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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1783813> since 2021-08-05T11:22:25Z

Published version:

DOI:10.1016/j.reactfunctpolym.2021.104893

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EFFECT OF EXPERIMENTAL CONDITIONS ON THE BINDING ABILITIES OF CIPROFLOXACIN-IMPRINTED NANOPARTICLES PREPARED BY SOLID-PHASE SYNTHESIS

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ABSTRACT: Imprinted nanoparticles present several advantages respect to bulk imprinted materials, but, when prepared by traditional methods, their usefulness is limited as the approaches are costly or require complex optimization steps, while the purification from template molecules is challenging. An innovative approach is the solid-phase synthesis. It consists in the covalent immobilization of the template onto a solid support, the polymerization of nanoparticles around the template, the clean-up from unproductive components and the final release of the imprinted nanoparticles, which are free of template and demonstrate high affinity for the target molecule. Here we report the use of ciprofloxacin as immobilized template to evaluate the effect of different experimental conditions in the solid phase polymerization (template scaffolding, polymerization mixtures, polymerization medium) and different rebinding conditions (buffer pH) on the binding properties. The results confirm that the solid phase synthesis approach is a flexible approach, where the experimental conditions are decisive for the binding properties. The results show that this approach is a powerful technique to easily prepare nanoparticles fully compatible with the aqueous environment, with reduced non specific binding ($\approx 10^4$ mol L⁻¹), high equilibrium binding constants (10^5 - 10^7 mol L⁻¹) and fast association rate constants ($\approx 10^6$ mol L⁻¹min⁻¹), values which are comparable to those of natural antibodies.

KEYWORDS: molecularly imprinted polymer; solid phase synthesis; ciprofloxacin; molecular recognition; ligand binding

1. INTRODUCTION

In recent years, the focus on molecularly imprinted polymers (MIPs) has progressively shifted from bulk materials characterized by micrometre-size dimensions and morphologies, to materials of much smaller dimensions. Imprinted nanoparticles, or “nanoMIPs”, present several practical advantages, including solubility in buffers and organic solvents, limited binding heterogeneity, reduced non-specific binding and improved mass transfer and binding kinetics due to larger

37 surface/mass ratio [1-5]. The synthesis of nanoMIPs can be attained through several different
38 approaches: high dilution [6,7], precipitation [8,9], distillation [10], mini- or micro-emulsion [11,12],
39 or controlled living radical polymerization [13,14]. Nevertheless, all these approaches show
40 severe drawbacks, as synthetic methods are rarely transferable from one template to another
41 without an optimization process, while the purification of nanoMIPs from the polymerization
42 mixture, including the total elimination of the template molecule, is often a difficult task.

43 An innovative approach to solving these issues is represented by the solid-phase synthesis
44 [15,16]. The polymerization process, illustrated in scheme 1, takes place in the interstitial space
45 between loosely packed glass beads covalently grafted with template molecules. Here, the growth
46 of cross-linked polymeric chains takes place in proximity of the glass surface, resulting in the
47 imprinting of the nascent nanoparticles by the grafted template molecules [17]. Once the
48 polymerization process is stopped, the non-covalent interaction between nanoMIPs and template
49 molecules is strong enough to allow any residual monomers, polymerization by-products and low
50 affinity polymer to be washed away. Finally, the high affinity nanoMIPs are recovered by washing
51 the glass spheres with a solution capable of breaking the non-covalent molecular interactions.

52 Solid-phase synthesis shows many advantages over traditional solution synthesis techniques.
53 First of all, because template molecules are covalently grafted onto the glass beads, no residual
54 template molecules are present in nanoMIPs, avoiding the bleeding effect that affects many
55 imprinted materials and prevents their practical use [18]. Grafted templates do not need to be
56 soluble in the polymerization solvent, eliminating any issue about solvent-template compatibility
57 [19]. Functionalized glass beads can be cleaned and reused many times, as long as the template
58 does not incur an irreversible denaturation or decomposition during the washing/elution steps
59 [20]. Template reusability has an obvious impact on the costs of synthesis, as it allows the use of
60 expensive molecules, while, in the case of toxic or harmful templates, confinement on the glass
61 surface eliminates any health risks from residual template during the recover step of the imprinted
62 nanoMIPs [21]. Because of the affinity separation step performed at the end of the polymerization
63 process, nanoMIPs can be easily separated from low affinity products. Thus, they show a more
64 homogenous and significantly higher affinity of the MIPs produced by solution synthesis [22]. Till
65 nanoMIPs remain attached to the solid support, binding sites are sterically protected, thus, post-
66 polymerization modifications are easily achievable [23].

67 Solid-phase synthesis quickly proved to be very versatile, and nanoMIPs targeting small
68 molecules [19,24,25], macrocyclic antibiotics [26,27], toxins [21,28], amino acids [19,29], peptides
69 [30,31], proteins [16,20,32], polysaccharides [33,34], nucleic acids [35], viruses [36], and whole
70 cells [37] have been described and used for the development of sensors and biomimetic assays.
71 In most of the examples reported here, nanoMIPs are prepared in an aqueous medium, using
72 N,N'-methylene-bis-acrylamide as a cross-linker and ammonium persulfate as a radical initiator,

73 but it is also possible to find several examples of nanoMIPs prepared in polar organic solvents,
74 using ethylene dimethacrylate or trimethylolpropane trimethacrylate as a cross-linker
75 [15,23,24,30,33].

76 The preparation of nanoMIPs by solid-phase synthesis seems to be a very versatile technique,
77 where the experimental conditions can be changed according to current needs. Thus, to get more
78 insights about the actual versatility of this innovative approach, the goal of this work is to directly
79 compare the binding properties of nanoMIPs prepared with the same template but in different
80 experimental conditions. For this purpose, we chosen as template a fluoroquinolone antibiotic,
81 ciprofloxacin, whose molecular imprinting has been widely described in the literature [38-40], but
82 of which the preparation of nanoMIPs has not been described so far through the solid-phase
83 synthesis technique. Ciprofloxacin was covalently bound to glass beads (scheme 2) provided or
84 not with a glutaraldehyde-based spacer arm ("long chain" / "short chain" beads) and nanoMIPs
85 were synthesized in polymerization mixtures based on different solvent (water vs. acetonitrile),
86 radical initiators (ammonium persulfate vs. AIBN), cross-linking agents (methylen-bis-acrylamide
87 vs. EDMA / TRIM) and functional monomers (acrylic acid / N-tert-butylacrylamide /
88 isopropylacrylamide vs. methacrylic acid). Finally, the binding properties were assessed by
89 measuring adsorption isotherms and binding kinetics of the resulting nanoMIPs in aqueous
90 medium at different pHs.

92 2. EXPERIMENTAL

93 **2.1. Materials.** Glass beads were Spheriglass-2429 70-100 μm average particle size (Potters,
94 UK). Ciprofloxacin was Supelco (Milan, Italy). Acrylic acid (AA), 3-(aminopropyl)trimethoxysilane
95 (APTMS), ammonium persulphate (APS), azo-bis-isobutyronitrile (AIBN), 1-ethyl-3-(3-
96 dimethylaminopropyl)carbodiimide (EDC), ethylenediamine, ethylene dimethacrylate (EDMA),
97 glutaraldehyde (50% aqueous solution), hexamethyldisilazane (HMDS), N-hydroxysuccinimide
98 (NHS), N-isopropylacrylamide (NIPAm), methacrylic acid (MAA), N,N'-methylen-bis-acrylamide
99 (BIS), morpholinethansulphonic acid (sodium salt, MES), sodium borohydride, N-tert-
100 butylacrylamide (TBAAm), N,N,N',N'-tetramethylethylenediamine (TEMED) and trimethylolpropane
101 trimethacrylate (TRIM) were Sigma-Merck (Milan, Italy). Solvents and all other chemicals were
102 purchased from Sigma-Merck (Milan, Italy). All the solvents were of HPLC grade, whereas all
103 chemicals were of analytical grade. The water used was ultra-purified in Purelab Prima System
104 from Elga (Marlow, UK). Polymerisation inhibitors were removed by filtration through activated
105 basic alumina. Antibiotic stock solutions were prepared by dissolving 25 mg of the substance in
106 25 mL of water/methanol 1+1 (v/v) then stored in the dark at $-20\text{ }^{\circ}\text{C}$.

107
108 **2.2. Glass beads amination.** In a 100-mL round-bottomed flask, 25 g of glass beads in 20 mL of

109 1 mol L⁻¹ aqueous NaOH and boiled for 1 h. Then, they were diluted with 50 mL of ultrapure water
110 and filtered on a 0.22 μm nylon membrane. The glass beads were washed with 100 mL of 1 mol
111 L⁻¹ aqueous HCl and with ultrapure water till neutrality. Then they were rinsed twice with acetone
112 and dried at 60 °C overnight.

113 The dried glass beads were transferred in a 1-L round-bottomed flask and dispersed in 500 mL
114 of toluene, removing water by azeotropic distillation. Then, the flask was cooled to room
115 temperature, 10 mL of APTMS were added, and the mixture let to react overnight. The glass
116 beads were filtered on a 0.22 μm nylon membrane and washed with 3x50 mL of toluene.

117 To end-cap the residual silanols, the glass beads were transferred in a 250-mL round-bottomed
118 flask and dispersed in 50 mL of toluene, removing water by azeotropic distillation. Then, the flask
119 was cooled to room temperature, 1 mL of HMDS was added to the dispersion and the mixture let
120 to react overnight. The end-capped glass beads, named “short-chain beads” (SC-beads) were
121 filtered on a 0.22 μm nylon membrane, rinsed twice with acetone and dried at 60 °C overnight.
122 After silanization, the amino groups available on the silanized glass beads surface were
123 determined by Kaiser’s method [41] as 1,1 μmol g⁻¹.

124 To introduce the glutaraldehyde-based spacer arm, 10 g of SC-glass beads were transferred in a
125 25-mL glass vial, dispersed in 10 mL of a freshly prepared 5% (v/v) glutaraldehyde solution in
126 phosphate buffer (10 mmol L⁻¹, pH 7.4) and incubated at 25 °C for 2 h. Then, 0.5 mL of freshly
127 distilled ethylenediamine was added and the flask was incubated at 25 °C for 2 hours. The pH of
128 the mixture was adjusted to pH 10, 20 mg of sodium borohydride were added and after 1 h the
129 glass beads, named “long-chain beads” (LC-beads) were filtered on a 0.22 μm nylon membrane,
130 washed with ultrapure water, rinsed twice with acetone and dried at 60 °C overnight.

131

132 **2.3. Template immobilization.** In 25-mL glass vials 20 mg of ciprofloxacin (0.06 mmol) were
133 dissolved in 20 mL of MES buffer (10 mmol L⁻¹, pH 4.7), 104 mg of NHS (0.9 mmol) and 140 mg
134 of EDAC (0.6 mmol) were added and the solutions incubated at 4 °C for 60 min. Then, they were
135 transferred in 100-mL flasks containing 10 g of aminated glass beads (SC or LC) in 40 mL of PBS
136 (0.1 mol L⁻¹, pH 7.4). The suspensions were incubated at room temperature overnight, filtered on
137 a 0.22 μm nylon membrane, washed with ultrapure water, rinsed twice with acetone, dried under
138 vacuum at room temperature and stored in the dark at 4 °C.

139

140 **2.4. Synthesis of nanoMIPs.** The polymerization mixtures were prepared in according with the
141 literature [22], with minor modifications and adjusting the dilution of monomers to avoid formation
142 of unwanted lumps of polymer.

143 For nanoMIPs prepared in acetonitrile (acnSC-MIP and acnLC-MIP), 0.946 mL of MAA (11.15
144 mmol), 1.027 mL of EDMA (5.45 mmol), 1.019 mL of TRIM (3.19 mmol) and 50 mg of AIBN (0.30

145 mmol) were dissolved in 20 mL of acetonitrile. Then, 5 mL of mixture were added to 50-mL
146 polypropylene SPE cartridges containing 2.5 g of SC- or LC-glass beads. The cartridges were
147 purged with nitrogen for 5 min, sealed and left to polymerize at 60 °C for 10 min in a roller-
148 equipped incubator. The supernatant was drained by vacuum aspiration, the dry cartridges were
149 cooled to 4 °C and polymerization by-products and low-affinity nanoMIPs were washed with 5x2
150 mL of ice-cold acetonitrile. High affinity nanoMIPs were collected by eluting the cartridges with
151 5x2 mL of methanol - acetic acid 9+1 (v/v). The eluate was evaporated in a rotavap, weighted,
152 and stored at 4 °C.

153 For nanoMIPs prepared in water (wSC-MIP and wLC-MIP), 20 mg of NIPAm (0.177 mmol), 33
154 mg of TBAm (0.259 mmol, predissolved in 1 mL of ethanol), 11 µL of AA (0.160 mmol) and 1 mg
155 of BIS (0.0065 mmol) were dissolved in 50 mL of ultrapure water. Then, 5 mL of mixture were
156 added to 50-mL polypropylene SPE cartridges containing 2.5 g of SC- or LC-glass beads. The
157 cartridges were purged with nitrogen for 5 min, 3 µL of TEMED and 100 µL of 30 mg mL⁻¹ aqueous
158 solution of APS were added and the polymerization was carried out at room temperature for 1 h
159 in a roller-equipped incubator. The supernatant was drained by vacuum aspiration, the dry
160 cartridges were cooled to 4 °C and polymerization by-products and low-affinity nanoMIPs were
161 washed with 5x2 mL of ice-cold water. High affinity nanoMIPs were collected by eluting the
162 cartridges with 5x2 mL of hot water. The eluate was lyophilized, weighted and stored at 4 °C.

163 Not-imprinted polymers (NIPs) were prepared by precipitation polymerization in the same
164 experimental conditions in terms of composition of the polymerization mixture, quantity of solvent
165 and polymerization time, but without the presence of functionalized glass beads. After the
166 polymerization, the slightly opalescent solution was filtered on 0.22 µm nylon membranes to
167 eliminate larger polymers, dried (synthesis in acetonitrile) or lyophilized (synthesis in water),
168 weighted, and stored at 4 °C.

169

170 **2.5. Coupling of nanoMIPs to glass beads.** In 4-mL glass vials 2 mg of nanoMIPs were
171 dissolved under sonication in 2 mL of MES buffer (10 mM, pH 4.7), 10 mg of NHS (0.087 mmol)
172 and 14 mg of EDAC (0.058 mmol) were added and the solutions incubated at 4 °C for 60 min.
173 Then, they were transferred in 25-mL flasks containing 2 g of LC-glass beads in 8 mL of PBS (0.1
174 mol L⁻¹, pH 7.4). The suspensions were incubated at room temperature overnight, filtered on 0.22
175 µm nylon membranes, washed with ultrapure water, rinsed twice with acetone, dried under
176 vacuum at room temperature and stored in the dark at 4 °C.

177

178 **2.6. HPLC method.** Reverse phase HPLC analysis was used for fluoroquinolones determination.
179 The HPLC apparatus (Merck-Hitachi, Milan, Italy) was a LaChrom Elite system composed of a
180 programmable binary pump L-2130, an auto-sampler L-2200, a fluorescence detector L-7485,

181 provided with EZChrom Elite software for the instrumental programming, data acquisition and
182 data processing. The column used was a 100 mm × 4.6 mm Chromolith RP-18 (Merck, Milan,
183 Italy). The mobile phase was water/acetonitrile 85+15, formic acid 0.5% (v/v). Elution were
184 performed in isocratic conditions at a flow rate of 0.7 mL min⁻¹. The sample volume injected was
185 5 μL, and the fluorescence wavelength were λ_{ex}=280/λ_{em}=440 nm. Ciprofloxacin solutions
186 between 10 and 500 ng mL⁻¹ were prepared in the eluent immediately before use. The solutions
187 were analysed in triplicate and mean peak areas were plotted against ciprofloxacin concentration.
188 The calibration plot was drawn by using a weighted linear regression (weight = 1/conc).

189
190 **2.7. Determination of binding properties.** To measure binding isotherms, about 40 mg of glass
191 beads supporting nanoMIPs were exactly weighed in 4 mL flat bottom amber glass vials. Then,
192 1.0 mL of solutions containing increasing amounts of ciprofloxacin ranging from 25 to 400 ng were
193 added. The vials were incubated overnight at room temperature under continuous agitation on a
194 horizontal rocking table. Then, the solutions were filtered on 0.22 μm nylon membranes and the
195 free amounts of ciprofloxacin were measured by HPLC analysis. Each experimental point was
196 assessed as the average of three repeated measures.

197 To measure binding kinetics, about 40 mg of glass beads supporting nanoMIPs were exactly
198 weighed in 4 mL flat bottom amber glass vials. Then, 1.0 mL of solutions containing 50 ng of
199 ciprofloxacin were added and the vials were incubated for time intervals between 0.5 and 8
200 minutes at room temperature under continuous agitation on a horizontal rocking table. Then, the
201 solutions were immediately filtered on 0.22 μm nylon membranes, and the free amounts of
202 ciprofloxacin were measured by HPLC analysis. Each experimental point was assessed as the
203 average of three repeated measures.

204 Binding parameters were calculated by using SigmaPlot 12 (Systat Software Inc., Richmond, CA,
205 USA). Non-linear least square fitting was applied to the averaged experimental data. Binding
206 isotherm parameters were calculated by using a Langmuir binding isotherm model:

207
208
$$B = \frac{B_{max}K_{eq}F}{1 + K_{eq}F}$$

209
210 where B is the ligand bound to the polymer, F the ligand not bound to the nanoMIP, K_{eq} the
211 equilibrium binding constant and B_{max} the binding site density.

212 Binding kinetics parameters were calculated by using a 1st order kinetic model:

213
214
$$B = B_{eq}[1 - \exp(-k_{ass}t)]$$

215

216 where B is the ligand bound to the nanoMIP at time t, B_{eq} the ligand bound to the polymer at
217 equilibrium and k_{ass} the association kinetic constant.

218 To assure robust results, weighted (1/y) Pearson VII limit minimization was chosen as the
219 minimization method. To avoid being trapped in local minima, which would give incorrect results,
220 minimizations were carried out several times by using different initial guess values for the binding
221 parameters.

222

223 3. RESULTS AND DISCUSSION

224 **3.1. Binding properties of nanoMIPs.** Under all the experimental conditions considered, the
225 solid-phase synthesis produced nanoMIPs fully soluble in water, resulting in transparent and
226 colourless solutions, without any perceivable turbidity. Yields calculated respect to the amount of
227 monomers in the polymerization mixtures were: 5.4 mg (2.5%) for acnLC-MIP, 5.0 mg (2.3%) for
228 acnSC-MIP, 1.9 mg (29%) for wLC-MIP, and 1.5 (23%) for wSC-MIP. Dynamic light scattering
229 measurements performed on nanoMIPs are reported in figure 1. They show particles with
230 diameters on the order of magnitude of hundreds of nanometres (acnLC-MIP: 166 ± 87 , acnSC-
231 MIP: 147 ± 96 , wLC-MIP: 255 ± 147 , wSC-MIP: 198 ± 73). As the binding properties of nanoMIPs
232 towards ciprofloxacin can be obtained from the analysis of their equilibrium binding isotherms, an
233 efficient separation between free and bound ligand is mandatory. So, we had to devise an
234 experimental approach that made this separation simple and fast, as slow methods like
235 ultrafiltration or dialysis did not represent a viable way. We have therefore chosen to support the
236 nanoMIPs on the same glass beads used for their synthesis in order to easily separate by filtration
237 the grafted beads – carrying the bound ligand – from the solution which contains the free ligand.
238 Preliminary experiments showed that bare glass beads, HDMS-silanized beads, and beads
239 functionalized with a spacer arm based on aminated glutaraldehyde (LC-beads) were unable to
240 bind ciprofloxacin in an aqueous medium in a pH range between 4 and 8, while LC-beads grafted
241 with NIPs – as reported in figure 2 – showed a limited binding, with calculated equilibrium binding
242 constants in the order of magnitude of 10^4 L mol⁻¹ at pH 6 (synthesis in water: $K_{eq} = 2.3\pm 1.1\times 10^4$
243 L mol⁻¹; synthesis in acetonitrile: ($K_{eq} = 8.9\pm 1.1\times 10^4$ L mol⁻¹). It must be noted that in the case of
244 the solid phase synthesis technique, it is obviously not possible to prepare a “nanoNIP” strictly
245 following the same approach. This problem can be addressed by using nanoMIPs prepared with
246 structurally different templates [22]. However, in the literature there are examples of MIPs which
247 show unexpected molecular recognition properties towards molecules completely unrelated to
248 the template [42-44]. For this reason, on the assumption that different polymerization methods
249 have only limited effects on the binding properties of NIPs [45,46], we chosen to use NIPs
250 prepared by precipitation polymerization with the same formulation used for the preparation of
251 nanoMIPs. Therefore, it is plausible that whatever observed absorption of ciprofloxacin by the

252 grafted beads is attributable mainly to the interaction of the antibiotic molecules with nanoMIPs,
253 thus excluding the presence of any other non-specific binding.

254 The binding parameters obtained from binding isotherm (figures 3-4) and association kinetics
255 plots (figures 5-6) are reported in tables 1-2\). They confirm the versatility of the solid-phase
256 synthesis approach as, regardless of the polymerization conditions, nanoMIPs strongly bind
257 ciprofloxacin in buffered water, with equilibrium binding constants (K_{eq}) ranging from 10^5 to 10^7 L
258 mol^{-1} . It is noteworthy that these values are about 100-1000 times higher than those reported in
259 the literature for ciprofloxacin-imprinted polymers prepared by bulk polymerization [47,48], and
260 they approach the average affinity values reported in the literature for natural antibodies directed
261 towards small organic molecules [49]. The increased affinity for ciprofloxacin can be explained on
262 the basis that the solid-phase polymerization technique allows to easily separate low affinity
263 nanoMIPs from higher affinity ones by simply washing the glass beads once the polymerization
264 is finished.

265 Equilibrium binding constants (K_{eq}) can be dissected into the association (k_{ass}) and dissociation
266 (k_{dis}) kinetic rate constants, such that $K_{eq}=k_{ass}/k_{dis}$. It may therefore be interesting to examine the
267 values of these rate constants in the case of nanoMIPs. As reported in figure 7, it is possible to
268 observe a marked inverse proportionality between the values of k_{ass} and k_{dis} , where the values of
269 k_{dis} decreases compared to the values of k_{ass} . It follows that the resulting value of K_{eq} depends
270 simultaneously on both the association and dissociation rate constants. The k_{ass} values are in the
271 order of magnitude of 10^6 L mol^{-1} min^{-1} (0.60-4.24), comparable to those reported in the literature
272 for antibodies directed towards organic molecules (10^6 - 10^7 L mol^{-1} min^{-1}) [50]. This is not
273 surprising, as it means that ciprofloxacin associates to the binding sites with kinetic rates
274 comparable to natural antibodies, indicating the same diffusion-controlled process. On the
275 contrary, nanoMIPs dissociate faster than natural antibodies, with k_{dis} values located in a range
276 from 0.07 to 1.26 min^{-1} , markedly differing from the average value of 0.01-0.1 min^{-1} reported in the
277 literature for natural antibodies [50].

278

279 **3.2. Effect of spacer arm on ciprofloxacin binding.** In analogy with solid-phase peptide
280 synthesis techniques [51], the presence/absence of a spacer arm between the surface of glass
281 beads and the covalently grafted template may influence the growth of the nanoMIP structure
282 through steric hindrance effects. For this reason, we decided to covalently bound ciprofloxacin to
283 aminated glass beads provided or not with a glutaraldehyde-based spacer arm ("long chain" /
284 "short chain" beads). Concentrated aqueous solutions of glutaraldehyde are known to
285 spontaneously polymerize to form mixtures of linear polymers of varying length [52]. Thus,
286 glutaraldehyde-grafted glass beads ensure that the template is placed sufficiently far from the
287 glass surface to minimize steric hindrance effects.

288 The comparison of equilibrium binding constants for pairs of nanoMIPs synthesized onto SC- or
289 LC-beads shows small but systematic differences. NanoMIPs prepared in acetonitrile onto LC-
290 beads (acnLC-MIP) have less affinity than nanoMIPs prepared in acetonitrile onto SC-beads
291 (acnSC-MIP), while nanoMIPs prepared in water onto LC-beads (wLC-MIP) have greater affinity
292 than nanoMIPs prepared in water onto SC-beads (wSC-MIP). However, a more in-depth analysis
293 that takes into account the uncertainty on the calculated value of the constants shows no
294 statistically relevant differences (t-test: $\alpha=0.05$, $n=10$, $t=0.13-1.72$) between pairs. Therefore, it is
295 not possible to say with certainty that the presence of a spacer arm on the glass beads has an
296 influence on the affinity of the resulting nanoMIPs. The same can be observed comparing the
297 association rate constants of nanoMIPs synthesized onto SC- or LC-beads, as no statistically
298 relevant differences (t-test: $\alpha=0.05$, $n=8$, $t=0.24-2.02$) between pairs can be observed. It indicates
299 that the presence of a spacer arm on the glass beads has not an influence on the velocity of
300 association of the resulting nanoMIPs.

301 On the contrary, the comparison of binding site density (B_{max}) for pairs of nanoMIPs synthesized
302 onto SC- or LC-beads shows large and systematic differences confirmed by statistical analysis
303 (t-test: $\alpha=0.05$, $n=10$, $t=2.46-25.25$). NanoMIPs prepared in acetonitrile or water onto LC-beads
304 (acnLC-MIP, wLC-MIP) have higher binding site density than nanoMIPs prepared in acetonitrile
305 or water onto SC-beads (acnSC-MIP, wSC-MIP). The grafting protocol on glass beads is identical
306 for all the nanoMIPs considered, so it is reasonable to assume that the quantity of nanoMIPs
307 actually grafted is the same. Consequently, different B_{max} values must depend on the experimental
308 conditions of nanoMIP preparation. Since it does not seem to be a significant difference between
309 nanoMIP prepared in water and acetonitrile (see section 3.3), it can be concluded that it is the
310 presence of the spacer arm to control the density of the binding sites, probably through a steric
311 hindrance effect between the growing polymer and the glass surface.

312

313 **3.3. Effect of polymerization conditions on ciprofloxacin binding.** As stated in the
314 introduction, nanoMIPs can be obtained by solid-phase synthesis using very different
315 polymerization mixtures. Polymerization in aqueous environment typically involves the use of
316 polar functional monomers, N,N'-methylene-bis-acrylamide as a cross-linker and ammonium
317 persulphate as a radical initiator. On the contrary, polymerization in an organic environment –
318 typically acetonitrile – involves the use of less polar functional monomers, using ethylene
319 dimethacrylate or trimethylolpropane trimethacrylate as cross-linkers and radical initiators such
320 as AIBN or RAFT agents. It is therefore possible that nanoMIPs produced from significantly
321 different polymerization mixtures can exhibit different binding properties towards the same ligand.
322 The comparison of binding parameters for nanoMIPs synthesized in acetonitrile (acnLC-
323 MIP/acnSC-MIP) or water (wLC-MIP/wSC-MIP) shows a strong dependence from the pH of the

324 rebinding buffer. This dependence can be traced back to the solvent in which the nanoMIPs are
325 prepared and the protonation state of template and functional monomers. In fact, in both the
326 polymerization mixtures it is present a pH-sensitive functional monomer (methacrylic acid in
327 acetonitrile-based mixtures, acrylic acid in water-based mixtures) and ciprofloxacin presents two
328 substituents subject to acid-base equilibria: a secondary nitrogen on the piperazinyl ring
329 ($pK_a=8.74$) and a carboxylic group on the quinolone structure ($pK_a=6.09$) [53].

330 About equilibrium binding constants, as reported in figure 8, when the pH of the buffer increases,
331 the values are decreasing for nanoMIPs prepared in water, while they increase for those prepared
332 in acetonitrile. Concerning the first one, the concentration of acrylic acid is 3.2 mmol L^{-1} ,
333 corresponding to a calculated pH of about 3.4. In these conditions, the protonated form of the acid
334 prevails, ruling out ionic interactions with the protonated secondary nitrogen but not hydrogen
335 bond-based interactions with the grafted template. Thus, when nanoMIPs prepared in water
336 rebind ciprofloxacin, binding is strongest at pH 4, where carboxyls in the polymer structure are
337 fully protonated and hydrogen bonding is possible, but it decreases at higher pHs, where
338 carboxyls deprotonate progressively, losing the ability to establish hydrogen bonds. Concerning
339 the synthesis in acetonitrile, grafted template and methacrylic acid are in their neutral forms, but
340 an ion pair could form anyway between the acid and the secondary nitrogen. Thus, when
341 nanoMIPs prepared in acetonitrile rebind ciprofloxacin, an acidic buffer suppresses the ion pair
342 interaction (methacrylic acid is protonated and neutral, secondary nitrogen on ciprofloxacin is
343 positively charged), while neutral or basic buffers stabilizes the ion pair interaction (methacrylic
344 acid is deprotonated and negatively charged, secondary nitrogen on ciprofloxacin is yet positively
345 charged), thus increasing the binding affinity.

346 About the association rate constants, as reported in figure 9, the values show the same trend as
347 the equilibrium binding constants, decreasing when pH increases in the case of nanoMIPs
348 prepared in water, and increasing when pH increases in the case of nanoMIPs prepared in
349 acetonitrile. These trends can be explained in the light of what has been said in the case of the
350 equilibrium constant: nanoMIPs show an increasing loss of binding ability due to the progressive
351 deprotonation of polymeric carboxyls (nanoMIPs prepared in water) or the suppression of ion-
352 pairs (nanoMIPs prepared in acetonitrile), causing a slowing of the association and an
353 acceleration of the dissociation processes. It is presumably due to the progressive deformation
354 of the binding site, which becomes less tight and therefore less able to bind and retain the
355 ciprofloxacin molecule.

356 The effect of the formulation of the polymerization mixture on the density of binding sites is
357 reported in figure 10. A statistically significant increase in values passing from pH 4 to pH 6 is
358 observed for all nanoMIPs (t-test: $\alpha=0.05$, $n=10$, $t=3.64-15.5$), while a further increase from pH 6
359 to pH 8 – although observable – is not significant (t-test: $\alpha=0.05$, $n=10$, $t=0.25-2.08$). This

360 increase therefore occurs when the nanoMIPs are in a non-acidic environment. A possible
361 explanation consists in the establishment of electrostatic repulsion between deprotonated
362 carboxyls, which could cause an expansion of the polymer structure, with consequent greater
363 accessibility of binding sites otherwise hidden.

364 365 **4. CONCLUSIONS**

366 The experimental results reported here confirm that the solid phase synthesis of molecularly
367 imprinted polymers is a very flexible approach, where the experimental conditions such the nature
368 of the polymerization mixture (N,N'-methylene-bis-acrylamide vs. ethylene dimethacrylate /
369 trimethylolpropane trimethacrylate) or the polymerization environment (water vs. acetonitrile) are
370 decisive in defining the binding properties of the resulting nanoMIPs through different non-
371 covalent interactions that can be established between the polymer in formation and the
372 immobilized template during the polymerization process. Moreover, these results show also that
373 the solid phase synthesis approach is a powerful technique to easily prepare nanoMIPs fully
374 compatible with the aqueous environment, with reduced non specific binding ($<10^3$ L mol⁻¹), high
375 equilibrium binding constants (10^5 - 10^7 L mol⁻¹) and fast association rate constants ($\approx 10^6$ L mol⁻¹
376 min⁻¹), values which are comparable to those of natural antibodies.

377 In conclusion, if compared to traditional imprinted polymers, the enhanced binding properties of
378 nanoMIPs prepared by solid phase synthesis make these nanomaterials very promising
379 recognition elements for applications in fields where aqueous compatibility, low non specific
380 binding, high affinity and fast binding kinetics are basic requirements.

381
382 **AUTHOR CONTRIBUTIONS:** Conceptualization, C.B.; methodology, C.B.; investigation, S.C.
383 and M.C.; data curation, F.D.N.; writing—original draft preparation, C.B.; writing—review and
384 editing, L.A. All authors have read and agreed to the published version of the manuscript.

385
386 **FUNDING:** This research did not receive any specific grant from funding agencies in the public,
387 commercial, or not-for-profit sectors.

388
389 **ACKNOWLEDGMENTS.** The authors thank Prof. V. Maurino (Department of Chemistry –
390 University of Torino) for dynamic light scattering measurements of nanoMIPs

391
392 **CONFLICTS OF INTEREST.** The authors declare no conflict of interest.

393
394 **DATA AVAILABILITY STATEMENT.** The raw and processed data required to reproduce these
395 findings are available on request.

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574 **TABLES**

575 **Table 1:** calculated binding equilibrium parameters (\pm standard error) for ciprofloxacin
576 measured on nanoMIPs at pH 4, 6, and 8.

577

polymer	buffer pH	$K_{eq}, \times 10^{-6} \text{ L mol}^{-1}$	$B_{max}, \text{ nmol g}^{-1}$
acnLC-MIP	4	0.82 ± 0.15	2.94 ± 0.01
	6	3.20 ± 0.35	3.62 ± 0.01
	8	6.35 ± 0.83	3.51 ± 0.01
acnSC-MIP	4	1.05 ± 0.17	2.02 ± 0.01
	6	4.91 ± 0.52	3.03 ± 0.01
	8	7.49 ± 0.38	3.12 ± 0.09
wLC-MIP	4	15.40 ± 1.26	1.65 ± 0.01
	6	3.25 ± 0.24	1.80 ± 0.02
	8	0.21 ± 0.07	3.63 ± 0.01
wSC-MIP	4	12.16 ± 1.23	0.63 ± 0.01
	6	3.33 ± 0.31	2.31 ± 0.00
	8	0.27 ± 0.18	1.82 ± 0.01

578

579

580 **Table 2:** calculated association and dissociation rate parameters (\pm standard error) for
581 ciprofloxacin measured on nanoMIPs at pH 4, 6, and 8.

582

polymer	buffer pH	$k_{\text{ass}}, \times 10^{-6} \text{ L mol}^{-1} \text{ min}^{-1}$	$k_{\text{dis}}, \text{ min}^{-1}$
acnLC-MIP	4	1.36 ± 0.21	1.66 ± 0.41
	6	2.89 ± 0.44	0.90 ± 0.17
	8	3.60 ± 0.55	0.57 ± 0.11
acnSC-MIP	4	1.93 ± 0.60	1.84 ± 0.64
	6	2.97 ± 0.52	0.61 ± 0.12
	8	3.94 ± 0.11	0.53 ± 0.03
wLC-MIP	4	3.81 ± 0.23	0.25 ± 0.03
	6	2.70 ± 0.20	0.83 ± 0.09
	8	0.60 ± 0.08	2.83 ± 1.01
wSC-MIP	4	4.24 ± 0.54	0.35 ± 0.06
	6	2.26 ± 0.29	0.68 ± 0.11
	8	0.71 ± 0.20	2.62 ± 1.87

583

584

585 **FIGURE CAPTIONS**

586

587 **Scheme 1:** schematic representation of the solid phase synthesis method.

588

589 **Scheme 2:** covalent conjugation of ciprofloxacin to aminated glass beads

590

591 **Figure 1:** DLS of nanoMIPs prepared in acetonitrile (acnLC-MIPs: red, acnSC-MIPs: yellow) and
592 water (wLC-MIPs: green, wSC-MIPs: blue)

593

594 **Figure 2:** binding isotherm plots for NIPs in buffer pH 6. Red circles: synthesis in water; blue
595 circles: synthesis in acetonitrile.

596

597 **Figure 3:** binding isotherm plots for nanoMIPs prepared in acetonitrile. Circles: acnLC-MIPs;
598 triangles: acnSC-MIPs. Red symbols: rebinding in buffer pH 4; green symbols: rebinding in buffer
599 pH 6; blue symbols: rebinding in buffer pH 8.

600

601 **Figure 4:** binding isotherm plots for nanoMIPs prepared in water. Circles: wLC-MIPs; triangles:
602 wSC-MIPs.. Red symbols: rebinding in buffer pH 4; green symbols: rebinding in buffer pH 6; blue
603 symbols: rebinding in buffer pH 8.

604

605 **Figure 5:** association kinetic plots for nanoMIPs prepared in acetonitrile. Circles: acnLC-MIPs;
606 triangles: acnSC-MIPs. Red symbols: rebinding in buffer pH 4; green symbols: rebinding in buffer
607 pH 6; blue symbols: rebinding in buffer pH 8.

608

609 **Figure 6:** association kinetic plots for nanoMIPs prepared in water. Circles: wLC-MIPs; triangles:
610 wSC-MIPs.. Red symbols: rebinding in buffer pH 4; green symbols: rebinding in buffer pH 6; blue
611 symbols: rebinding in buffer pH 8.

612

613 **Figure 7:** dissociation rate constants (k_{dis}) vs. association rate constants (k_{ass}) plot. Error bars
614 indicate 1 standard error unit.

615

616 **Figure 8:** effect of buffer pH on the equilibrium binding constant (K_{eq}). Red bars: rebinding at pH
617 4; green bars: rebinding at pH 6; blue bars: rebinding at pH 8. Error bars indicate 1 standard error
618 unit.

619

620 **Figure 9:** effect of buffer pH on the association rate constant (k_{ass}). Red bars: rebinding at pH 4:

621 green bars: rebinding at pH 6; blue bars: rebinding at pH 8. Error bars indicate 1 standard error
622 unit.

623

624 **Figure 10:** effect of buffer pH on the binding site density (B_{\max}). Red bars: rebinding at pH 4:
625 green bars: rebinding at pH 6; blue bars: rebinding at pH 8. Error bars indicate 1 standard error
626 unit.