investigate the effects that aqueous buffer composition has on the specific
99 rebinding and/or non-specific adsorption of template protein molecules into hydrogel-based
100 MIPs compared with control non-imprinted polymers (NIPs). We will demonstrate that MIP
101 surfaces can also be affected (e.g. positively in terms of their binding affinity for target
102 protein) depending on the chemical nature of the buffer and its pH.

104 **2. Experimental**

105 **2.1 Materials and reagents**

106 Acrylamide (AA), N-hydroxymethylacrylamide (NHMA), N-iso-propylacrylamide (NiPAm),
107 N,N-methylenebisacrylamide (bis-AA), ammonium persulphate (APS), N,N,N,N-
108 tetramethylethyldiamine (TEMED), sodium dodecyl-sulphate (SDS), glacial acetic acid

109 (AcOH), tris(hydroxymethyl)-amine (Tris-base), tris(hydroxymethyl)-amine hydrochloride
110 (Tris-HCl), phosphate buffered saline (PBS) tablets (137 mM NaCl; 27 mM KCl; 10 mM
111 Na₂HPO₄; 1.76 mM KH₂PO₄), succinic acid, bovine haemoglobin (BHb), bovine liver
112 catalase (BCat), and equine heart myoglobin (EMb) were all purchased from Sigma-Aldrich,
113 Poole, Dorset, UK. Sieves (75µm) were purchased from Endecotts Ltd. and Inoxia Ltd., UK.
114 Pooled plasma and serum samples from human volunteers were used in the biocompatibility
115 studies.

116

117 **2.2 Spectrophotometric analysis**

118 Calibration curves in MilliQ water, buffer solutions, and 10% AcOH:SDS were prepared for
119 BHb, BCat and EMb. Spectral scans revealed peak wavelengths for BHb in MilliQ water and
120 10% AcOH:SDS to be 405 nm and 395 nm respectively. All buffer solutions for BHb
121 exhibited a peak wavelength at 406 nm, with the exception of succinate buffer pH 2.9, which
122 exhibited a peak wavelength at 367 nm. Peak wavelengths for BCat in MilliQ water, Tris
123 buffer and 10% AcOH:SDS were found to be 405 nm, 404 nm and 392 nm respectively. Peak
124 wavelengths for EMb in MilliQ water, Tris buffer and 10% AcOH:SDS were found to be 410
125 nm, 408 nm and 396 nm respectively. Analysis and subsequent determination of protein
126 concentration in appropriate media was performed at specific peak wavelengths using a UV
127 mini-1240 CE spectrophotometer (Shimadzu Europa, Milton Keynes, UK).

128

129 **2.3 Hydrogel production method**

130 Hydrogel MIPs were synthesised by separately dissolving AA (54 mg), NHMA (77 mg),
131 NiPAm (85.6 mg) and bis-AA as cross-linker (6 mg), (8.5 mg) and (9.5 mg) respectively
132 along with template protein (12 mg) in 1mL of either PBS or MilliQ water. The solutions
133 were purged with nitrogen for 5 minutes, followed by an addition of 20 µL of a 10% (w/v)

134 APS solution and 20 μL of a 5% (v/v) TEMED solution. Polymerisation occurred overnight
135 at room temperature giving final crosslinking densities of 10%. MilliQ water is used as a
136 hydrogel preparation standard, except when investigating the effect of changing the gel
137 preparation conditions and optimising template rebinding using PBS. For every MIP created
138 a non-imprinted control polymer (NIP) was prepared in an identical manner but in the
139 absence of protein.

140

141 **2.4 Hydrogel conditioning**

142 After polymerization, the gels were granulated separately using a 75 μm sieve. Of the
143 resulting gels, 0.5 mL were transferred into 1.5 mL centrifuge eppendorf tubes and
144 conditioned by washing with five 1 mL volumes of either MilliQ water or buffer solution (50
145 mM Tris pH 7.4; 50 mM succinate pH 7.4; 50 mM succinate pH 2.9; 150 mM PBS pH 7.4;
146 and PBS pH 4.7). This was followed by five 1 mL volumes of a 10% AcOH:SDS eluent (pH
147 2.8). A further five 1 mL washes of either MilliQ water or buffer solution were conducted to
148 remove any residual AcOH:SDS eluent and equilibrate the gels. The PBS and succinate
149 buffer solutions used in this study were adjusted to pH 4.7 and pH 7.4 using 1 M HCl and 1
150 M NaOH respectively. Each conditioning step was followed by a centrifugation using an
151 eppendorf mini-spin plus centrifuge for 3 minutes at 6000 rpm (RCF: 2419 x g). All
152 supernatants were collected for analysis by spectrophotometry.

153

154 **2.5 Rebinding optimisations**

155 Once the gels (0.5 mL) were equilibrated, a 1 mL protein solution 'load' prepared in either
156 MilliQ water or buffer solution, containing 3 mg of protein was added to the target MIPs and
157 NIP controls. The MIPs and NIPs were then washed with four 1 mL volumes of either MilliQ
158 water or buffer solution 'wash', followed by five 1 mL washes of 10% AcOH:SDS 'elute'.

159 Each step (load, wash, and elute) was followed by a centrifugation as previously described,
160 and again all supernatants were collected for analysis by spectrophotometry.

161

162 **3. Results and discussion**

163 **3.1 Rebinding Optimisations**

164 The molecular imprinting effect or imprinting efficiency throughout this work was
165 characterised by the rebinding capacity exhibited by the protein-specific MIP in relation to
166 the control NIP. This can be represented as a selectivity ratio (α) using Eq. (1).

167

$$168 \quad \alpha = \frac{[specific\ binding]_{MIP}}{[specific\ binding]_{NIP}} \quad (1)$$

169

170 Using this equation, the selectivity ratio, α , is generally determined using the specific-bound
171 protein concentrations recovered in the 10% SDS:AcOH supernatant ‘elute’ fractions. This
172 heavily relies on the 10% SDS:AcOH being 100% efficient in removing/eluting protein;
173 however only a 90% efficiency has been reported previously [6]. Therefore, in order to
174 evaluate precisely how much protein is retained by the MIP, an alternative approach is to
175 calculate the specific-bound protein by subtracting the recovered non-specific protein in the
176 ‘load’ and ‘wash’ fractions from the initial protein additions. Table 1 illustrates the
177 experimental data in terms of selectivity ratios (α) calculated using the latter method.
178 Interestingly, the resulting MIP selectivity shows a varying affinity for different protein-
179 polymer combinations depending on the hydrophobicity of the polymer. It can be seen that
180 MIPs based on polyacrylamide (polyAA) showed the most promising results in terms of
181 imprinting efficiency closely followed by poly N-hydroxymethylacrylamide (polyNHMA),
182 then poly N-iso-propylacrylamide (polyNiPAm).

183

184 Fig.1 illustrates the quantified imprinting effect for BHb in polyAA, polyNHMA and
185 polyNiPAm hydrogel MIPs. Distinctive MIP and NIP loading characteristics are seen in the
186 differing degrees of template BHb protein recovered in supernatant phases after protein
187 loading in water (Load), water washing (Wash) and 10% SDS:AcOH elution (Elute) phases.
188 It is evident that the template BHb is easily recognised and bound by recognition sites in the
189 specific cavity-based MIPs from the negligible amounts of unbound BHb observed in the
190 Load supernatant, while the Elute fractions comprise high concentrations of the specifically
191 rebound protein. This strongly suggests that the template BHb has been specifically bound
192 within MIP specific cavities. Furthermore, the total amount of protein recovered in the
193 MIP_{polyAA} (1.5 mg) after rebinding and elution with 10% SDS:AcOH is less than the amount
194 used for rebinding (3 mg), suggests some irreversible rebinding of protein to the MIP. The
195 NIPs however, remain unselective and essentially reject the protein as they lack imprinted
196 cavities and only allow for non-specific adsorption of protein.

197

198 It should be noted that for all MIPs approximately 50% of the imprinted template was
199 recovered and quantified using spectrophotometric analysis during the initial conditioning
200 washes (MilliQ water and/or buffer, and the 10% ratio (w/v) of SDS:AcOH). The time
201 allowed for template removal is specified within the washing procedures, and the last wash
202 fractions were not observed to contain any protein. Therefore we are confident that the
203 remaining 50% of the template protein did not continue to leach out during rebinding studies.
204 An issue to address is that the diffusivity of proteins in cross-linked polymer matrices is
205 rather slow when looking at surface binding [16-17]. Generally polymer geometry, polymer
206 hydration, cross-linker density, protein size and temperature all play a role in the time needed
207 for a protein to diffuse into the polymer matrix and to reach equilibrium [17]. For instance,
208 the molecular weight of BHb is approximately four times higher than that of EMb [16-17].

209 Thus the difference in size leads to a retarded movement of BHb in the cross-linked polymer
210 matrix and therefore more time is expected to be required in order to reach equilibrium.
211 However, MIPs exhibited here are ground down (75 μ m) and exhibit a bulk gel effect.
212 Therefore the required incubation time was validated using polyAA-gels before affinity was
213 properly assessed. It should be noted that equilibrium of BHb and EMb upon incubation with
214 their corresponding MIPs was reached at different times. However after 20 min overall
215 loading time, concentrations for BHb in the supernatant did not continue to decrease,
216 suggesting that equilibrium binding had been reached. Therefore an optimised time (20 min)
217 was applied for protein equilibrium binding.

218

219 In order to alter MIP binding selectivity, buffer solutions were incorporated, and specifically
220 chosen as they are used in biological (antibody-based) assays, to stabilise proteins. Their
221 application in conjunction with MIPs that could potentially replace antibodies was therefore
222 of value if MIP selectivity can be improved as a function of stabilising the protein
223 conformation by judicious choice of buffer medium. Table 2 lists selectivity ratios (α) for the
224 BHb-MIP_{polyAA} depending on the various buffer and pH variation for MIP preparation,
225 conditioning series (washing water/buffer), and BHb protein loading. Experiments 1 to 10
226 summarise the effect of changing solution conditions between water and PBS and the
227 resultant selectivity is given in decreasing order. Initially the presence of PBS is beneficial;
228 however a comparison of experiments 1 and 6, despite identical preparation and conditioning,
229 shows a 27:1 and a 7:1 selectivity respectively. This is due to dilution of the PBS ionic
230 strength within the MIP when loading in water and suggests that the loading stage is more
231 crucial in affecting selectivity than either the preparation or the conditioning stages. This shift
232 is possibly causing an expansion in the gel during the loading phase due to the simultaneous
233 movement of buffer ions out of the gel, and water and protein into the gel. The movement of

234 water into a gel (by osmosis) to dilute the ionic strength within the gel is a well-documented
235 phenomenon [18]. The osmotic pressure of the gel is a function of the salt present within the
236 gel and varies with the nature of the salt in the order of the lyotropic series. The lyotropic
237 series is a classification of ions in order of their ability to salt out (dissolve) or salt-in
238 (precipitate) proteins and hydrogel polymers. While early members of the series such as
239 phosphate decrease the solubility of non-polar molecules and cause salting out, later members
240 in the series increase the solubility of non-polar molecules (salting-in) the driving force for
241 water diffusion into the gel is the concentration gradient of phosphate ions within the gel and
242 outside the gel. It is likely that during this state of dynamic macro and nano-structural
243 change in the MIP due to ingress of water, the protein although in the native state is unable to
244 significantly bind with the selective cavities in the MIP [19]. The ionic strength impact of the
245 PBS on MIP selectivity is further confirmed when using diluted PBS (experiments 7 and 8)
246 and only PBS in MIP preparation (experiments 5 and 10). However, when we investigated
247 reloading in sodium chloride solution only (no phosphate salts), the selectivity ratio was 1:1.
248 Therefore, it would appear that the phosphate buffer salts are contributing to the improved
249 selectivity by retaining the pH at 7.4 and providing a more stable protein environment.
250

251 Experiments 11 and 12 show the effect of using a Tris buffer at pH 7.4 for the conditioning
252 and loading phases, the only difference being the medium used for initial preparation of the
253 MIP. Interestingly the MIP prepared in water gives a much elevated selectivity ratio of 128:1
254 compared with MIP prepared in PBS (30:1 selectivity). Both are superior to the other
255 conditions studied and it would appear that conditioning and loading with Tris is having a
256 beneficial effect on the selective binding of BHb to the MIP compared with the NIP. It is
257 possible that although the pH is constant at 7.4 the initial PBS ions embedded within the MIP
258 structure are competing with the Tris molecules for favourable interactions with the MIP

259 matrix. A similar effect can also be seen when comparing experiments 1 and 3, where PBS
260 yields a higher selectivity when initially preparing the MIP in water rather than in PBS. A
261 comparison of the buffers' structures in Table 3 illustrates the three undissociated hydroxy
262 groups present in the Tris buffer ($pK_a = 8.1$). It is plausible that the latter are able to
263 hydrogen bond with both MIP and template BHb protein. This in turn aids in stabilising the
264 native protein structure in the fine protein-selective cavity within the MIP and provides ideal
265 conditions for the protein to selectively bind with the MIP at an optimum pH of 7.4. This
266 hypothesis is validate by control Tris buffer experiments 13 and 14 at lower (5.4) and higher
267 (9.4) pH values which both exhibit lower selectivity values of 60:1.

268

269 Experiments 15-18 illustrate the presence of succinate buffer ($pK_a = 4.2$), and at either high
270 (7.4) or low (2.9) pH MIP selectivity is low. However, a lower selectivity is exhibited for
271 succinate at pH 2.9, experiment 4 also demonstrated a radical reduction in MIP selectivity
272 when introducing acidified PBS (pH 4.7). This suggests that at lower acidified pH levels
273 there are negative effects associate with protein binding. Under the acidified conditions for
274 the PBS, Tris and succinate buffer systems (pH 4.7, 5.4, and 2.9 respectively) $-NH_2$ groups in
275 both MIP_{polyAA} ($pK_a = 7.9$) and template BHb protein ($pI = 6.8$) are protonated [22]. The
276 protein and MIP itself are therefore able to undergo subtle conformational changes or
277 molecular relaxation under such acidic conditions. This combination of charge repulsion
278 between cationic MIP and cationic protein as well as the ensuing structural changes in both
279 under acidic conditions may be contributing to the diminished selectivity of the MIP.
280 Interestingly, this is also supported by Uysal et al [21] who demonstrated at best a 2:1
281 selectivity ratio for BHb MIP:NIP when they prepared and utilised at pH 4.0.

282

283 **3.2 Tris buffer conditioning**

284 Notable for its ability to drastically improve MIP selectivity, Tris buffer (pH 7.4) conditioning
285 was applied to polyAA, polyNHMA and polyNiPAm gels for the selective imprinting of
286 bovine haemoglobin (BHb), equine myoglobin (EMb) and bovine catalase (BCat). It should
287 be noted that MIP preparation throughout this section remained constantly in MilliQ water.
288 Fig. 2 illustrates a comparison between an EMb-MIP_{polyAA} under MilliQ water and Tris buffer
289 conditions. It is observed that less non-specific protein is present within the MIPs, and a
290 higher protein concentration is observed in the 'elute' supernatants. This suggests that a
291 higher degree of specifically bound protein is attained with Tris buffer (pH 7.4) conditions.
292 The NIPs were also less capable of non-specifically binding BHb and a higher detection of
293 protein in the Load phase was noticed. Furthermore, all protein could be accounted for;
294 accumulation of the protein recovered in the different wash fractions mirrored the same
295 amount of protein that was originally added to the polymers.
296
297 Table 4 illustrates the optimised and improved selectivities and demonstrates that the Tris
298 buffer is clearly having a strong beneficial effect on the selective binding of protein to the
299 MIP. Conformational stability of proteins have been known to increase if anionic buffers are
300 used above the pI of the protein (and conversely, if cationic buffers are used below the pI)
301 [20]. At optimum pI, proteins contain carboxyl and amide groups existing as $-\text{NH}_3^+$ and $-\text{COO}^-$
302 $-\text{COO}^-$. Above their pI however, proteins become negatively charged and the groups exist as $-\text{NH}_2$
303 $-\text{NH}_2$ and $-\text{COO}^-$, (see Table 4). This overall negative net charge induces more favourable and
304 complementary hydrogen bonding interactions, resulting in increased specific as well as non-
305 specific binding. The Tris buffer (pKa of 8.1) with its three un-dissociated hydroxyl groups at
306 pH 7.4 seems suitable for improving MIP selectivity by providing optimum imprinting for
307 specific and reducing non-specific binding interactions. It is plausible that the Tris buffer is
308 aiding in stabilising the native protein structure in the fine protein-selective architecture

309 imprinted within the MIP. Moreover, MIP selectivities demonstrate a varying affinity for
310 different protein-polymer combinations. Interestingly, the selectivity is highest for BHb-
311 MIP_{polyAA}, followed by BHb and EMb-MIP_{polyNHMA}, while BCat has the lowest selectivity for
312 all MIPs. This provides some hints to the properties in terms of polymer and protein
313 interactions. It has been observed that with smaller size proteins a higher crosslinking density
314 is necessary [14]. EMb (17.5kDa) a quarter that of the size of BHb (64.5kDa) would therefore
315 require a MIP with a higher crosslinking density, otherwise protein cavities cannot be
316 optimised in order to reach full imprinting capacity. The opposite is also true for larger
317 proteins. For example, BCat (250kDa) is much larger and a much lower crosslinking density
318 is required. Control experiments have shown these densities to be optimum at 15% and 5%
319 for EMb and BCat respectively. It should be noted that for consistency all hydrogels in this
320 paper were produced using a standard 10% crosslinking density. Therefore the low selectivity
321 exhibited by the MIPs towards BCat can be attributed to the latter, as only a few cavities will
322 be present due to a high crosslinking density. Moreover, MIP selectivity is highest for
323 polyAA, followed by polyNHMA and polyNiPAm gels. This can be attributed to the
324 hydrophobicity of the polymers; although polyNHMA is the most hydrophilic and
325 polyNiPAm the most hydrophobic, the results are suggesting that the neutral polyAA is
326 providing ideal binding affinities for this set of proteins within Tris buffer conditions.

327

328 To further expand on the Tris buffer effect (pH 7.4), two series of gel conditioning
329 experiments (MilliQ water and Tris buffer pH 7.4) were conducted for a polyAA MIP.

330 Fig. 3 illustrates the positive effects of Tris introduction to the MIPs at both the conditioning
331 and loading phases. However, when MilliQ water is introduced into the Tris conditioning
332 series at the loading phase a staggering 0.8 selectivity was observed (Fig. 3, Table 5). This
333 was exhibited by both MIP and NIP and resulted in an unusually high amount of protein

334 being irreversibly bound. Over 98% of the protein irreversibly bound to the NIP, while only
335 83% bound to the MIP (Fig. 3). The reason for this high degree of irreversible binding to the
336 NIP could be due to a change in conformation of the protein due to a change in pH. Since
337 Tris is of pH 7.4 and conditioning series have stabilised the gels, a MilliQ water load (pH 5.4)
338 would therefore induce conformational changes, and in turn alter the protein binding
339 characteristics. Another explanation could be that the Tris buffer is causing gel contraction
340 around the reloaded protein and trapping it within the polymer network [21-22]. Table 5
341 illustrates the experimental data collected and resulting MIP:NIP selectivity ratios (α)
342 depending on the variant Tris buffer and MilliQ water conditioning series for MIP washing
343 and re-loading. It can be seen that the full Tris buffer conditioning series demonstrates its
344 superiority in terms of improving the imprinting efficiency resulting in a distinctively high
345 selectivity ratios (α) compared with the standard MilliQ water conditioning strategy.

346

347 **3.3. Biocompatibility study**

348 In order to assess their suitability in real biological samples, polyAA-BHb HydroMIPs were
349 investigated for their potential application for biological diagnostics using human plasma and
350 serum matrices to assess for potential interferents that could affect template protein
351 rebinding. Reload samples of diluted plasma or serum (1/10 or 1/100) in either MilliQ water
352 or Tris buffer (pH 7.4) were spiked with a 3mg/ml of BHb template. Figure 4 illustrates
353 the % of BHb rebinding in terms of MIP imprinting efficiency after Tris buffer (pH 7.4) pre-
354 conditioning (Figure 4 a) and MilliQ water pre-conditioning (Figure 4 b) studies. The MIPs
355 were tested for protein rebinding from the bio sample first in the medium that was used for
356 pre-conditioning and then in the medium not used for pre-conditioning. The effects such of
357 cross-loading in alternate media are also presented in Figs 4 a and b. The % of rebound BHb
358 is calculated by subtracting the non-specific BHb bound in the NIP from the specific BHb

359 bound to the MIP, divided by the initial reload concentration (3mg/ml) x 100. By subtraction
360 of protein binding in the NIP control from the MIP this correction allows us to effectively
361 demonstrate the absolute imprinting or rebinding efficiency exhibited by our MIPs and eliminate
362 any non-specific polymer matrix effect.

363

364 Varying percentages of BHB binding can be seen in Figure 4 depending on either the serum
365 or plasma studies and also within the different dilution factors. This can most likely be
366 attributed to the potential interference of the bio sample, i.e. globulins and clotting proteins
367 [19]. It can be seen that a 1/10 dilution of both serum and plasma has a hindrance on the
368 rebinding efficiency of BHB, and that the plasma has a higher hindrance effect than that of
369 serum. However, it is still clear to see that Tris buffer conditioning followed by reloading in
370 Tris diluted samples demonstrates its superiority in terms of MIP rebinding efficiency
371 compared with the standard MilliQ water conditioning and reloading from water-diluted
372 samples. This can also be seen, to an even greater extent, in the alternate media cross-loading
373 studies. When MilliQ water is introduced into the Tris conditioning series at the loading
374 phase (Figure 4a) less than 10% of protein was specifically bound. This was due to the
375 unusually high amount of protein irreversibly binding in the NIP as well as MIP (resulting in
376 this poor comparative rebinding efficiency) as previously discussed in the previous section.

377

378 The effective demonstration of our optimised buffer conditioning strategy in terms of MIP
379 rebinding efficiency using real biological human plasma and serum samples and the
380 sensitivity, specificity and stability of protein MIPs would make attractive future rapid
381 diagnostics and also potentially as a simple screening tool in the analytical community.

382

383 **4. Conclusions**

384 We have found that the selectivity of the MIP can be increased or decreased post-preparation
385 depending on the buffer composition and conditions used and that it is the buffer conditioning
386 of the MIP at the re-loading stage which primarily determines MIP selectivity. We were able
387 to achieve new high MIP selectivities when preparing and reloading the MIP under Tris (pH
388 7.4) buffer conditions, compared with either PBS, water only or succinate buffer.
389 Acidification had a detrimental effect on MIP selectivity, likely due to protonation of both
390 protein and MIP itself and the ensuing electrostatic repulsion, not allowing the protein to
391 occupy MIP cavities. Our results also suggest a cautionary message to avoid loading in water
392 only especially after conditioning the MIP or NIP with Tris buffer or PBS (pH7.4). By
393 optimising buffer composition and pH conditions, we are achieving new record high
394 selectivities for the MIP when compared with NIP. Biocompatibility studies have also
395 demonstrated the effective use of our optimised buffer conditioning strategy in terms of MIP
396 rebinding efficiency using real biological human plasma and serum samples. This study, in
397 turn, has major implications in improving the selectivity of analytical tools for solid phase
398 extraction and biosensors which are based on hydrogel-based molecularly imprinted
399 polymers.

400

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