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Dual-Oriented Solid-Phase Molecular Imprinting: Toward Selective Artificial Receptors for Recognition of Nucleotides in Water

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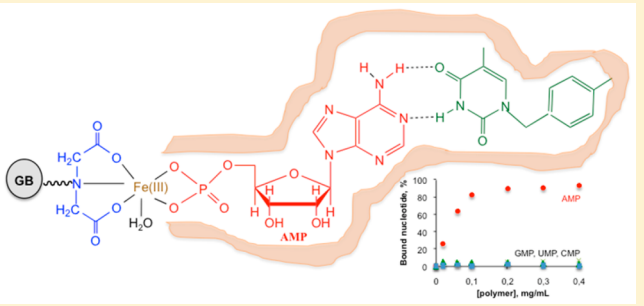
Supporting Information

ABSTRACT: We describe the synthesis of water-soluble molecularly imprinted polymer nanoparticles (MIP-NPs) as a new artificial host receptor for the recognition of adenosine monophosphate (AMP), used herein, as a model nucleotide. MIP-NPs were prepared by solid-phase synthesis on glass beads (GB) using, for the first time, immobilized Fe(III)-chelate as an affinity ligand to orientate the AMP via its phosphate group. A polymerizable thymine monomer which can induce complementary base-pairing with the adenine moiety of the nucleotide was synthesized and incorporated in the polymerization mixture to constrain the AMP in a dual-orientated configuration. The MIP-NPs were remarkably selective toward AMP as they did not bind other nucleotides, GMP, UMP, and CMP. This strategy of using the phosphate group of AMP as a hinge enables unhindered pairing of the nucleobase with its corresponding complementary base monomer and can be extended to the preparation of specific MIP receptors for other key nucleotides in aqueous conditions.



1. INTRODUCTION

Nucleotides are ubiquitously present in biological systems and are highly important biomolecules as they are the building blocks of nucleic acids (RNA and DNA). Additionally, they play essential roles in cell signaling and metabolism and participate in numerous enzymatic reactions. Consequently, nucleotides are one of the most targeted anionic compounds, and many different artificial host systems that can work in aqueous conditions have been developed.[1,2] The majority of nucleotide receptors are based on ion-pairing of the phosphate moiety with polycationic polyamines and polyguanidines. Calixarenes and cyclodextrins have also been used as they present hydrophobic pockets for the nucleobase and/or the sugar.[1,2] Another very promising type of artificial receptors is molecularly imprinted polymers (MIPs). MIPs, often referred as synthetic antibodies, are obtained by the copolymerization of functional and cross-linking monomers around a template molecule.[3−5] The template molecule can be the target analyte or a derivative thereof. Removal of the template leaves cavities complementary in shape, size, and functional groups orientation in the polymer network, which are able to rebind the template molecule with high affinities and selectivities comparable to those of natural antibodies. MIPs have considerable advantages over antibodies as they possess a higher chemical, physical, and thermal stability, ease of obtention, and low cost.

However, nucleotides are very hydrophilic molecules due to the presence of a ribose and a phosphate functionality, which render them very soluble in water. This property is not really compatible with traditional imprinting method which preferentially takes place in apolar and aprotic organic solvents.[6] Hence, imprinting of nucleosides (nucleotides without the phosphate group), often used as template for the preparation of MIP for nucleotides' recognition, requires their prior conversion to their acetylated or acylated forms.[7−9] Sometimes, a functional monomer, 2,6-bis(acrylamido)pyridine (BAApy), is added to make multiple hydrogen bonds with the nucleobase.[10] Though this strategy results in MIPs with specific binding in these solvents, no binding was observed in water.[8,10] Attempts to obtain water-compatible MIPs for targeting the nucleotide adenosine 5′-monophosphate (AMP), for instance, have also been reported. Commonly used functional monomers were based on boronic acid, associated with acrylamide to target the cis-diol function of ribose and the nucleobase, respectively.[7,11] Others used cationic monomers like (3-acrylamidopropyl)trimethylammonium chloride[12] and 2-(dimethylamino)ethyl methacrylate[13] to target the phosphate group by making electrostatic interactions. Silica-based MIPs have also been reported.[14] Most of these MIPs showed cross-

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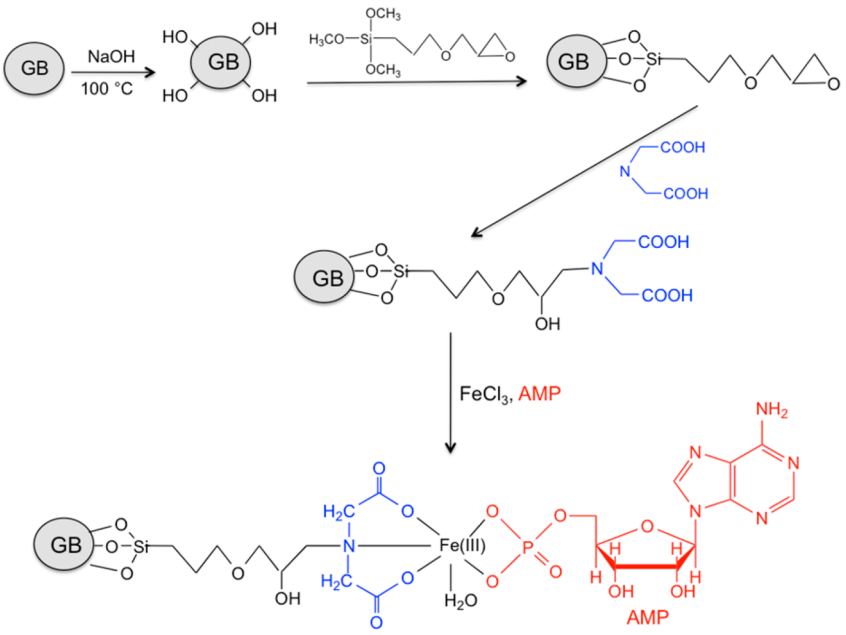

Figure 1. Immobilization of AMP on GBs using Fe^{3+} chelate. The phosphate group interacts with Fe^{3+} to form a four-membered ring (adapted from ref 23).

selectivities with other nucleo(s/t)ides and were mostly synthesized as films or in bulk which generally give rise to heterogeneous polymers with a diverse population of binding sites ranging from high to low affinity, a common feature of MIPs prepared by the self-assembly approach. Moreover, the presence of residual template even after extensive washing remains a problem.[3,6]

To overcome these problems and also to respond to the growing interest[15,16] for nanosized, "monoclonal-type" MIPs which are soluble in water, we[17−19] and others[20] have developed a solid-phase synthesis approach where the template is immobilized via an affinity ligand on glass beads as solid support. This configuration allows an oriented immobilization of the template upon which MIP-NPs are synthesized. The GBs play the role of both a reactor and a separation column since the MIP-NPs are synthesized and purified *in situ*. After synthesis and washing of unreacted reagents and nanoparticles that polymerized distantly from the immobilized template, only uniform MIP-NPs with homogeneous (since all have the same orientation) binding sites are eluted.

Herein, we describe the solid-phase synthesis of MIP-NPs targeting AMP. Metal-chelate (Figure 1) and *p*-aminobenzamidine (PAB) (Figure S1) were used as affinity ligands to immobilize AMP on the glass beads. For the metal-chelate setup, we exploited the strong coordinate bonding between the phosphate of AMP and Fe^{3+}, itself complexed with iminodiacetic acid (IDA).[21] In order to confer high selectivity to the MIP, a functional monomer bearing a complementary thymine base, 1-(vinylbenzyl)thymine (VBT)[22] (Figure 2), so as to form hydrogen bonds with the adenine base on the AMP, was synthesized and included as a comonomer in the polymerization mixture.

A high amount of *N*-isopropylacrylamide (NIPAm) was included in the MIP mixture to impart thermoresponsiveness and allows its facile liberation from the immobilized AMP. The resulting MIP-NPs were very specific for AMP as no binding was observed with the NIP. No binding was observed with guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP), and cytidine 5′-monophosphate (CMP) (structures in Figure 3), indicating the high selectivity of the MIP-NPs for its template.

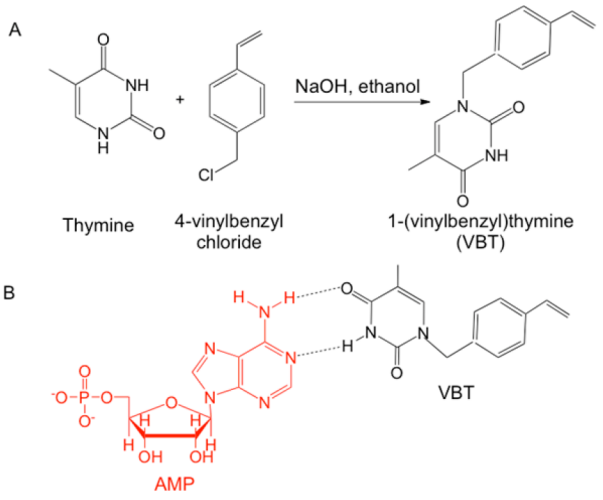

Figure 2. (A) Synthesis of the functional monomer 1-(vinylbenzyl)-thymine (VBT). (B) Pairing between adenine of AMP and thymine of VBT.

Interestingly, the MIP-AMP could bind adenosine to the same extent as AMP. Recently, a solid-phase approach was employed for synthesizing MIPs for nucleoside targeting.[24] 2′-Deoxyadenosine (dA) was used as template, and a polymerizable 2′-deoxyuridine was incorporated in the MIP so as to induce complementary base-pairing. Unfortunately, dA was linked to the glass beads by its amine group, hence rendering the base-pairing face partially inaccessible to the normal hydrogen-bonding geometry. In this work, we used instead a nucleotide as template to enable its immobilization via its phosphate moiety, hence leaving the nucleobase sterically nonhindered for recognition with the complementary base pair. This configuration indeed leads to more selective MIPs, and at

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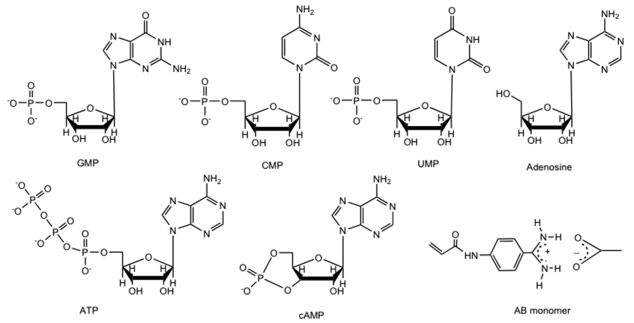

Figure 3. Chemical structures of molecules employed in this study.

the same time the MIP can advantageously recognize both the nucleotide and its associated nucleoside.

In contrast, MIP-NPs obtained with *p*-aminobenzamidine as affinity ligand (Figure S1) were not as selective as those obtained with the metal chelate (Figure 1). This difference will be discussed in terms of ^{31}P NMR studies of the interaction of AMP with a benzamidine-based monomer.

2. EXPERIMENTAL SECTION

Materials and Instruments. HPLC solvents were purchased from Biosolve Chimie (Dieuze, France). All other solvents and chemicals were of analytical grade and purchased from VWR International (Fontenay sous Bois, France) or Sigma-Aldrich (St. Quentin Fallavier, France), unless otherwise stated. Adenosine 5′-monophosphate disodium salt (AMP), guanosine 5′-monophosphate disodium salt (GMP), uridine 5′-monophosphate disodium salt (UMP), cytidine 5′-monophosphate disodium salt (CMP), adenosine 5′-triphosphate disodium salt hydrate (ATP), adenosine 3′,5′-cyclic monophosphate sodium salt monohydrate (cAMP), adenosine, and $FeCl_3 \cdot 6H_2O$ were purchased from Sigma-Aldrich. Glass beads (GBs) of diameter 0.1 mm were obtained from Roth Sochiel E.U.R.L (Lauterbourg, France). Deuterated NMR solvents and NMR tubes were purchased from Euriso-top (France) and Norell (USA), respectively. Buffers were prepared with Milli-Q water, purified using a Milli-Q system (Millipore, Molsheim, France). Absorbance measurements were done on a CARY60 UV−vis spectrophotometer (Agilent Technologies). ^{1}H and ^{31}P NMR experiments were performed on a 400 MHz Bruker spectrometer at 25 °C.

Analysis of the Interaction of AB with AMP Using ^{31}P NMR Spectroscopy. AB monomer (Figure 3), the polymerizable form of *p*-aminobenzamidine, was synthesized as its acetate salt by reacting PAB with acryloyl chloride, as previously described (details in the Supporting Information).[25] The stoichiometry and strength of the AB−AMP complex were determined by chemical shift analysis using the methods of continuous variation (Job plot)[26] and of titration,[27,28] respectively. For the Job plot, solutions of AB (M) and AMP (T) in a mixture of CD_3OD/D_2O (4/1), at a constant total concentration of 2 mM and with a template molar fraction, $\chi_T = [T]/([T] + [M])$, varying from 0 to 1 by steps of 0.1, were prepared. The total volume of each sample was 0.6 mL. Titration experiments were done to determine the association constant. A stock solution of 40 mM AB was prepared and added (from 0 to 10 equiv) to a fixed amount of 4 mM concentration of AMP.

Synthesis of 1-(Vinylbenzyl)thymine. The synthesis of 1-(vinylbenzyl)thymine (VBT) (Figure 2A) was performed as previously described.[22] Briefly, 5 g (39.6 mmol) of thymine was dissolved in 50 mL of NaOH solution (2.42 M). The mixture was heated to 65 °C under magnetic stirring. 25.7 mg (0.12 mmol) of the inhibitor 2,6-di-*tert*-butyl-4-methylphenol and 50 mL of ethanol were added and left under stirring for a further 15 min, followed by the addition of 5.6 mL (39.7 mmol) of 4-vinylbenzyl chloride. The mixture was refluxed at 85 °C for 18 h and then left to cool to room temperature, and the ethanol was evaporated under vacuum. The suspension was diluted with 150 mL of water, and the pH was adjusted to 7.0 so as to obtain a precipitate. The precipitate was filtered and vacuum-dried overnight. It was then solubilized in 180 mL of dichloromethane by stirring for 3 h. The mixture was filtered in order to remove undissolved components and the dichloromethane concentrated under vacuum to 50 mL. 150 mL of hexane was added, causing the precipitation of 1-(vinylbenzyl)-thymine. The final product was stirred for 1 h, filtered, and vacuum-dried. Yield was 47%. ^{1}H NMR (400 MHz, DMSO-d_6): 7.63 (broad qd, 1H), 7.47−7.26 (d, 4H), 6.72 (dd, 1H), 5.82 (dd, 1H), 5.25 (dd, 1H), 4.82 (s, 2H), 1.75 (broad d, 1H). The ^{1}H NMR spectrum is shown in Figure S2.

Determination of the Lower Critical Solution Temperature (LCST) of the Polymerization Mixture. The polymerization mixture was prepared in a glass vial by mixing NIPAm (41.19 mg, 91 mol %) and VBT (3.88 mg in 1 mL of methanol, 4 mol %) as functional monomers and *N*,*N*-ethylenebis(acrylamide) (EbAm) (3.36 mg, 5 mol %) as cross-linker in a total volume of 9.64 mL of 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) so that the total monomer concentration is 0.5% (w/w). The MES buffer will herafter be referred as buffer A. 3.4 mg of potassium persulfate (KPS)/0.3 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (7.5/1 molar ratio; the amount of KPS was 3% mol/mol with respect to polymerizable double bonds) as initiation system was then added. The mixture was purged with nitrogen for 10 min. The polymerization mixture was left to polymerize overnight in a water bath at 37 °C. The LCST of the polymer was determined via turbidity measurement (cloud point determination)[29] using a UV−vis spectrophotometer fitted with a circulation bath and single cell Peltier accessory. The transmittance of the polymer solution (diluted 5 times with buffer A), contained in a 1 cm path glass cuvette at a fixed wavelength of 720 nm (due to light scattering), was recorded as a function of temperature (heating rate: 1 °C/min). The polymer solution was heated from 25 to 40 °C, and the LCST was taken as the temperature at which the solution transmittance reached 50%, which corresponds to ∼32 °C (Figure 4).

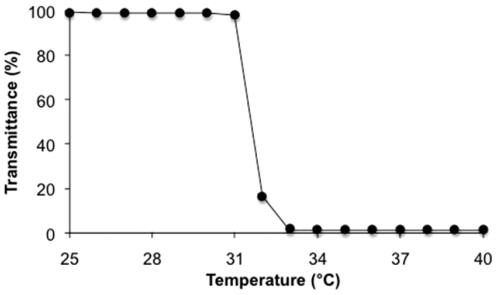

Figure 4. Transmittance−temperature curve of NIP-NPs in 25 mM MES buffer, pH 5.5.

Immobilization of IDA-Fe^{3+} Metal-Chelate on Glass Beads. All GBs were first activated by boiling in 4 M NaOH (typically 100 g of GBs in 100 mL of NaOH) for 10 min. After activation, they were washed with water and acetone and then dried in an oven at 50 °C. The GBs were then functionalized with (3-glycidyloxypropyl)-trimethoxysilane (Glymo) so as to introduce iminodiacateic acid (IDA), as previously described.[18] Briefly, 60 g of activated GBs was added to a mixture of 100 mL of anhydrous toluene and 100 mL of Glymo in a round-bottom flask and refluxed at 110 °C for 17 h. The Glymo-coupled glass beads (Glymo-GBs) were collected and washed with toluene and acetone, three times each solvent. After drying, the Glymo-GBs were immersed into 500 mL of a 0.75 M IDA solution (containing 0.34 M NaCl and 2.0 M Na_2CO_3, pH 11.0) and stirred at 70 °C for 17 h. The final GBs were obtained after washing five times with water.

Solid-Phase Synthesis of MIP-NPs for AMP. The solid-phase synthesis of MIP-NPs was carried out in a glass column equipped with a thermostated jacket (XK 26/40, GE Healthcare, Fontenay sous Bois, France), connected to a circulation thermostated water bath (Bioblock Scientific polystat 5, Fischer Scientific, France). All solvents were

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pumped through the column using a peristaltic pump at a flow rate of 2 mL/min. The column was packed with 20 g of functionalized IDA-GBs and washed with 10 volume column (VC) (200 mL) of water. Then 10 VC of iron(III) chloride (50 mM), prepared in water, was passed through the column. Unbound Fe^{3+} was removed by passing 10 VC water, and the column was equilibrated with 200 mL of buffer A.[21,30] Afterward, 20 mL of a solution of AMP (4 mg/mL buffer A) was loaded on the column. The flow-through was collected and passed through the column again for 1 h. Excess AMP was removed by passing 200 mL of buffer A, until the absorbance at 259 nm was close to zero.

The polymerization solution was prepared by mixing NIPAm (123.57 mg, 91 mol %), VBT (11.63 mg, 4 mol %), and EbAm (10.09 mg, 5 mol %) in 29.1 mL of buffer A. The solution was purged with nitrogen for 30 min. KPS (10.2 mg in 200 μL of buffer A)/TEMED (0.8 μL) was added, and this mixture was percolated through the column. The temperature was set to 39 °C, i.e., >LCST, for overnight. After polymerization, the column was washed with 150 mL (3 × 50 mL) of buffer A at 39 °C and then left to cool down to room temperature. The MIP-NPs were eluted with 20 mL (4 × 5 mL) of buffer A at room temperature.

Control nonimprinted nanoparticles (NIP-NPs) and MIP-NPs devoid of thymine group were synthesized using the same protocol as described above, except that AMP was not immobilized on the GBs and VBT was absent in the polymerization mixture, respectively. All eluted fractions were subjected to absorbance measurements at 259 nm for verifying eventual leakage of AMP by using its calibration curve (Figure S3A). The absence of Fe^{3+} (see protocol in the next section) was verified by using the calibration curve of the iron(III)−EDTA−H_2O_2−NH_3 complex at 513 nm (Figure S4).

Particle Size Determination. The hydrodynamic size of the MIP-NPs was measured directly on the eluate fractions from the column by dynamic light scattering (DLS) at 25 °C, using a Zeta-sizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK). DLS analysis was performed on all eluted fractions, and MIPs having similar sizes and dispersities (first three fractions, total 15 mL) were pooled. For transmission electron microscopy (TEM) imaging, an aliquot of the pooled fraction was dialyzed overnight against Milli-Q water. TEM images were captured using a JEM-2100F (JEOL, Japan). The TEM grid was a 300 mesh carbon-coated copper grid from AGAR Scientific (Stansted, UK).

Determination of Fe^{3+} in the Washing and Eluted Fractions. The determination of iron(III) was adapted from Poeder and co-workers.[31] 100 μL of each fraction was added to 790 μL of a 100 mM ethylenediaminetetraacetic acid (EDTA) solution, followed by 60 μL of H_2O_2 (35%) and 50 μL of ammonia solution (28%). The absorbance of the iron(III)−EDTA−H_2O_2−NH_3 complex which has a deep violet coloration was measured at 513 nm. A calibration curve prepared with 0.1−1 mM $FeCl_3$ in water in a final volume of 1 mL was used for quantification (Figure S4).

Equilibrium Binding Studies and Selectivity Test. A cleanup procedure of the MIP-NPs was essential before doing the binding experiments. The pooled MIP-NPs fractions eluted from the column (15 mL) were subjected to repeated (4 times) concentration and dilution with buffer A, using an Amicon Ultra-15 centrifugal filter unit with MWCO 30 kDa (Millipore, Molsheim, France) in order to remove tiny particles, which interfere with the absorbance values of the nucleotides, during read-out on a spectrophotometer. The final retentate was then diluted to 15 mL with buffer A, and this constituted our stock of working MIP-NPs. Its concentration (mg/mL) was determined by taking 2 mL and centrifuging at 40000*g* for 1 h at 40 °C to precipitate the polymer NPs. The centrifuge machine was left for 30 min to equilibrate to 40 °C prior to use. After discarding the supernatant, the precipitate was resuspended in 2 mL of water and lyophilized. The dry MIP-NPs were weighed with a precision balance; this allows to determine the concentration of the MIP-NPs (mg/mL) and the yield of polymerization, calculated as mg of MIP-NPs per g of GBs.

1 mM stock solutions of AMP, GMP, CMP, UMP, ATP, adenosine, and cAMP were prepared in buffer A and kept at 4 °C. The polymer particles (0.02−0.4 mg/mL) were pipetted in separate 2 mL polypropylene microcentrifuge tubes, and the analytes (final concentration 50 μM) were added. The final volume was adjusted to 1 mL with buffer A, and the mixture was incubated overnight on a tube rotator in a thermostated chamber at 40 °C. The samples were centrifuged at 41415*g* for 1 h, and the supernatant was placed in a quartz cuvette for recording of their absorbance. The amount of analyte bound to the polymers was calculated by subtracting the amount of unbound analyte from the initial amount of analyte added (λ_{max} = 259 nm for AMP, adenosine, ATP, and cAMP, λ_{max} = 252 nm for GMP, λ_{max} = 271 nm for CMP, and λ_{max} = 262 nm for UMP). The calibration curves of the different analytes are shown in Figure S3A−G.

3. RESULTS AND DISCUSSION

Initial Strategy Using Amidinium Moiety To Orientate Phosphate Groups. Because of their ubiquitous nature, nucleotides have relevant roles in biological processes and are among the most targeted anionic molecules in supramolecular chemistry for developing biomimetic artificial receptors.[1,2] Natural receptors like proteins selectively recognize the phosphate moiety of nucleotides via ammonium (lysine amino acid) and guanidinium (arginine amino acid). Therefore, polyammonium-based and more recently guanidinium-based host receptors targeting AMP, ADP, and ATP with high binding constants of up to 10^7 M^{-1} in aqueous media have been reported.[1,2] Like guanidine,[32] amidine groups are superbases with pK_a of ∼12 in water[33] and can form strong stoichiometric electrostatic interactions with carboxylates[34] and phosphates.[35] Recently, we have synthesized a benzamidine-based monomer ((4-acrylamidophenyl)(amino)methaniminium acetate (AB)) (Figure 3), which forms a 1:1 AB-carboxylate template complex with a high association constant (K_a > 900 M^{-1}),[34] in CD_3OD/D_2O (4/1), as determined by ^1H NMR spectroscopy.[25,36] The resulting MIPs displayed high specificity and selectivity for their target molecules in aqueous conditions.

Encouraged by the results obtained with carboxylate targets, we hypothesized that AB would also form strong interaction with phosphate moieties. Thus, in our initial work, we synthesized MIPs for AMP by precipitation polymerization using AB, VBT, and ethylene glycol dimethacrylate as functional and cross-linking monomers in CH_3OH/H_2O (4/1) as porogen (see details in Supporting Information). Thermal polymerization, instead of UV light, was employed to avoid photodimerization of the thymine moieties.[37] Prior to synthesis, the strength and stoichiometry of the AB−AMP complex were studied in CD_3OD−D_2O (4/1), using ^{31}P NMR spectroscopy (see details in Supporting Information). The Job plot and titration experiments predict the formation of a 1:2 AMP:AB complex (Figure S5B) with an overall binding constant, $\beta_{12} = K_{a1}K_{a2}$, of 300 M^{-2} (Figure S5C), where K_{a1} and K_{a2} are the association constants of the interaction of AB with the first and second interaction site of AMP, respectively. Unexpectedly, the association constant was relatively low (K_a must be ≥900 M^{-1} to establish 1:1 stoichiometric noncovalent interactions)[34] and suggests that the conformation adopted by AMP during interaction with AB may not completely favor binding of the phosphate with the amidinium moiety but might form weaker Π−Π interaction between the aromatic ring of AB with the nucleobase of AMP. VBT contains a complementary thymine residue and was included in the polymerization mixture to mimic the base pair in DNA and hence favor pairing with the adenine of AMP by hydrogen bonding. It was synthesized from thymine and 4-vinylbenzyl chloride in one step, with a yield of 47%, using a procedure adapted from a

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previous report[22] (Figure 2A). The resulting MIPs were very specific for AMP (negligible NIP binding) but were not at all selective since CMP and GMP were bound to the same extent as AMP in water (Figure S6A). This nonselectivity indicates that pairing was not or poorly achieved. The reason, as suggested above, might be due to nonspecific interactions like Π−Π binding, which occurs between AB and the two nucleobases. By using a solid-phase strategy (Figure S1) where the immobilized amidinium moiety was first constrained to present an orientation favorable for phosphate interaction with AMP, then followed by subsequent interaction of VBT to react with the nucleobase of AMP, improved the selectivity to some extent; the MIP did not bind CMP at all, but bound GMP and AMP, though AMP to a higher extent, indicating that the MIP could not distinguish between the two purine nucleotides (Figure S6B).

We then switched to another affinity ligand, devoid of aromatic groups. The new ligand was based on immobilized Fe(III) ion which enabled the coupling of the nucleotide via its phosphate group.[21]

Solid-Phase Synthesis of MIP-NPs Using IDA-Fe^{3+} as Affinity Ligand. Figure 1 shows the steps used for the immobilization of AMP on GBs. Briefly, GBs were activated by boiling in NaOH, followed by silanization with Glymo and coupling with IDA, as previously described.[18,38] IDA-GBs were then packed in a column, equipped with two adapters for regulation of the bed volume and a thermostated jacket. A solution of 50 mM $FeCl_3$ in water was then pumped through. After washing the excess Fe^{3+} with water and equilibration with buffer A, AMP was loaded on the column. AMP adsorbs to immobilized Fe^{3+} at pH 5.5, through its free terminal phosphate group.[21] The strong affinity of Fe^{3+} for phosphorylated biomolecules (peptides, proteins, amino acids) was already reported.[23,30] At this stage, it is important to point out that phosphate buffer, which was precedently employed as equilibrium and working buffer,[17−19,38] was substituted here by MES buffer to avoid interference of the phosphate groups of the buffer with that of AMP for binding with Fe^{3+}.

A polymerization mixture, composed of NIPAm, VBT, and EbAm (91/4/5, molar ratio) and the initiator system (KPS, TEMED) in buffer A, was then passed on the column. NIPAm is a functional monomer,[39] capable of hydrogen bond interaction, due to the presence of oxygen and nitrogen atoms[40] but at the same time was used herein as the major component in this polymer recipe so as to obtain thermoresponsive MIP-NPs. The lower critical solution temperature of the polymer had been previously determined via turbidity measurement (cloud point determination) by measuring the transmittance versus temperature using a UV−vis spectrophotometer and found to be ∼32 °C (Figure 4). The imprinted polymer was synthesized at 39 °C, and the growing polymeric nanoparticles, which are in the collapsed state at this temperature, encapsulate the immobilized AMP during polymerization.

After polymerization, the column reactor was washed with buffer A at 39 °C to remove unreacted reagents and particles that polymerized distantly from the immobilized template. The reactor was then washed with the same buffer at room temperature (25 °C), allowing the MIP-NPs to swell and be eluted. The yield of polymerization of the MIP-NPs was 0.65 ± 0.17 mg per g of GBs (n = 3), comparable to values previously obtained with MIP-NPs synthesized by the solid-phase method, using Glymo coupling and metal-chelate as affinity ligand.[18,38]

No leakage of Fe^{3+} and AMP was detected in the MIP-NPs (limit of quantification (LOQ): 100 μM for Fe^{3+} (Figure S4) and 5 μM for AMP (Figure S3A)). Control NIP-NPs and MIP-NPs without VBT were synthesized following the same protocol as described above but in the absence of AMP and in the presence of AMP but without VBT in the polymerization mixture, respectively.

Physicochemical Characterization of MIP-NPs. MIP-NPs were obtained as a transparent solution, and no aggregation was observed for a period of six months when stored in a refrigerator. The hydrodynamic size and morphology of the MIP-NPs were analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Figure S7), respectively. The diameters of MIP-NPs, NIP-NPs, and MIP-NPs without VBT were 55.9 ± 2.6, 57.5 ± 0.8, and 69.8 ± 4.7 nm (n = 3), with a polydispersity index (PDI) of ∼0.3, respectively, as measured by DLS at 25 °C. The MIP-NPs appear as spherical particles in the TEM image.

Evaluation of the Binding Characteristics of MIP-NPs. The recognition behavior of MIP-NPs for AMP (50 μM) was determined by equilibrium binding studies in free solution. Figure 5A shows the high specificity of the MIP as no binding

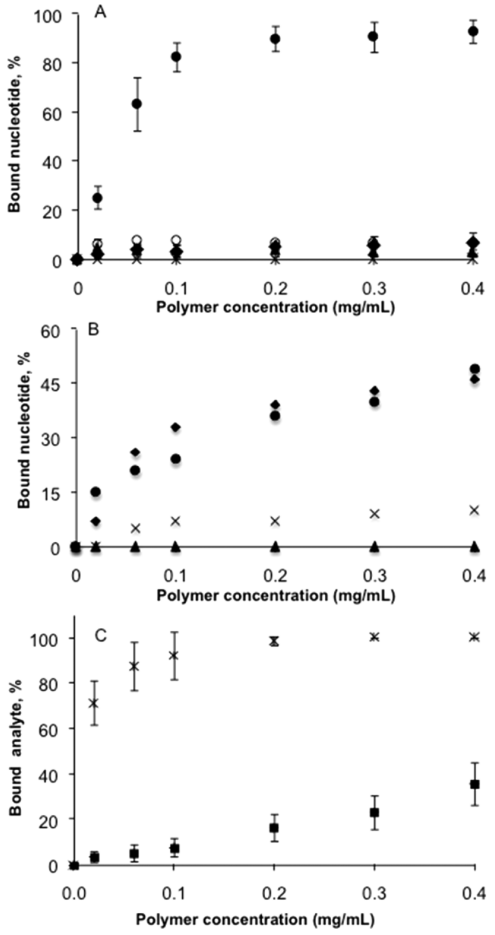

Figure 5. Equilibrium binding isotherms in buffer A of 50 μM AMP (circle), GMP (diamond), CMP (triangle), and UMP (cross) on MIP-AMP (filled) and NIP-AMP (open). Polymers were obtained in the presence (A) and absence (B) of VBT; (C) adenosine (star) and ATP (square). Data are means from three independent experiments with three different batches of polymers. The error bars represent standard deviations.

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of the NIP toward AMP was observed. The MIP was also very selective toward AMP as it did not bind GMP, CMP, and UMP. This selectivity is remarkable as the two purine nucleobases, AMP and GMP, are quite similar in size, shape, and hydrophobicity. It is important to note that the selectivity was partially lost when VBT was not present in the polymerization mixture (Figure 5B). The MIP could not differentiate between AMP and GMP, but it did not bind CMP or UMP, indicating that the thymine moiety in the polymer plays an essential role in the recognition procedure. The binding capacity of the MIP for AMP was determined by plotting a graph of B (bound) versus F (free) (Figure S8). The nonlinear fitting of the data to a single-site Langmuir binding isotherm showed a maximum binding capacity (B_{max}) of 1350 nmol/mg of MIP and a dissociation constant (K_d) of 74.5 μM, indicating that high binding capacity and affinity were achieved by using the dual-oriented solid-phase imprinting strategy.

Interestingly, the MIP could recognize adenosine (Figure 5C) to the same extent as AMP. Adenosine plays an important role in neuroprotection and brain functions activity and is a biomarker of cancer.[8,24] On the other hand, negligible and no binding were observed with ATP (Figure 5C) and cAMP, respectively. The bent conformation adopted by ATP due to its three phosphate groups and the additional phospholinkage in cAMP, probably prevent the fitting of their adenine base with the thymine group within the MIP. Furthermore, the two −OH groups of the ribose moiety are very apt to form hydrogen bonding with NIPAm[40] which gave additional structuration to the binding cavity, explaining why cAMP cannot go in.

4. CONCLUSIONS

Template-free water-soluble MIP-NPs targeting AMP were prepared by solid-phase synthesis using for the first time immobilized Fe(III)-chelate as affinity ligand to attract the AMP by its phosphate group. A polymerizable thymine monomer, which is complementary to the adenine moiety of the nucleotide, was synthesized and incorporated in the MIP polymerization mixture so as to constrain the AMP in a dual-orientated configuration. The resulting MIP was very selective toward AMP as it did not bind other nucleotides, GMP, CMP, and UMP in buffer. This remarkable selectivity of the MIP for its target molecule in the presence of surrounding water molecules and counterions which compete with the donor and acceptor sites indicates the creation of high fidelity binding sites within the polymer. Noteworthy is the fact that the choice of the affinity ligand is of prime importance as immobilized *p*-aminobenzamidine which contains an aromatic moiety did not lead to selective MIPs, probably due to Π−Π stacking with the nucleobase of AMP.

Interestingly, the MIP could bind adenosine, AMP without the phosphate group, to the same extent as AMP. Using the phosphate group of the AMP as a hinge enables free pairing of the nucleobase with a complementary base-pair residue monomer. This strategy can be easily extended to the preparation of selective water-soluble MIPs for other nucleotides, nucleosides, and potentially oligonucleotides, provided that there is no limitation of the synthetic availability of the complementary polymerizable base monomer. We believe that the development of cost-effective and chemically and physically stable artificial receptors like MIPs will be of great interest to different research areas of medicine, biochemistry, and separation science for recognition, sequestration, detection, and delivery of nucleotides and nucleosides in aqueous media.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.7b01782.

Immobilization of PAB on GBs, solid-phase synthesis of MIP-NPs for AMP, on PAB-immobilized GBs and equilibrium binding studies of these MIP-NPs with AMP and other nucleotides, ^{31}P NMR studies of AB−AMP complex, ^{1}H NMR spectrum of VBT, calibration curves of nucleotides and $FeCl_3$, determination of size, morphology, and binding capacity of MIP-NPs obtained on IDA-Fe^{3+} (PDF)

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Notes

The authors declare no competing financial interest.

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