Effect of experimental conditions on the binding abilities of ciprofloxacin-imprinted nanoparticles prepared by solid-phase synthesis

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 (≈10^4 mol L^{-1}), high equilibrium binding constants (10^5-10^7 mol L^{-1}) and fast association rate constants (≈10^6 mol $L^{-1}min^{-1}$), 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

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

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

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

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

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

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

2. EXPERIMENTAL

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. RESULTS AND DISCUSSION

3.1. Binding properties of nanoMIPs. Under all the experimental conditions considered, the solid-phase synthesis produced nanoMIPs fully soluble in water, resulting in transparent and colourless solutions, without any perceivable turbidity. Yields calculated respect to the amount of monomers in the polymerization mixtures were: 5.4 mg (2.5%) for acnLC-MIP, 5.0 mg (2.3%) for acnSC-MIP, 1.9 mg (29%) for wLC-MIP, and 1.5 (23%) for wSC-MIP. Dynamic light scattering measurements performed on nanoMIPs are reported in figure 1. They show particles with diameters on the order of magnitude of hundreds of nanometres (acnLC-MIP: 166±87, acnSC-MIP: 147±96, wLC-MIP: 255±147, wSC-MIP: 198±73). As the binding properties of nanoMIPs towards ciprofloxacin can be obtained from the analysis of their equilibrium binding isotherms, an efficient separation between free and bound ligand is mandatory. So, we had to devise an experimental approach that made this separation simple and fast, as slow methods like ultrafiltration or dialysis did not represent a viable way. We have therefore chosen to support the nanoMIPs on the same glass beads used for their synthesis in order to easily separate by filtration the grafted beads – carrying the bound ligand – from the solution which contains the free ligand. Preliminary experiments showed that bare glass beads, HDMS-silanized beads, and beads functionalized with a spacer arm based on aminated glutaraldehyde (LC-beads) were unable to bind ciprofloxacin in an aqueous medium in a pH range between 4 and 8, while LC-beads grafted with NIPs – as reported in figure 2 – showed a limited binding, with calculated equilibrium binding constants in the order of magnitude of 10^4 L mol^{-1} at pH 6 (synthesis in water: K_{eq} = 2.3±1.1x10^4 L mol^{-1}; synthesis in acetonitrile: (K_{eq} = 8.9±1.1x10^4 L mol^{-1}). It must be noted that in the case of the solid phase synthesis technique, it is obviously not possible to prepare a “nanoNIP” strictly following the same approach. This problem can be addressed by using nanoMIPs prepared with structurally different templates [22]. However, in the literature there are examples of MIPs which show unexpected molecular recognition properties towards molecules completely unrelated to the template [42-44]. For this reason, on the assumption that different polymerization methods have only limited effects on the binding properties of NIPs [45,46], we chosen to use NIPs prepared by precipitation polymerization with the same formulation used for the preparation of nanoMIPs. Therefore, it is plausible that whatever observed absorption of ciprofloxacin by the

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

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

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

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

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

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

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

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

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

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

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

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

4. CONCLUSIONS

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

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

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CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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

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TABLES

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

polymer	buffer pH	K_{eq}, x 10^{-6} L mol^{-1}	B_{max}, 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

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

polymer	buffer pH	k_{ass}, x 10^{-6} L mol^{-1} min^{-1}	k_{dis}, 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

FIGURE CAPTIONS

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

Scheme 2: covalent conjugation of ciprofloxacin to aminated glass beads

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

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

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

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

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

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

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

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

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

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

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