1 **Enhanced selectivity of hydrogel-based molecularly imprinted polymers (HydroMIPs)**

2 **following buffer conditioning.**

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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 catalyse (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

## 1. Introduction

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

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

3

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.

103

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 $Na_2HPO_4$; 1.76 mM $KH_2PO_4$), 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   $$\alpha = \frac{[specific\ binding]_{MIP}}{[specific\ binding]_{NIP}} \hspace{3cm} (1)$$

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

8

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 (75um) 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 ($\alpha$) 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

10

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 (pKa = 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 (pKa = 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 $MIP_{polyAA}$ (pKa = 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 catalyse (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 $-NH_3^+$ and -

302 $COO^-$ . Above their pI however, proteins become negatively charged and the groups exist as -

303 $NH_2$ and $-COO^-$, (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 ($\alpha$)

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 ($\alpha$) 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 rebind 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 then 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**

16

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.

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