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Molecularly imprinted polymer of 5-methyluridine for solid-phase extraction of pyrimidine nucleoside cancer markers in urine

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ABSTRACT

Normal and modified urinary nucleosides represent potential biomarkers for cancer diagnosis. To selectively extract modified nucleosides, we developed a molecularly imprinted polymer (MIP) of 5-methyluridine as selective material for molecularly imprinted solid-phase extraction (MISPE). The MIPs were obtained from vinyl-phenylboronate ester derivative of the template, acrylamide and pentaerythritol triacrylate co-polymer, and were tested in batch and cartridge experiments with aqueous samples. Our results indicated that the imprinted polymer was selective for pyrimidine nucleosides with a K_d and a B_{max} of 46 μM and 18 μmol/g, respectively. Finally, a MISPE of the most common pyrimidine nucleoside cancer markers in urine sample was realized.

1. Introduction

The hydrolytic action of *ribonucleases* and *phosphatases* liberates normal and modified nucleosides during the ribonucleic acid (RNA) turnover. This is made post-transcriptionally by specific enzymes such as *RNA-methyltransferases* and *RNA-synthetases*[1] and leads to structural modifications of the nucleosides such as alkylation of the heterocycle, methylation of the sugar, heterocycle isomerization, reduction, thiolation, and deamination. These compounds cannot be used for the de novo RNA synthesis and thus circulate freely in blood before elimination in urines by the same excretion pathway than normal nucleosides.[2] It has been shown that urinary levels of natural and modified nucleosides in patients with stage 2–4 cancer were significantly elevated compared to human healthy as shown for leukemia, lymphoma,[3] lung cancer,[4] oesophagus,[5] breast,[6] and renal cell carcinoma.[7] RNA and particularly *t*RNA is a primary source of the modified nucleosides found in urine. A total of 93 natural and modified nucleosides including cytidine (**1**), uridine (**2**), guanosine (**3**), adenosine (**4**), 3-methylcytidine (**5**), 5-methylcytidine (**6**), *N4*-acetylcytidine (**7**), or pseudouridine (**8**) have been identified (Fig. 1), the later been found to be between 3 and 6 times more excreted in patient with severe combined immunodeficiency diseases, breast or ovary cancers, respectively.[8,9]

Thus, the quantitation of normal and modified urinary nucleosides should allow a premature cancer diagnosis and a monitoring during its evolution. During the last decade, we and others have used various techniques such as enzyme-linked immunoassay,[10] capillary electrophoresis,[11] cathodic stripping,[12] HPLC–MS,[13] or GC–MS[14] to analyze nucleosides in biological fluids. A common limitation is the pre-treatment of the biological sample, which can include an extraction, a hydrolysis or a derivation step and is prohibitive in terms of time and cost when applied to a large number of samples as in diagnosis bio-analyses.

In the present study, we have used the molecularly imprinting polymer (MIP) technology to set up a molecularly imprinted solid-phase extraction (MISPE) for selective extraction from urine of endogenous and modified pyrimidine nucleosides. The MIP concept, historically introduced by Wulff[15] and Mosbach[16] are speciality polymers generated via the interaction of functional monomers with a target molecule (template) and a cross-linking agent.[17] With the non-covalent self-assembly approach, the template, functional monomer, and cross-linking agents are equilibrated to generate a pre-polymerisation cluster utilizing hydrogen bond interactions, electrostatic attraction, and associated weak interactions. The mix is then polymerized to generate cavities and the template is subsequently removed via an effective extraction.

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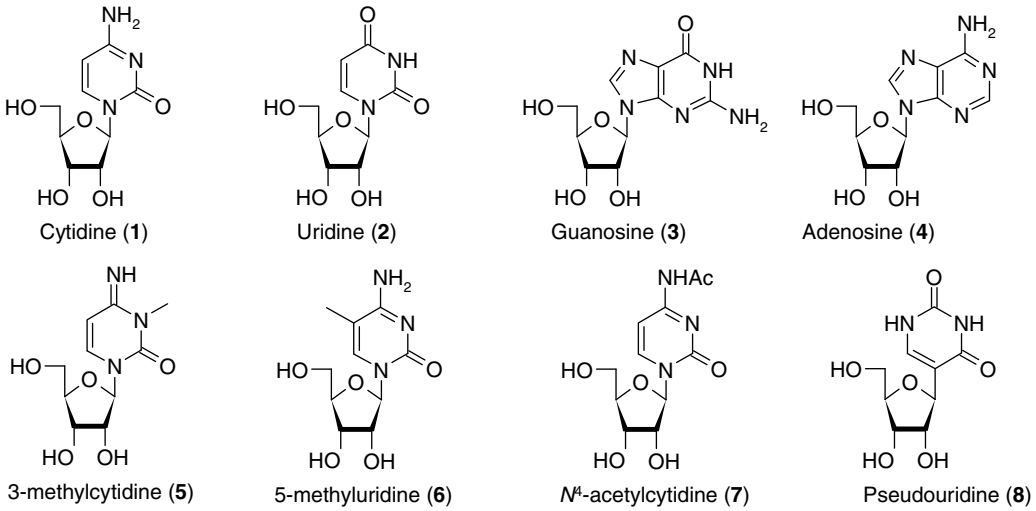

Figure 1. Studied natural and modified nucleosides.

The obtained cavities are supposed to have a complementary shape and electronic affinity to the template. For the imprinting of polar compounds, the classical approach consists in the derivation of polar compounds into less polar analogues, such as acetylated galactosides,[18] acetylated nucleosides and nucleotides.[19,20] Other polar molecules like *p*-nitrophenyl-α-β-L-furanoside[21] have been imprinted by a non-covalent approach as a cross-linked hydrogel.[22–24] For the unprotected sugar extraction, Wulff et al. have reported a covalent approach using a boronic acid ester of α-D-mannopyranoside as template.[25–28] The property of a boronic acid to bind diols has been also used to prepare polymeric gels for nucleotide adsorption using a boronic acid–hydroxyethylmethacrylate co-polymer.[29] MIP were developed as sensors on a quartz crystal microbalance electrode[30,31] or directly on an electrode[32] for the detection of adenosine monophosphate (AMP). Other pyrimidine derivatives have been imprinted by Hall et al.[33] using 2,6-bis(acrylamido)pyridine as monomer,[33–35] and a low complexation was exhibited toward uridine.[36]

Thus, the preparation of highly cross-linked non-polar polymer having a strong affinity for polar compounds (such as nucleosides) is still challenging. Starting with the 5-methyluridine (**9**) as dummy template (i.e., a template structurally similar to the compounds to be analyzed but different enough not to interfere with them), we have synthesized the corresponding polymerizable 2,3-diol boronate ester (**10**). We then turned our attention to complex the pyrimidine moiety by specific hydrogen bonds. The efficiency of the obtained polymer was evaluated as MISPE material for the selective extraction of selected natural and modified pyrimidine nucleosides in urine.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **10**, **11a**, and **12** (Fig. 2) was realized in our laboratory meanwhile acrylamide (**13**) and cross-linkers (**15–17**) were commercially available. These reagents were used to synthesize six MIPs with different compositions as summarized in Table 1.

2.2. Choice of monomers and NMR studies

To imprint the 5-methyluridine (**9**), we decided to covalently bind the ribose moiety applying the boronic acid methodology and the heterocycle by hydrogen-bonding interactions. To complex the nucleobase, several monomers have been reported such as the acrylic acid,[37,38] the acrylamide[39,40] (reported to induce a better affinity than methacrylic acid),[41] the 2,6-bis(acrylamido) pyridine.[36,42] In our case, we have synthesized the 2,6-bis(methacrylamido) pyridine monomer (**11a**) because of a lower tendency of self-association compared to the 2,6-bis(acrylamido) pyridine derivative (**11b**).[35] It is also a less reactive polymerizable monomer compared to the acrylamide analogue which contains two vinyl groups. In order to mimic the natural base-pairing interactions between adenosine and uridine, the 2,6-diamino-9-(3/4-vinylbenzyl) purine monomer[33] (**13**) was also evaluated. ^{1}H NMR titrations were conducted in order to monitor hydrogen bond interactions between the functional co-monomer (host) and compound **10a** (guest) by increasing the amount of host into a constant amount of guest. The complexation of **10a** with MAP (**11a**), DAVBP (**12**), and AA (**13**), respectively, induced a variation of the chemical shift of the imide proton of **10a** as reported in Figure 3.

Based on the literature on host–guest systems,[43–46] we assumed to have a 1:1 stoichiometry for the association. As previously published, the best complexing site for uracil moiety is the acceptor–donor–acceptor (ADA) triad obtained from the groups at *C*2, *N*3, and *C*4 position.[35,38,47] From the non-linear curve fitting of isoterms,[48] we can extract the apparent association constants (K_{app}) and the maximum complexation induced shifts (CISs), (see Table 2).

As shown with the K_{app} values, **10a** has a better association with acrylamide (**13**) than with DAVBP (**12**) or MAP (**11a**). The K_{app} for **11a** was at least twice lower than for **13**. These data were not in agreement with those reported for the association of *N*1-benzyluracil[33,35] with 2,6-bis(acrylamido)pyridine monomer (**11b**), which was found to exhibit a better interaction than the acrylamide (**13**) with the ADA system of uracil. The steric hindrance brought by the two methyl groups of **11a** but also found at the *C*5-position of the heterocycle and at the 2′,3′-position of the sugar could explained this result. The acrylamide (**13**), which has the biggest K_{app}, can interact with the nucleobase by two complexes (at *C4*–*N*3 or *C*2–*N*3), as reported for *N*1-methyluracil.[41] The maximum complexation induced shifts (CIS) is related to the length of the hydrogen bond and the dihedral angle of the bond.[49] From Table 2, it can be seen than the geometry of the complex with DAVBP was different than the two others since no self-association was detected (results not shown).

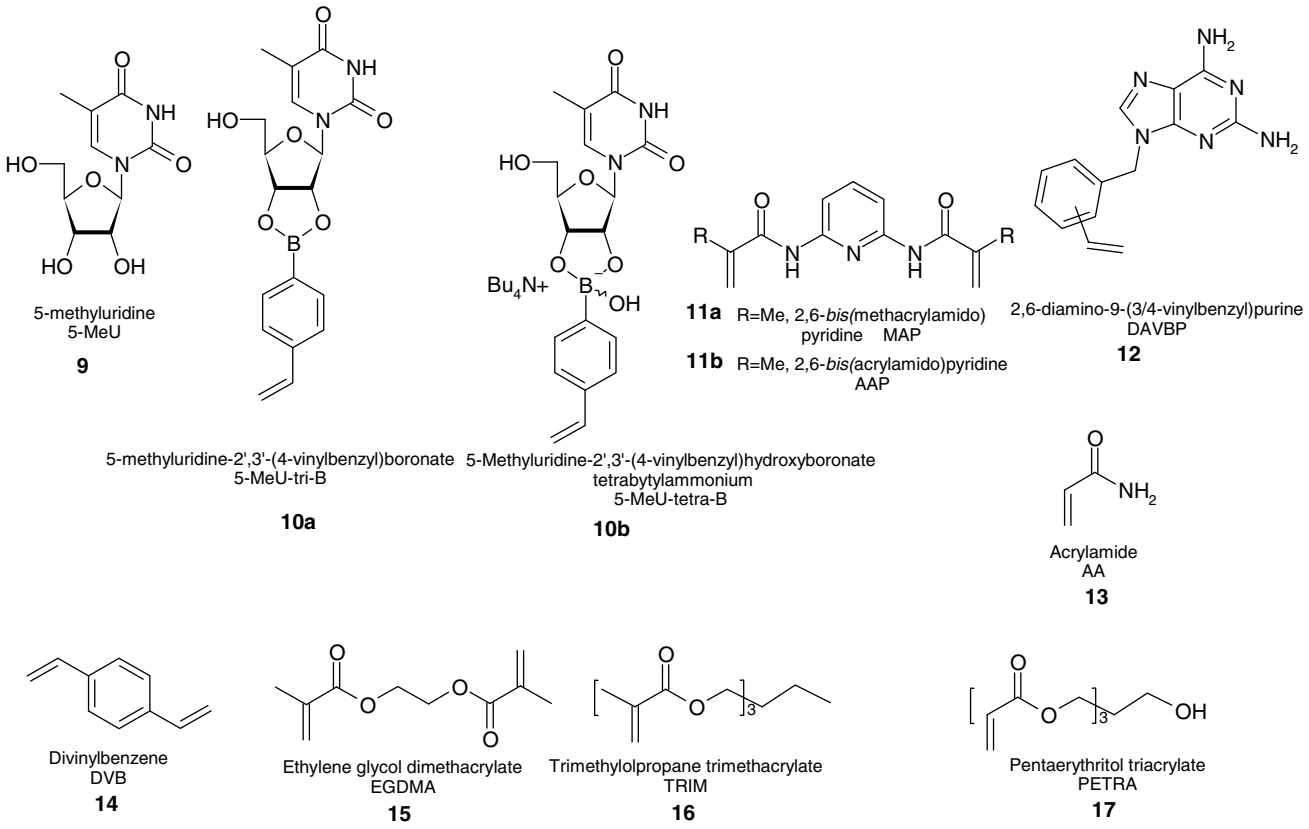

Figure 2. Templates, functional monomers, and cross-linkers used for MIP optimization.

Table 1
Composition of the polymers synthesized[a]

Polymer	A	B	C	D	E	F
Monomer (quantity)	MAP (2 mmol)	DAVBP (2 mmol)	DAVBP (2 mmol)	AA (4 mmol)	AA (4 mmol)	AA (4 mmol)
Cross-linker (20 mmol)	EGDMA	EGDMA	DVB	EGDMA	TRIM	PETRA
Porogen volume (MeCN) (mL)	3.6	5.0	3.3	3.2	3.2	3.2

[a] Each polymers contain 1 mmol of template (**10**) and AIBN mass was 40 mg (for abbreviation see Fig. 2).

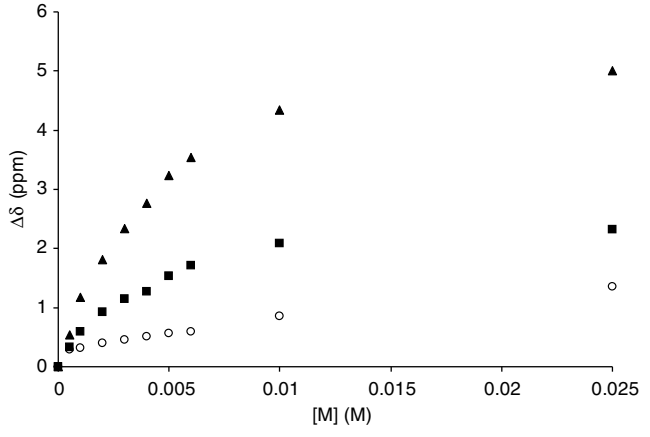

Figure 3. ^{1}H NMR complexation induced shifts ($\Delta\delta$) of the imide proton of **10a** versus total concentration of **11a** (open circles), **12** (filled squares), or **13** (filled triangle) in $CDCl_3$.

2.3. MIP synthesis

MIPs A–D were synthesized in the same conditions and varied from each other only by their composition (see Table 1). The rebinding studies (Table 3) were conducted following a protocol described in Section 4.6.

Table 2
Apparent association constants (K_{app}) and maximum complexation induced shifts ($\Delta\delta_{max}$) of the imide proton of **10a**

Functional monomer	K_{app} (M^{-1})	$\Delta\delta_{max}$ (ppm)
MAP (**11a**)	116 ± 36	1.75 ± 0.27
DAVBP (**12**)	271 ± 15	5.87 ± 0.13
AA (**13**)	312 ± 29	2.68 ± 0.09

From Table 3, the better rebinding was observed with the DAVBP-based MIP (polymer B) and not with the AA-based MIP (polymer D) as it could be expected from NMR data. Since the rebinding experiments were conducted in water and the ^{1}H NMR experiments in $CDCl_3$, the solvent should strongly disrupt the intramolecular hydrogen-bonding interactions. The major rebinding effect in water was due to the covalent bonding of 2′,3′-diol moiety of nucleosides with the boronic acid moiety, in agreement with the literature.[50] However, selectivity between uridine and cytidine derivatives was observed, in agreement with ^{1}H NMR experiments. It is interesting to note that no selectivity for the heterocycle was observed when a commercially available phenylboronic acid material was used.

Table 3
Rebinding assays on the synthesized MIP and NIP[a]

Rebinding (%)	A		B		C		D		E		F	
	MIP	NIP	MIP	NIP	MIP	NIP	MIP	NIP	MIP	NIP	MIP	NIP
Uridine	64	5	81	10	21	8	55	7	38	6	71	3
Cytidine	67	7	86	11	35	6	38	3	19	5	57	2
Guanosine	85	6	96	26	51	20	71	22	40	10	88	6

[a] Error on each point was less than 3%, $n = 3$, (see Table 1 for composition).

The polarity of the cross-linker was also investigated. The difference of rebinding rate between EGDMA-based MIP (polymer B) and DVB-based MIP (polymer C) highlighted the importance of the polarity of the material. Polymer C using DVB (**14**) as cross-linker was less polar than polymer B based on EGDMA (**15**) and exhibited a lower affinity (21%) for polar uridine compared to polymer B (81%). The best specificity for uridine, cytidine, and guanosine, was obtained using a AA-based MIP (polymers D–F). It has been shown that trimer cross-linkers (such as TRIM (**16**) and PETRA (**17**)) can increase the selectivity of the rebinding step.[51–54] TRIM- and PETRA-based MIP (polymers E and F, respectively) raised selectivity of MIP, with very good rebinding rate for the more polar PETRA cross-linker. Thus, we have shown that for the MIP of nucleosides, the rebinding and the selectivity were mainly governed by the polarity of the monomer and the cross-linkers. For highly polar compounds, the hydrogen-bonding capabilities were not the unique source of selectivity. Finally, based on the targeted specificity, the polymer F (AA-PETRA-based MIP) was chosen for all further experiments. The obtained hard porous materials have a surface area of 321 and 337 $m^2 g^{-1}$ for MIP and NIP respectively, with a cauliflower form (Fig. 4). Their degree of swelling in volume in water was estimated to be approximately 10%.

2.4. Influence of the nature and concentration of the counter-ion

Since 5-methyluridine (**9**) was used as the dummy template, all further experiments were carried out with uridine (**2**). In anhydrous aprotic solvents, a boronic acid derivative forms with 1,2-diol a trigonal boronate ester (**10a**). In aqueous solution, trigonal boronate is unstable and is either hydrolyzed to the starting compounds or ionized to form an anionic tetrahedral boronate (**10b**).

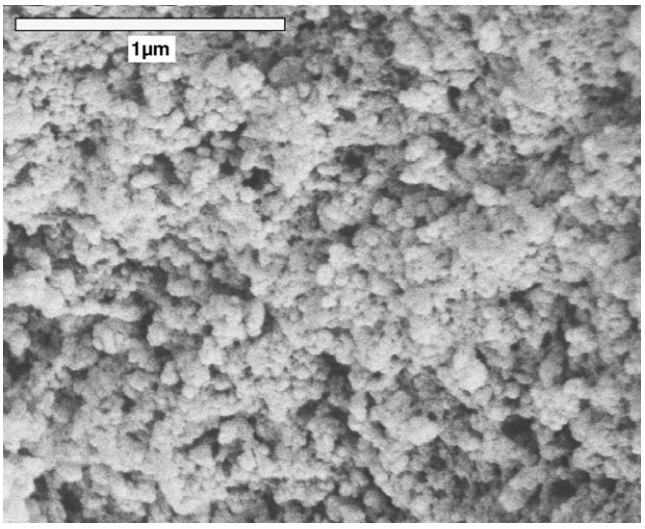

Figure 4. SEM image of the obtained MIP.

Under acidic and neutral pH, the trigonal **10a** is major whereas tetrahedral **10b** is mainly observed for pH > pK_a (e.g., 8.8).[55] It is only under this last conformation (tetrahedral) that the diols can be well complexed.[56] The boronate ester bond is thus pH dependent and is stable in aqueous solutions at pH above 9, so all further experiments were achieved at pH 10.

The first unsuccessful assay of rebinding experiments was done with a 0.1 M sodium hydroxide solution adjusted to pH 10 with 0.1 M HCl. Following the works by Smith et al.,[57] *tetra*-butyl ammonium hydroxide (TBAH) was added in the rebinding solutions (in 1000/1 molar ratio versus the rebound compound) and under this condition, 74% of uridine (**2**) was rebound within 24 h. The *N*3 of uridine has a pK_a value of 9.5,[58] so at pH 10 the heterocycle is negatively charged. At that pH, uridine forms an ion pair with the quaternary ammonium. The access of the nucleoside to the cavity of the polymer could be thus facilitated by an ion pairing mechanism. This was confirmed by the absence of complexation observed when sodium hydroxide was only used. We then turned our attention to the length of the alkyl chain of the quaternary ammonium ion (Fig. 5A). As expected, the longer the alkyl chain, the higher the rebinding capacity of uridine. When the chains became longer than butyl, problems of solubility in water and loss of rebinding rate occurred. The last studied parameter to show the ion pairing behavior of the uridine binding was the influence of the counter-ion concentration (Fig. 5B). As expected, an increase of the concentration of TBAH led to an increase of the rebinding of the MIP. With concentrations higher than a molar ratio of 1000/1, a loss of selectivity can be observed. Under these conditions, the rebinding was almost immediate (data not shown).

2.5. Isotherm

The Scatchard representation was next explored to determine the number and type of binding sites.[34] The Scatchard plot of MIP F displayed a strongly curved shape, reflecting that uridine was adsorbed on either imprinting sites or non-imprinting sites, (Fig. 6). In fact, the Scatchard plots indicate that MIP F shows high and low concentration regimes that are nearly linear, interconnected with strongly curved regions in the mid-concentration range. As the Scatchard plot for NIP is curvilinear, it indicates that a one site model should afford a good fit.

B_{max} and K_d constants were determined according to Scatchard's representation (Table 4). B_{max} represents the number of sites on the polymer and K_d the affinity for it. K_d values for both sites highlight the interaction between the polymer and uridine; a high affinity is correlated with a low K_d. Since covalent rebinding is stronger than hydrogen-bonding interactions, it is clear that the high affinity sites come from boronate ester and by the way from selective sites. The second sites (low affinity sites) probably come from the excess of acrylamide introduced in the formulation of the MIP. As expected from the excess of acrylamide, we can observe that there were twice more non-selective sites than selective ones. Nevertheless, the excess of acrylamide is necessary to favor the hydrogen-bonding interactions.

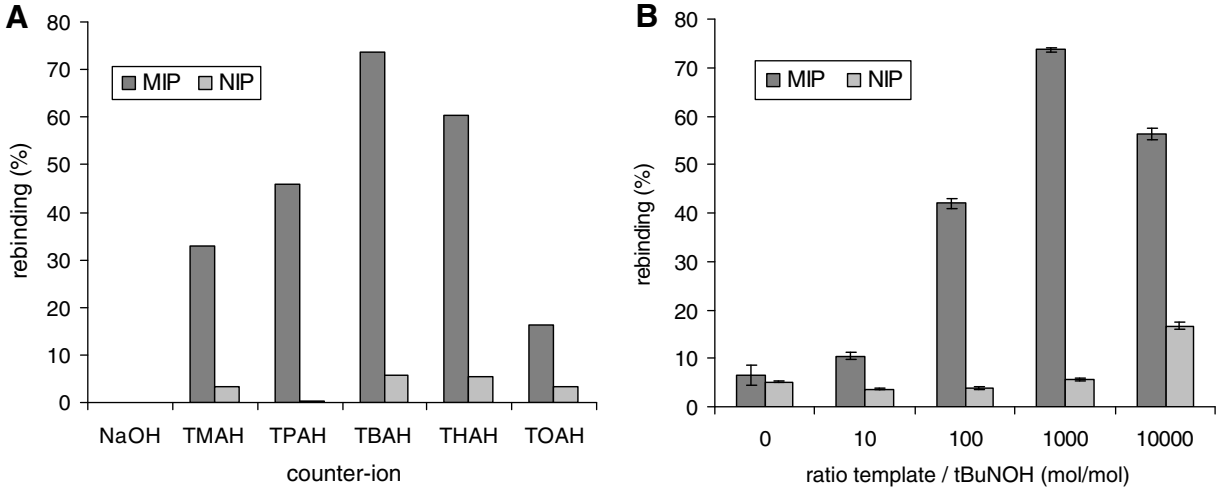

Figure 5. Influence of the nature (A) and the concentration (B) of the counter-ion on the rebinding of uridine. TMAH, (Me)4NOH; TPAH, (Pr)4NOH; TBAH, (*n*-Bu)4NOH; THAH, (*n*-Hex)4NOH; TOAH, (*n*-Oct)4NOH.

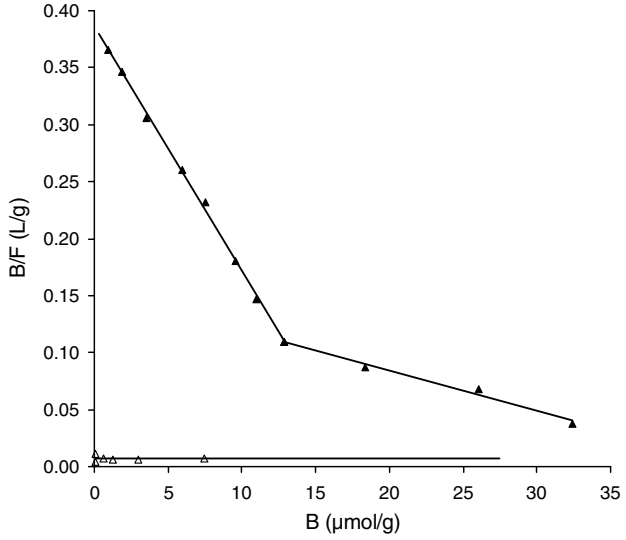

Figure 6. Scatchard representation of the binding of uridine on MIP F (filled triangle) and NIP F (open triangle).

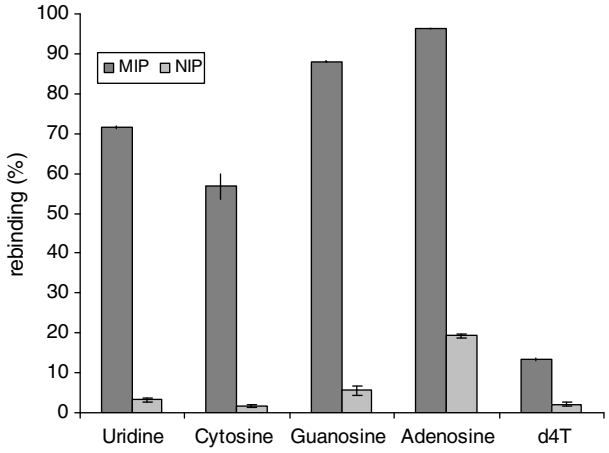

Figure 7. Rebinding rate of different nucleosides on MIP F and NIP F packed in a SPE cartridge (100 mg).

Table 4
Constants from Scatchard plot for high and low affinity sites on MIP F

High affinity sites		Low affinity sites	
B_{max} (μmol/g)	K_d (μmol/L)	B_{max} (μmol/g)	K_d (μmol/L)
18	46	44	283

With those optimized conditions for the MIP preparation, we have evaluated this material in a cartridge for solid-phase extraction of nucleosides of interest (MIPSE).

2.6. MIP selectivity

The imprinting effect can be observed when non-specific interactions between the template and the MIP are decreased. We firstly evaluated the rebinding of some natural nucleosides, for example, cytidine (**1**), uridine (**2**), guanosine (**3**), and adenosine (**4**), on MIP and NIP packed in a SPE cartridge (100 mg) (Fig. 7). The 3′-deoxy-2′,3′-didehydrothymidine (d4T), a nucleoside lacking the 2′,3′-diol groups, were also evaluated. It appears that purine nucleosides—guanosine (**3**) and adenosine (**4**)—were more retained on the MIP than pyrimidine nucleosides—cytidine (**1**) and uridine (**2**). These data could be explained by the difference of polarity of the selected nucleosides as well as by non-specific interactions. Uridine (**2**) was more retained compared to cytidine (**1**), which was in opposition with the log*P* values at pH 10 (−2.88 for **2** and −2.05 for **1**), respectively). These data showed an imprinting effect for compounds with similar polarities. Moreover, the contribution of the boronate ester bonds was clearly demonstrated by the poor rebinding of d4T. Finally, nearly no rebinding was observed on NIP.

2.7. MISPE of selected nucleosides

In order to optimize the MISPE conditions, a first experiment was done with the selected nucleosides in water sample (Fig. 8). Typically, 10 μg of selected nucleosides in water (1 mL) were mixed with 25 mM acetate buffer pH 10 and TBAH and then percolated on 100 mg of the 5-methyluridine–MIP cartridge. The same experiment was carried out on NIP. A first TBAH washing was done with buffer in order to elute the unbound polar compounds. A second washing was done with acetonitrile to (a)

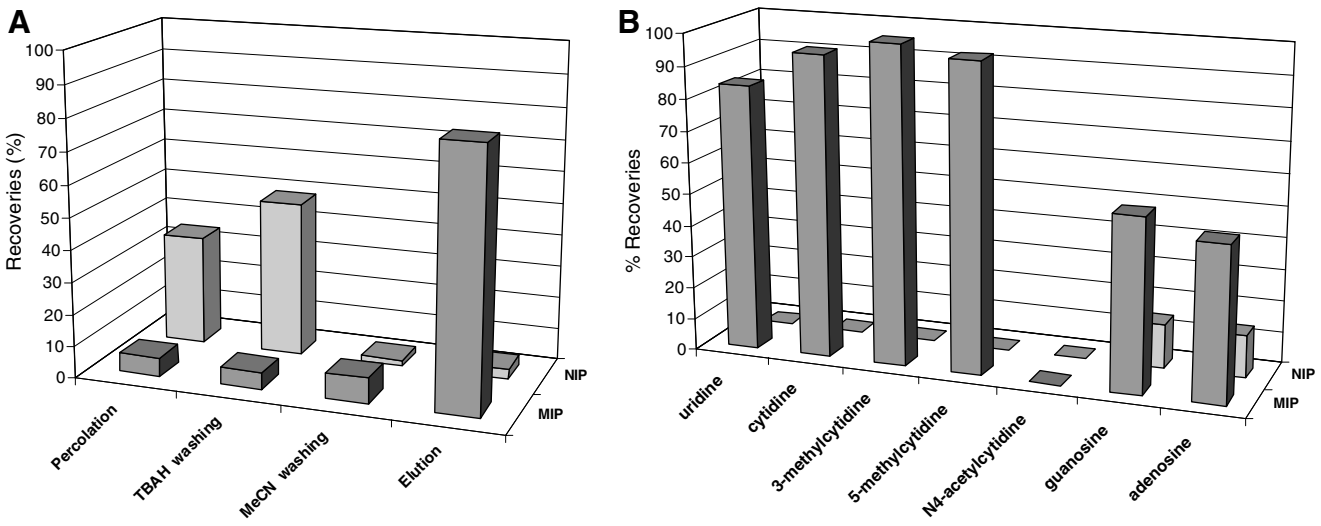

Figure 8. Specificity of MIP F in MISPE experiment. (A) Elution profiles obtained for 100 mg of 5-methyluridine MIP F and NIP and (B) recoveries for other nucleosides in the same conditions. For conditions, see Section 4.

remove unbound non-polar compounds and (b) to increase the selective interactions by favoring the hydrogen bounding at the heterocycle moiety. Then, 1 mL of water/methanol/acetic acid (50:45:5) was percolated to release the adsorbed nucleosides. At each steps, the collected solutions were carefully analyzed by LC–UV. Figure 8A represents the elution profile obtained for uridine (**2**) on MIP F and NIP. The extraction recoveries were 80% for the MIP while only 3% for the NIP. This process was applied to other natural and modified nucleosides (Fig. 8B). The extraction recoveries for all cytosine nucleosides (**1**, **5**, and **6**) were quantitative excepted for *N*4-acetylcytidine (**7**), which at pH 10, was hydrolyzed to cytidine (**1**). For all other nucleosides, the extraction recoveries were ranging from 49% to 80%. This data emphasized the selectivity of the prepared MIP for pyrimidine nucleosides compared to purine ones.

Finally, the extraction from urine of selected nucleosides, including modified nucleosides as cancer markers was realized by the prepared MIPSE. The pH value of urine being around 6, it is necessary to adjust to pH 10 by a buffer solution of ammonium acetate. TBAH was also added to the sample in order to load the ion pair on the MISPE cartridge. Figure 9 reports the UV chromatograms corresponding to the injections of the standard mixture (Fig. 9A), spiked-urine with nucleosides (Fig. 9C) and MISPE extracted nucleosides from spiked-urine (Fig. 9B). As shown in Figure 9C and B, the nucleosides were strongly extracted (thus retained) by the prepared MISPE. In Fig. 9B, the increase of peak 1 corresponding to the cytidine (**1**) is due to the hydrolysis of *N*4-acetylcytidine (**2**).

3. Conclusion

For the first time, this study described the synthesis of a highly specific molecularly imprinted polymer of pyrimidine nucleosides. The role of the polarity of polymer has been here

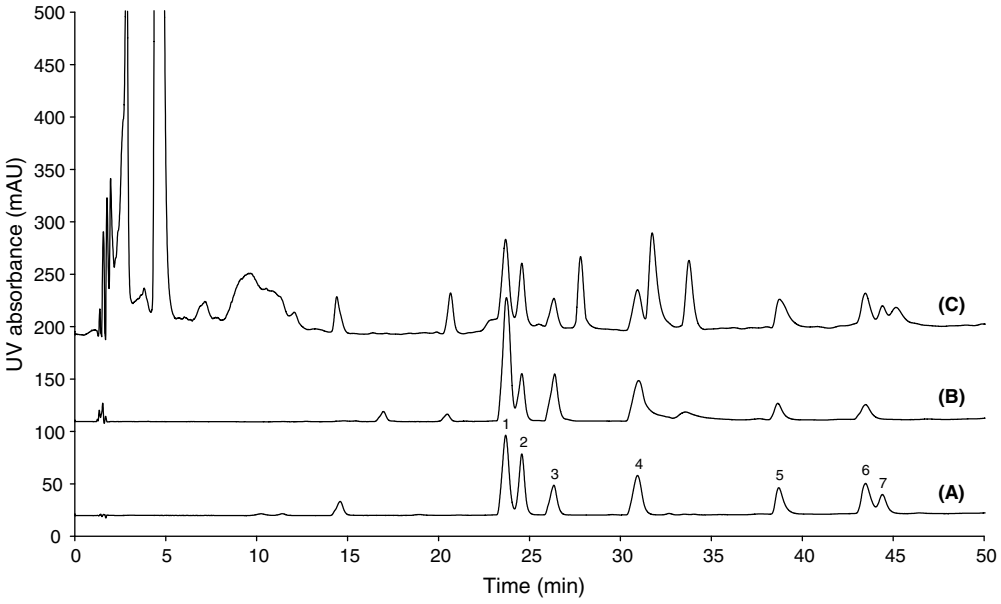

Figure 9. Chromatograms corresponding to (A) standard solution (1 ppm), (B) elution profile after MISPE from urine-spiked, and (C) urine-spiked with 1 ppm of nucleosides. Peak identification: **1**, cytidine; **2**, uridine; **3**, guanosine; **4**, adenosine; **5**, 3-methylcytidine; **6**, 5-methylcytidine; **7**, *N*4-acetylcytidine. For conditions, see Section 4.

clearly highlighted and the use of an ion pairing agent to increase the rebinding has been depicted closely. Finally, the use of this polymer as stationary phase for MISPE has been demonstrated. The potential of MISPE for the direct extraction of cancer marker pyrimidine nucleosides from urine has then largely demonstrated.

4. Experimental

4.1. Materials and reagents

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), azo-bis-isobutyronitrile (AIBN), methylmethacrylate (MMA), acrylamide (AA), divinylbenzene (DVB), and 5-methyluridine were purchased from Acros (Noisy-Le-Grand, France). 4-Vinylphenylboronic acid was purchased from Aldrich (Saint-Quentin-Fallavier, France). The acetonitrile was distillated on calcium hydride. All chemicals and solvents were analytical or HPLC grade and were used without further purification. The ultra-pure water was provided by a UHQ system (Elga, High Wycombe, UK).

4.2. Instrumentation

4.2.1. NMR

All NMR spectra were realized on a Brücker Advance 2 450 MHz instrument, (Wissenbourg, France) at room temperature. The template (5 mg) was dissolved in deuterated chloroform (1 mL). If necessary, monomer was added corresponding to 1 molar equivalent for the 2,6-bismethacrylamido-pyridine 2 (MAP, 3.31 mg) and 2,6-diaminopurine-7-vinylbenzyl 3 (DAPVB, 3.62 mg) both functional monomers reacting on the 2 carbonyl functions and NH from the template or 2 equiv (1.92 mg) of AA because each monomer react on 1 carbonyl. These data were analyzed with the Mestrec software ver 4.9.6.0 (Mestrelab Research, Santiago de Compostela, Spain).

4.2.2. Scanning electron microscopy

Imprinted polymers were deposited onto silicon slides, and coated with 5 nm of gold using a thermal evaporator (Denton DV-502A, Denton Vacuum, Moorestown, NJ, USA). Scanning electron micrographs (SEM) were obtained at 4 kV with Hitachi S4500 equipped with a Field Emission Gun (Hitachi, Japan).

4.2.3. BET surface measurements

A 60 mg quantity of polymers was degassed overnight at 100 °C to remove adsorbed gases and moisture. Surface areas analyses were performed at 77 K by Brunauer–Emmett–Teller (BET) on an ASAP 2020 surface area and porosity analyser (Micromeritics Instrument Corporation, Creil, France).

4.2.4. HPLC

The instrumentation used to perform this work included a VWR—Hitachi LaChrom Elite (Tokyo, Japan) HPLC system composed by a quaternary pump equipped with a degasser, an autosampler programmed to inject 20 μL, a column oven set at 25 °C and a diode array detector acquiring spectra in the 200–300 nm wavelength range. Analysis was performed in isocratic mode by using a Hypercarb column (5 μm, 100 × 4.6 mm, Thermo Electron, Shandon, UK) and acetonitrile/25 mM ammonium acetate buffer pH 5.5 mixture (15:85, v/v) as eluent. The flow rate was 1 mL/min. These data were computed at a wavelength of 254 nm using EZChromElite ver 3.1.7 software (Agilent Technologies, Pleasanton, CA, USA).

4.3. Synthesis of templates

4.3.1. Synthesis of 2′,3′-(4-vinyl)phenylboronate-5-methyluridine (10a)

4-Vinylphenylboronic acid (148 mg, 1 mmol) was dissolved in anhydrous 1,4-dioxanne (20 mL). 5-Methyluridine (258 mg) was added and the mixture was refluxed for 10 h. The solution was then co-evaporated with toluene under reduced pressure. Yield: 97%; ^{1}H NMR δ (ppm) in $CDCl_3$: 9.79 (s, 1H, NH), 7.78 (d, J = 8.1 Hz, 2ArH), 7.44 (d, J = 8.1 Hz, 2H, ArH), 7.15–7.13 (m, 2H), 6.74 (q, J = 17.7, 10.9 Hz, 2H), 5.85 (d, J = 17.7 Hz, 1H), 5.53 (d, J = 2.7 Hz, 1H), 5.43–5.28 (m, 3 H), 5.95 (s, 3H).

4.3.2. Synthesis of 2′,3′-(4-vinyl)phenylboronate-5-methyluridine *tetra*-butylammonium hydroxide (10b)

Compound **10a** (370 mg, 1 mmol) was dissolved in 5 mL of anhydrous methanol and 1 mL of 1 M *tetra*-butylammonium hydroxide in methanol was added. The mixture was shaked for 10 min and the solvent was evaporated under reduced pressure at 30 °C. Yield: 98%; ^{1}H NMR δ (ppm) in $CDCl_3$: 7.78 (d, J = 8.05 Hz, 2H, ArH), 7.44 (d, J = 8.05 Hz, 2H, ArH), 7.15–7.13 (m, 2H), 6.74 (q, J = 17.7, 10.88 Hz, 2H), 5.85 (d, J = 17.7 Hz, 1H), 5.53 (d, J = 2.68, 1 H), 5.43–5.28 (m, 3H), 5.95 (s, 3H).

4.4. Synthesis of monomers

4.4.1. Synthesis of 2,6-bis(methacrylamido)pyridine MAP (11a)

The synthesis of 11a follows an already reported procedure.[38] 2,6-Diamino-pyridine (109 mg, 1 mmol) was solubilized in dry pyridine (4 mL) at 0 °C. Methacryloyl chloride (587 μL) was added on a period of 15 min and the mixture was stirred for 30 min at this temperature then for 3 h at room temperature. The mixture was evaporated with toluene. The obtained brown solid was dissolved in a solution of ammonium acetate saturated and the mixture was extracted twice with methylene chloride. The organics phases were dried on magnesium sulfate and then evaporated. The oil was purified on silica column using methylene chloride/methanol (99:1, v/v). Yield: 78%; ^{1}H NMR δ (ppm) in $CDCl_3$: 8.01 (s, 2H, NH), 7.92 (d, J = 7.85 Hz, 2H, ArH), 7.64 (t, J = 8.15 Hz, 1H), 5.81 (s, 2H), 5.47 (s, 2H), 2.01 (s, 6H, CH_3).

4.4.2. Synthesis of 2,6-diamino-9-(3/4-vinylbenzyl) purine DAVBP (12)

2,6-Amino-purine (150 mg, 1 mmol) was solubilized in DMF (6 mL). Cesium carbonate (1.075 g, 3.3 mmol) was then added. The mixture was heated at 90 °C, *m* and *p*-isomers of vinylbenzyl chloride (171 μL, 1.2 mmol) were then added. The reaction was kept at this temperature 10 h. The mixture was filtrated to remove the salts and was evaporated with toluene. The oil was purified on silica column by methylene chloride/methanol (95:5, v/v). Yield: 40%; ^{1}H NMR δ (ppm) in $CDCl_3$: 7.47 (s, 1H, Hpurine), 7.37 (d, J = 8.2 Hz, 2H, ArH), 7.21 (d, J = 8.2 Hz, 2 H, ArH), 6.69 (dd, J = 10.9, 17.6 Hz, 1H, 1Hvinyl), 5.73 (d, J = 17.6 Hz, 1H, 1Hvinyl), 5.46 (s, 2H, NH_2), 5.26 (d, J = 10.9 Hz, 1H, 1Hvinyl), 5.19 (s, 2H, CH_2), 4.77 (s, 2H, NH_2).

4.5. Synthesis of MIP and NIP

The template was introduced in a glass vial and dissolved in acetonitrile. The functional monomer, the cross-linker, and the initiator (AIBN) were then added. The tube was degassed for 5 min by nitrogen then sealed. The polymerization was photochemically initiated by a standard laboratory UV light source (BLX E-365 5 × 8W, 365 nm, Vilbert Lourmat, Marne la vallée, France) for 24 h and the tubes were then heated for 2 h at 70 °C to complete the polymerization. During the first 2 h of polymerization, the tubes were

turned of 90° each 15 min. The obtained polymer was removed from the vial, crushed and soxhlet extracted in methanol/acetic acid 70:30 overnight. The particles were drilled and sized to 25 μm.

For MIPs with DAPVB the tube were heated in acetonitrile before introduction of AIBN until the dissolution and directly polymerized on UV because of a poor insolubility of monomers in the solvent. Porogen solvent mass was equivalent to cross-linker and functional monomer together. The molar amount of AIBN was corresponding to 1% molar amount of all compounds. For each MIP, a corresponding non-imprinted polymer (NIP) was synthesized in the same conditions but without vinyl-phenylboronate ester template. By using a dummy template (imprinting 5-methyluridine and rebinding uridine) no interference was possible in the analysis since the analyte and the template were different.

4.6. Batch rebinding studies

A mass of 5 mg of polymers was placed in 1.5 mL polypropylene microtubes and incubated in 0.5 mL of ammonium acetate buffer solution 10 mM, pH 10, containing 10 mg/L of nucleosides and 54 μL/10 mL of *tetra*-butylammonium hydroxide 40% (w/v) in water for 24 h with constant shaking. The polymer was then centrifuged for 10 min at 18,000g (microfuge 22R centrifuge, Beckman Coulter, Krefeld, Germany) and the concentration of nucleosides in supernatant was measured by HPLC after filtration on disposable 0.20 μm cellulose acetate syringe filter supplied by Iwaki Glass (Tokyo, Japan).

4.7. Preparation of doped dog urine sample

A volume of 1 mL of urine was introduced in 10 mL flask and 1 mL of buffer solution of ammonium acetate 25 mM, pH 10, containing nucleosides at a concentration of 100 ppm was added to obtain a final concentration of 10 ppm. A volume of 54 μL of Bu_4NOH (40% wt in water) was introduced in order to obtain a solution of 80 mM and the volume was adjusted with a ammonium acetate solution 25 mM at pH 10. The solution was directly extracted on MISPE cartridges without others treatments.

4.8. Molecularly imprinted solid-phase extraction (MISPE)

MISPE cartridges were prepared by adding 100 mg of dried and sieved polymer in an empty solid-phase extraction cartridge between two glass frits. The cartridges were activated by methanol then conditioned for 5 min with 2 mL of 10 mM ammonium acetate, pH 11.5, buffer solution containing 800 mM of TBAH. Solution of urine to analyze was loaded on the cartridge (1 mL). Then, the cartridge was washed successively with 10 mM ammonium acetate, pH 11.5, buffer solution containing 800 mM of TBAH, 1 mL of acetonitrile and the analytes were eluted with 1 mL of 1 M HCl in water/methanol/acetic acid 50:45:5, v/v/v.

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