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Molecularly Imprinted Polymers as Antibody Mimics in Automated On-Line Fluorescent Competitive Assays

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An automated molecularly imprinted sorbent based assay (MIA) for the rapid and sensitive analysis of penicillin-type *β*-lactam antibiotics (BLAs) has been developed and optimized. The polymers were prepared using penicillin G procaine salt as template (PENGp) and a stoichiometric quantity of a urea-based functional monomer to target the single oxyanionic species in the template molecule. Highly fluorescent competitors (emission quantum yields of 0.4−0.95), molecularly engineered to contain pyrene labels while keeping intact the 6-aminopenicillanic acid moiety for efficient recognition by the cross-linked polymers, have been tested as analyte analogues in the competitive assay. Pyrenemethylacetamido penicillanic acid (PAAP) was the tagged antibiotic providing for the highest selectivity when competing with PenG for the specific binding sites in the molecularly imprinted polymer (MIP). Upon desorption from the MIP, the emission signal generated by the PAAP was related to the antibiotic concentration in the sample. The 50% binding inhibition concentration of penicillin G standard curves was at 1.81×10^{-6} M PENG, and the detection limit was 1.97×10^{-7} M. The sensor showed a dynamic range (normalized signal in the 20 to 80% range) from 6.80×10^{-7} to 7.21×10^{-6} M (20−80% binding inhibition) PENG in acetonitrile:HEPES buffer 0.1 M at pH 7.5 (40:60, v/v) solutions. Competitive binding studies demonstrated various degrees of cross-reactivity with penicillin-type *β*-lactam antibiotics such as ampicillin (71%), oxacillin (66%), penicillin V (56%), amoxicillin (13%), and nafcillin (46%) and a lower response to other isoxazolyl penicillins such as cloxacillin (27%) and dicloxacillin (16%). The total analysis time was 14 min per determination, and the MIP reactor could be reused for more than 150 cycles without significant loss of recognition. The automatic MIA has been successfully applied to the direct analysis of penicillin G in spiked urine samples with excellent recoveries (mean value 92%). Results displayed by comparative analysis of the optimized MIA with a chromatographic procedure for penicillin G showed excellent agreement between both methods.

Immunological methods are commonly applied in clinical, environmental, agricultural, food, and forensic laboratories, as they provide a very powerful analytical tool for a wide range of analytes, such as proteins, hormones, toxins, antibiotics, etc. Immunoassays are very sensitive and selective, do not require skilled workers or sophisticated instrumentation, can run many analyses simultaneously, and are generally cost-effective for large sample loads. However, reagent stability, the high cost, and difficulties associated with antibody production, together with the need to use laboratory animals, are often cited as problems. In addition, the production of antibodies for toxic compounds or immunosuppressants is particularly difficult because of their adverse action on the metabolism and the immune system.

Molecularly imprinted polymers (MIPs) have unique properties that make them suitable for use in immuno-type assays. They can be engineered to exhibit good sensitivity and specificity for various analytes of medical, environmental, and industrial interest. They are also highly robust, showing excellent operational stability under a wide variety of conditions.[1] However, in comparison to biological assays using antibodies as selective recognition elements, molecularly imprinted sorbent-based assays (MIAs) have several shortcomings, some of which have been addressed in recent years, such as the limited water compatibility, slow kinetics, the dominance of radioactive tracers as analyte probes, and the need to separate the MIP with the bound probe from the unbound probe in solution after incubation.[2,3]

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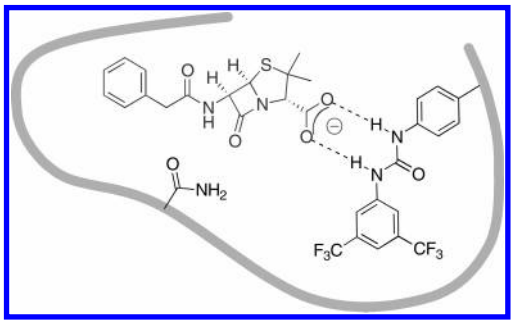
Heterogeneous phase immunosensors, in combination with flow techniques, can be automated easily and combine the sensitivity and selectivity of immunoassays with the accuracy and simplicity of the flow methods.[4,5] These formats have advantages compared with other sensing formats: (i) it is possible to use the same reagents employed in microtiter plate formats, avoiding the synthesis of special reagents, (ii) the desorption of the captured immunocomplexes is very easy and effective, and (iii) the active working life of the immuno-support reaches a reusability of hundred of cycles. They can be applied in different fields, allowing automation and reducing sample manipulation. The principles behind these methods could, in principle, be applied in MIAs, provided that the appropriate labeled analyte derivatives are available and that the binding and regeneration kinetics are favorable.

Most of the labeled analytes described in the literature for MIAs are radioactive derivatives,[6] but fluorescent tags are also known.[7,8] The principal approaches that have found a greater application in the development of fluorescent based MIAs are based on the imprinting of the analyte and the use of either a related analyte-labeled derivative[9−12] or an unrelated probe for the competitive assays.[13−16] As we have shown previously,[17] in the first case, the spatial arrangement, nature, and size of the fluorescent tag must be tailored to the template structure to achieve efficient competition for the MIP binding sites. We have also demonstrated that the labeled conjugate showing the best performance in a MIP-based assay was also the one providing the highest sensitivity in an immunoassay based on the same type of measurements.[5] The second approach relies on the fact that the probe shows some similar functionality and size to the analyte and binds, although weakly, to the best imprinted binding sites. With these considerations, it may become a difficult task to find an unrelated probe with the above-mentioned characteristic. In fact, if the labeled conjugates are not selected properly, there is no guarantee that the sites interrogated with the probe are those with the best selectivity for the analyte.[2] In most cases, the MIAs developed to date usually require long incubation times in order to achieve efficient competition between the analyte and the fluorescent analogue, thus limiting the applicability of the polymers in combination with automatic flow techniques. Further, many of them have been tested only in organic solvents, again to promote efficient competition between the labeled analogue and the analyte for the polymeric binding sites, a great limitation for the analysis of aqueous samples.[12,13,16,17]

Scheme 1



Here, we wish to report the first example of an automated MIA compatible with aqueous samples. Targeting BLAs, a flow-through solid-phase competitive assay has been developed which exhibits excellent robustness and performance when applied to biological samples. This performance was enabled through the use of a stoichiometrically imprinted polymer showing good target binding in aqueous media (see Scheme 1). This polymer was prepared using penicillin G procaine salt as the template molecule and a stoichiometric quantity of a recently developed urea-based functional monomer to target the single oxyanionic species in the template molecule.[18−20] Ethyleneglycol dimethacrylate served as crosslinker and methacrylamide as an additional hydrogen-bonding comonomer.

In the assay, the analyte and a constant amount of labeled fluorescent analogue, [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-[(pyren-1ylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (PAAP), are allowed to compete for the binding sites of the MIP, which was packed into a reactor. After application of a desorbing solution, the fluorescence of the labeled derivative eluted from the sorbent is measured and related to the analyte concentration in the sample. The application of the desorbing solution allows the regeneration of the support without affecting its binding characteristics, thus allowing long-term application. The system has been fully automated, and several parameters affecting the sensor performance have been optimized, such as the binding solvent composition, the amount of polymer, tracer concentration, assay flow rates for reagent binding and elution, and the amount and nature of the desorbing solution. The method has been applied to the analysis of penicillin G in urine samples, and the results have been validated by HPLC with diode array detection (DAD).

EXPERIMENTAL SECTION

Chemicals. The urea-based functional monomer, *N*-[3,5-bis-(trifluoromethyl)phenyl]-*N*′-(4-vinylphenyl)urea (MPVU), was pre-

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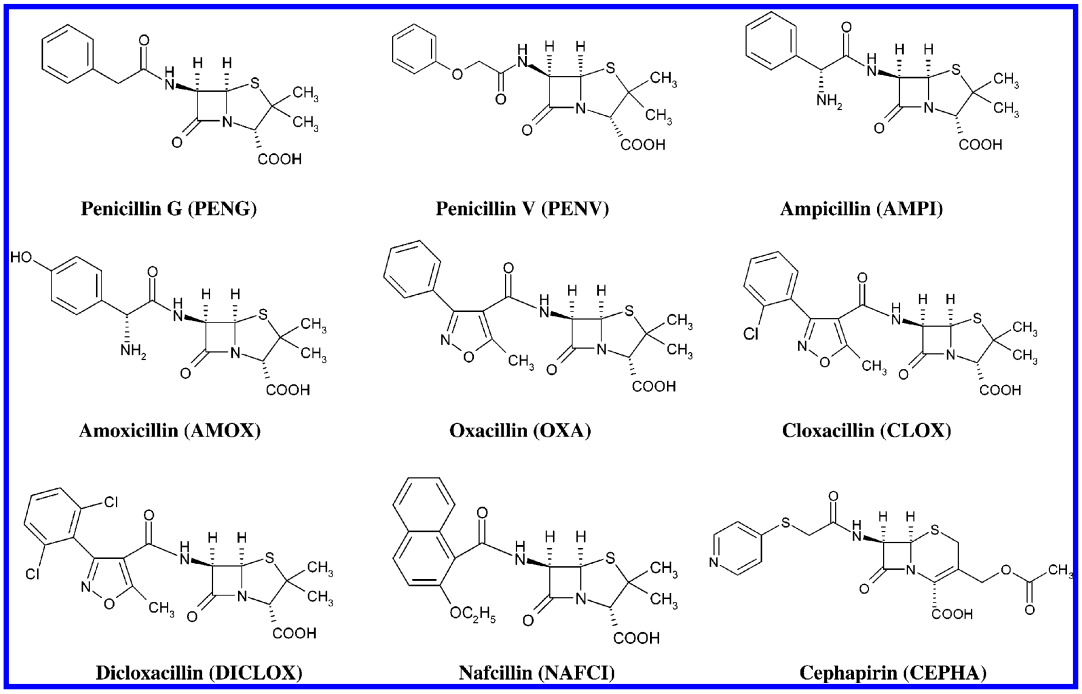

Figure 1. Chemical structures of the β-lactam antibiotics included in the study.

pared as described previously.[18] Ethyleneglycol dimethacrylate (EDMA) was purchased from Aldrich and purified prior to use as follows: EDMA was washed sequentially with 10% NaOH (aqueous), water, and then brine. After being dried over $MgSO_4$, it was distilled under reduced pressure to give inhibitor-free monomer. Methacrylamide was purchased from Sigma−Aldrich (St. Louis, MO). ABDV was obtained from Wako (Neuss, Germany) and used as received. The antibiotics (Figure 1) penicillin G potassium (PENG) and procaine (PENGp) salts, penicillin V potassium salt (PENV), amoxicillin (AMOX), nafcillin sodium salt (NAFCI), cloxacillin sodium salt (CLOX), dicloxacillin sodium salt (DICLOX), oxacillin (OXA), cephapirin (CEPHA), and ampicillin (AMPI) were supplied by from Bayer AG (Leverkusen, Germany).

HPLC grade acetonitrile and methanol were purchased from SDS (Peypin, France), and water for HPLC was purified with a Milli-Q system (Millipore, Bedford, MA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use. HEPES was supplied by Aldrich (Steinheim, Germany), and a buffer solution, pH 7.5, was prepared by dissolving 23.830 g in 1 L of purified water (0.1 M). Trifluoroacetic acid (TFA, +99%) was from Fluka (Buchs, Switzerland).

Synthesis of the Imprinted and the Nonimprinted Polymers. The polymers were prepared as described elsewhere.[19,20] Briefly, the template PENGp (286 mg, 0.5 mmol), functional monomer MPVU (186 mg, 0.5 mmol), methacrylamide (84 mg, 1 mmol), EDMA (3.8 mL, 20 mmol), and the free radical initiator ABDV (44 mg, 1% w/w total monomers) were dissolved in MeCN (5.6 mL). After dissolution, the solution was transferred to a glass tube, cooled to 0 °C, and then purged with N_2 for 10 min. After purging, the glass tube was sealed and polymerization initiated thermally by placing the tube in a water bath set at 40 °C. for 48 h to allow polymerization. The MIP monolith was removed from the tube and broken into smaller fragments. The template molecule was removed through the following sequential washing steps: MeOH (100 mL), MeOH/ 0.1 M HCl (aq) (9:1, v/v, 100 mL), and finally MeOH (100 mL). The wash solutions were combined and evaporated to dryness under reduced pressure, and the solid residue was then weighed and examined by ^{1}H NMR spectroscopy, showing that template removal was near quantitative (extract residue weight = 300 mg). Thereafter, the MIP was crushed and sieved, and particles in the size range 25−50 μm were collected for use in the SPE experiments. Prior to use, they were sedimented using MeOH/water (80:20, v/ v) in order to remove fine particles. A control, nonimprinted polymer was prepared in the same manner but with omission of the template molecule.

Synthesis of Pyrene-Labeled β-Lactam Antibiotics (BLAs). Pyrene-labeled BLAs and their analogues (Figure 2) were prepared from 6-APA or the corresponding β-lactam antibiotic and the succinimidyl esters (obtained from the commercial acids and *N*-hydroxysuccinimide) of pyrenebutyric or pyreneacetic acids (Aldrich; St. Louis, MO) in acetone/water/$NaHCO_3$.[21] In brief outline, after stirring the mixture overnight, the acetone was removed under reduced pressure and the aqueous mixture was subjected to repetitive extraction with diethyl ether in order to remove any unreacted succinimidyl ester. Then the aqueous phase was brought to pH 2.5 with 10% phosphoric acid and extracted several times with diethyl ether. The combined extracts were dried over anhydrous magnesium sulfate. After removal of the drying agent, the organic solvent was eliminated by rotavaporation to yield the target compound. The purity and structure of the labeled substrates was confirmed by elemental analysis and FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy, as well as mass spectrometry with electrospray ionization (ESI-MS) and will be reported elsewhere.[22] The following pyrene derivatives were synthesized in that way: [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-[(pyren-1-ylacetyl)-

(21) Orellana, G.; Aparicio Lara, S.; Moreno-Bondi, M.C.; Benito Peña, E. Synthesis of fluorescent derivatives of β-lactam antibiotics. Spanish Patent 2,197,811.

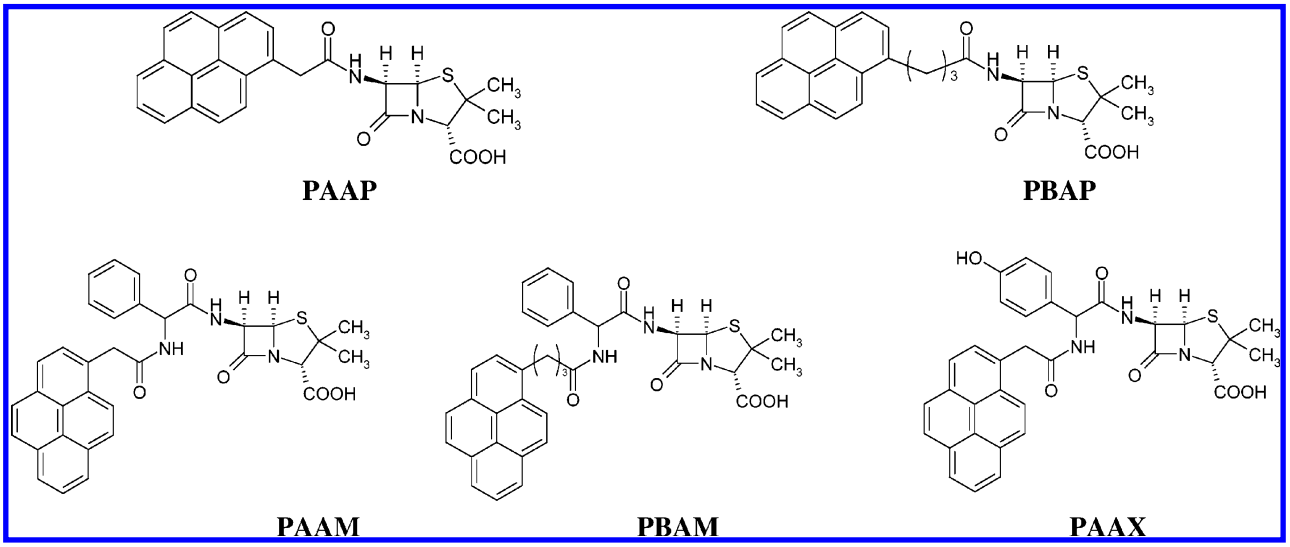

Figure 2. Chemical structures of the fluorescent derivatives of 6-aminopenicillanic acid.

amino]-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid (PAAP); [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-[(4-pyren-1-ylbutanoyl]amino]-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid (PBAP); [2*S*,5*R*,6*R*]-6-{[(2R)-2-amino-2-(4-hydroxyphenyl)ethanoyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (PAAX); [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-({(2R)-2-phenyl-2-[(pyren-1-yl-acetyl)amino]-ethanoyl}amino)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (PAAM); [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-({(2*R*)-2-phenyl-2-[(pyren-1-ylbutanoyl)amino]ethanoyl}amino)-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid (PBAM).

HPLC Evaluation of the Polymers. The MIPs were slurry-packed into stainless steel HPLC columns (150 mm × 4.6 mm i.d.) using methanol/water (80:20, v/v) as the solvent. HPLC evaluation of the polymers for recognition of the labeled BLAs was performed using an HPLC 1100 instrument (Agilent) equipped with a quaternary pump, an autoinjector, and a fluorescent detector. For these experiments, the following conditions were used: 1 mL/min flow rate, 20 μL sample volume, 400 μM analyte concentration, excitation at 341 nm, and detection at 396 nm. Methanol was used as the void marker and the retention factors (k) were calculated as $k = (t_R - t_0)/t_0$, where t_R is the retention time of the analyte and t_0 is the retention time of the void marker. Imprinting factors (IF) were calculated as $IF = k_{MIP}/k_{NIP}$.

Binding of PAAP to the MIP/NIP. To calculate the optimum amount of polymer to be used in the competitive assay, a constant amount of PAAP (250 nM) was incubated with increasing concentrations of MIP or NIP (1−25 mg mL^{-1}) and allowed to equilibrate for 24 h at room temperature. The experiment was repeated under the same conditions but in the presence of PENG (400 μM). After equilibration, the amount of PAAP remaining in the supernatant was measured by HPLC-FLD analysis. The analytical column was a LUNA C18 (2) (150 mm × 4.6 mm, 5 μm) column protected by a RP18 guard column (4.0 mm × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA). The mobile phase was 45:55 water/MeCN containing 0.08% phosphoric acid. Analyses were performed at a flow rate of 1.0 mL min^{-1} at room temperature. The injection volume was 20 μL, and the fluorescence detector wavelengths were set at 341 nm (excitation) and 396 nm (emission).

To evaluate the optimum concentration of PAAP for the competitive assays, MIP and NIP samples (19 mg) were incubated with 1 mL of increasing concentrations (1 nM to 250 nM) of PAAP in MeCN/aq HEPES buffer (pH 7.5) (40:60, v/v) in the presence and in the absence of 400 μM PENG. The supernatants were analyzed by HPLC-FLD as described above.

Measuring System. The automated flow injection manifold is similar to one described previously.[5] An eight-way distribution valve (Kloehn, Las Vegas, NV) equipped with a 2.5 mL syringe pump is connected to another eight-way distribution valve. The whole system is controlled using the Winpump software provided by Kloehn (Las Vegas, NV). The output of the pump is connected to a stainless steel column (Agilent, Germany) (20 × 2.1 mm), thermostated at 20 °C and packed with the MIP. The output flow is driven to a flow-through cell (100 μL, Starna, Germany) placed in the fluorometer sample holder. Fluorescence intensity measurements at 341 nm (exc.) and 396 nm (em.) were carried out in a Fluoromax 2 (Horiba-Jobin Yvon, Longjumeau Cedex, France), with the instrumental parameters and data processing controlled with the original software (Datamax). All solutions were thermostated at 20 °C using a Precisterm JP water bath (Selecta, Barcelona, Spain).

Fluorescent Competitive Assay. The measuring protocol is based on the principles of a competitive fluoroimmunoassay. All solutions were prepared in MeCN:aq. HEPES buffer (0.1 M, pH 7.5) (40:60, v/v). Initially, the sample (0.95 mL) was mixed in the syringe with a constant amount of PAAP (120 nM, 0.25 mL), and 1 mL of the solution was injected into the reactor at a flow rate of 0.75 mL min^{-1} to allow retention of the analytes on the MIP. The reactor was washed three times with 1 mL of HEPES buffer (0.1 M, pH 7.5) to remove all the unbound complexes. The analytical signal was generated upon dissociation of the PAAP molecules retained on the polymer after competition using 2.5 mL of a methanol solution at a flow rate of 1 mL min^{-1}.

Before a new measurement, the reactor was washed with 2.5 mL of HEPES buffer (0.1 M, pH 7.5) at a flow rate of 1 mL min^{-1}. A complete cycle for the whole automated assay procedure

(22) Orellana, G.; Aparicio Lara, S.; Moreno-Bondi, M. C.; Benito Peña, E. (Manuscript in preparation).

required approximately 14 min, including regeneration. The reactor showed great stability, and the system could be used for more than 150 measurements. Occasionally, the reactor had to be back-flushed with washing buffer to maintain a constant flow rate.

Experimental signals were normalized using the following expression:

$$\text{normalized response} = (B - B_\infty)/(B_0 - B_\infty) \qquad (1)$$

where B is the signal (fluorescence intensity) measured in the presence of the increasing analyte concentrations, B_∞ is the background fluorescence obtained in the presence of an excess of PENG, and B_0 is the signal in absence of antibiotic. The normalized response was plotted as a function of the analyte concentration (in logarithmic scale), and the experimental data were fitted to a four-parameter logistic equation (sigmoidal):

$$\text{normalized signal} = \frac{A_{\max} - A_{\min}}{1 + \left(\frac{[\text{analyte}]}{\text{EC}_{50}}\right)^b} + A_{\min} \qquad (2)$$

where $A_{\max}$ is the asymptotic maximum (maximum emission in absence of analyte), b represents the slope of the curve at the inflection point, EC_{50} is the analyte concentration at the inflection point (concentration giving 50% inhibition of $A_{\max}$), and $A_{\min}$ is the asymptotic minimum. The detection limit (LOD) was calculated as the analyte concentration for which the tracer binding to the antibody was inhibited by 10%, and the dynamic range (DR) of the method was evaluated as the analyte concentrations that produced a normalized signal in the 20−80% range.

Selectivity Studies. Cross-reactivity studies were carried out by measuring the competitive curves for other chemically related and nonrelated antibiotics under the optimized conditions in the range of 2×10^{-8} to 9×10^{-3} M. Cross-reactivity (CR) was calculated as the percentage between the EC_{50} value for PENG and the EC_{50} for the interfering compound,

$$\%\text{CR} = \frac{\text{EC}_{50}\ (\text{PENG})}{\text{EC}_{50}\ (\text{cross-reacting compound})} \times 100\% \qquad (3)$$

Sample Analysis and Validation. Urine samples were collected from a healthy individual not medicated with antibiotics for more than 6 months. Portions of 10 mL were spiked with an aqueous solution of PENG (final concentrations 4×10^{-6} and 8×10^{-6} M), and the pH was adjusted to 7.5 and made up to a final volume of 25 mL with HEPES (pH 7.5, final buffer concentration 0.1 M). For sample analysis, 6 mL of the solution was mixed with 4 mL of acetonitrile. The solution was filtered through a 0.45 μm nylon filter before use and injected in the measuring system. At least three replicate samples were analyzed to validate the results obtained.

For validation purposes, the samples were also analyzed by HPLC-DAD, 1100 instrument (Agilent) equipped with a quaternary pump, an auto-injector. The analytical column was a LUNA C18 (150 × 4.6 mm, 5 μm) protected by a RP18 guard column (4.0 × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA). A gradient program was used for the mobile phase, combining solvent A (water with 0.01% TFA) and solvent B (MeCN with 0.01% TFA) as follows: 0% B (3 min), 0−37% B (5 min), 37% B (11 min), 37−67% B (5 min), 67% solvent B (5 min). Analyses were performed at a flow rate of 1.5 mL min^{-1}, and the column temperature was kept at 35 °C. The injection volume was 200 μL, and the UV detector wavelength was set at 220 nm. For the recovery studies, matrix-matched calibration standards were prepared for each sample. All the analyses were carried out in triplicate.

RESULTS AND DISCUSSION

1. Chromatographic Evaluation. The ability of the molecularly imprinted polymers to retain the different β-lactam antibiotics (BLAs) as well as the template molecule (PENG) was investigated by comparing their retentions on the NIP and MIP. Figure 3 shows the retention factors (k) and the imprinting factors (IF) for the five labeled fluorescent BLAs and for the template molecule on the MIP and NIP using mobile phases containing acetonitrile: aqueous HEPES (0.1 M, pH 7.5) in the 50:50 to 0:100 (v/v) range. As has been described previously, pH 7.5 is required to ensure deprotonation of the carboxylic acid groups of the antibiotic and the fluorescent tracers, which is necessary for binding to occur to the polymeric urea moieties.[20] The evaluated fluorescent BLAs show strong retention on both MIP and NIP when a mobile phase of 100% aqueous HEPES (0.1 M, pH 7.5) is used (k_{MIP} and $k_{\text{NIP}} > 10$). This behavior can be attributed to the nonspecific hydrophobic interactions between the pyrene moiety of the labeled antibiotics and the bulk polymer matrices, which must be avoided in order to develop the competitive assay. The addition of up to 50% acetonitrile in the mobile phase reduces drastically the retention times of all the labeled analytes, as well as PENG, on both the MIP and NIP. However, the retention is always higher on the MIP. This behavior can be explained by considering that the increment of the acetonitrile content in the mobile phase leads to a decrease in hydrophobic interactions between the aromatic moiety of the dyes and the sorbents, favoring the specific recognition of the β-lactam part of the molecules by the PENG-imprinted sites of the MIP. Obviously, the interaction depends also on the size, shape, and geometry of the labeled BLA tested, and the best imprinting factor was obtained for the PAAP, which shows a three-dimensional structure quite similar to that of PENG.[17] The retention factor for PAAP using acetonitrile:aqueous HEPES 40:60 (0.1 M, pH 7.5) as mobile phase was very similar to that of PENG ($k_{\text{PAAP}} = 1.91$, $k_{\text{PENG}} = 1.25$). Moreover, in both cases the retention factors on the non-imprinted polymer (NIP) are very low ($k_{\text{PAAP}} = 0.42$, $k_{\text{PENG}} = 0.25$) and the corresponding imprinting factors were similar ($IF_{\text{PAAP}} = 4.57$ and $IF_{\text{PENG}} = 4.93$). Thus, we decided to use this solvent composition for the development of the MIA to ensure the efficient competition of the labeled BLA and PENG for the polymeric binding sites and to minimize the nonspecific interactions with the selective recognition polymer.

2. MIA Optimization and Analytical Characterization. To evaluate the optimum amount of MIP for the PenG assay, a fixed concentration of PAAP (250 nM) was incubated in batch for 24 h with increasing amounts of MIP in the absence and in the presence of a constant concentration of PenG (400 μM) in 1 mL of acetonitrile:aqueous HEPES (40:60, v/v) as the solvent. A

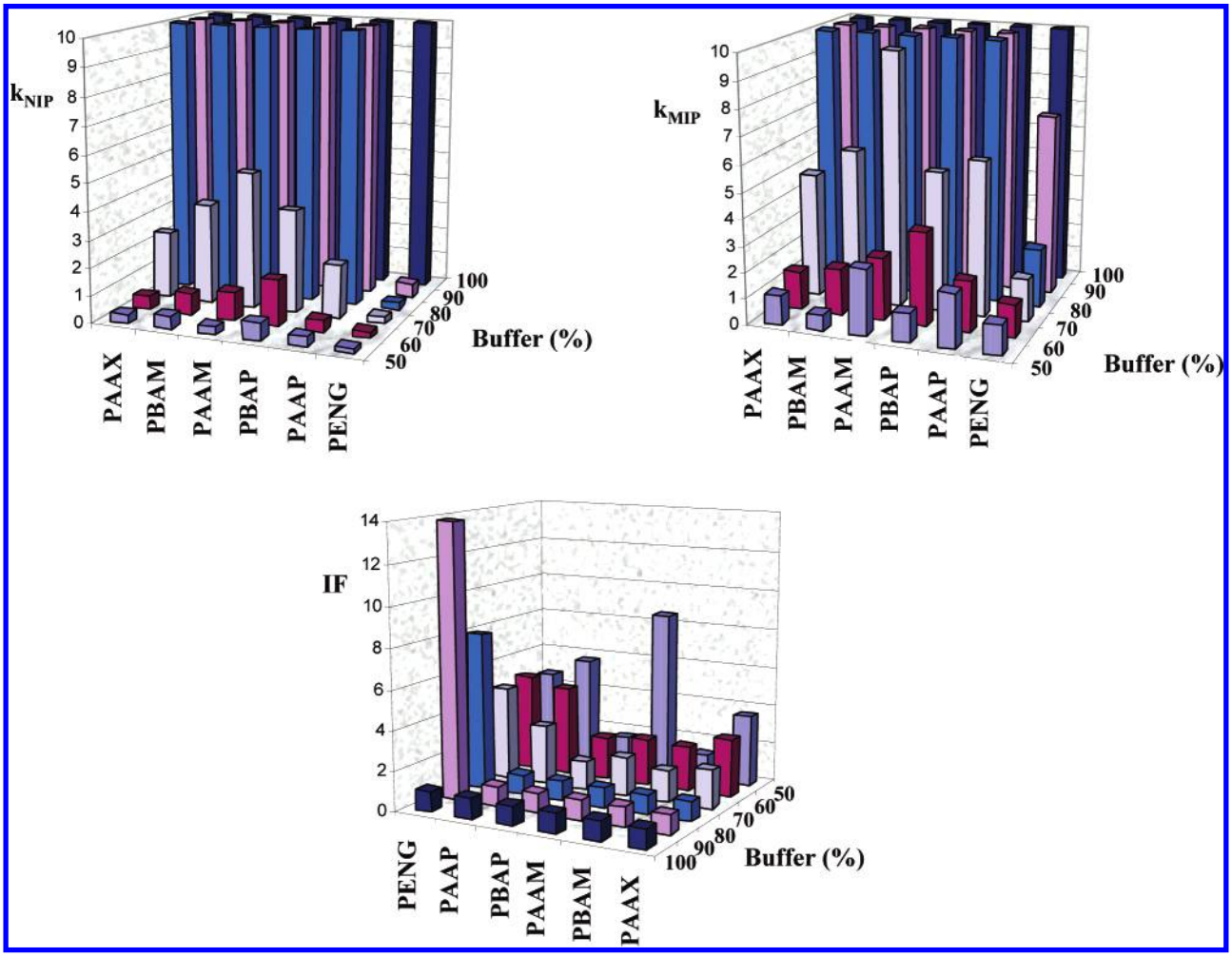

Figure 3. Retention factors (k) in (a) the MIP, (b) NIP, and (c) imprinting factors (IF) for the five fluorescent β-lactam antibiotics and the template molecule using mobile phases consisting of acetonitrile:aqueous HEPES (0.1 M, pH 7.5) in the 50:50 to 0:100 (v/v) range.

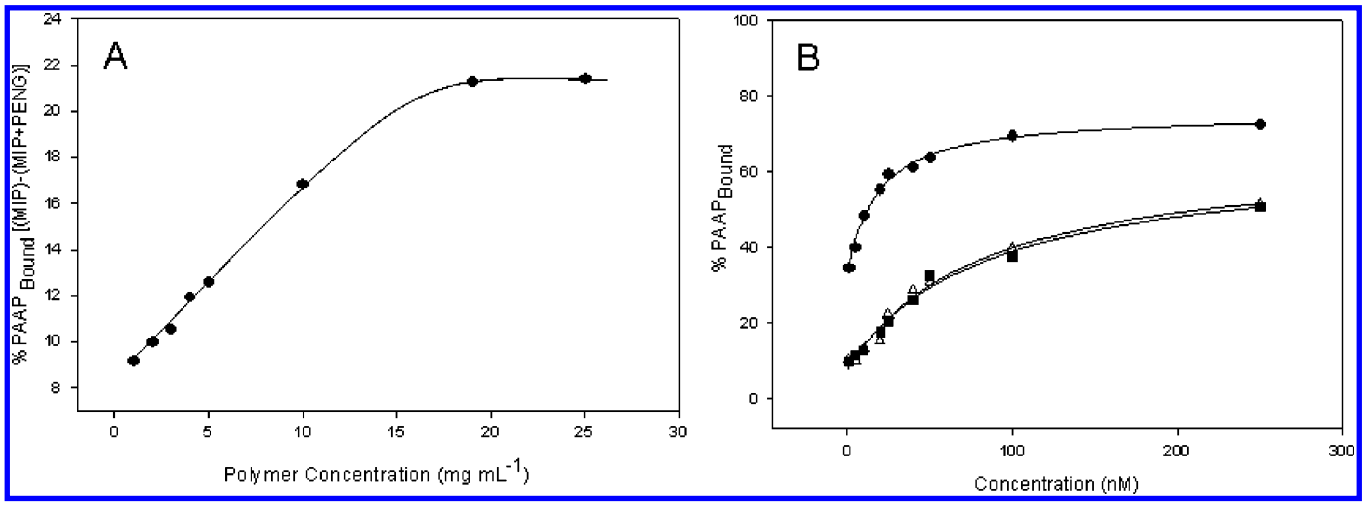

Figure 4. (a) Relative fraction of PAAP (%) bound to the MIP in the absence and in the presence of a constant amount of PenG (400 μM) as a function of the polymer concentration (1−25 mg mL^{-1}). PAAP (250 nM) in acetonitrile:aq. HEPES buffer (pH 7.5, 40:60 (v/v)) (n = 3; 24 h incubation). (b**)** Fraction of PAAP bound (%) to a constant amount of polymer (19 mg in 1 mL of acetonitrile:aq. HEPES buffer (pH 7.5, 40:60 (v/v)), as a function of the fluorescent probe concentration (1 nM to 250 nM), after 24 h incubation: MIP (●) and NIP (■) in the absence of PENG and MIP in the presence of 400 μM PENG (△).

control experiment was carried out using the NIP in place of the MIP. As shown in Figure 4a, PAAP competes with PENG for the polymeric binding sites, as the labeled BLA is displaced from the MIP in the presence of the template molecule. The curve obtained in the presence of PENG can be superimposed on that obtained for the NIP in the absence of the template, demonstrating that the labeled BLA is displaced by PENG from the specific binding sites. The largest difference in the fraction of PAAP bound to the

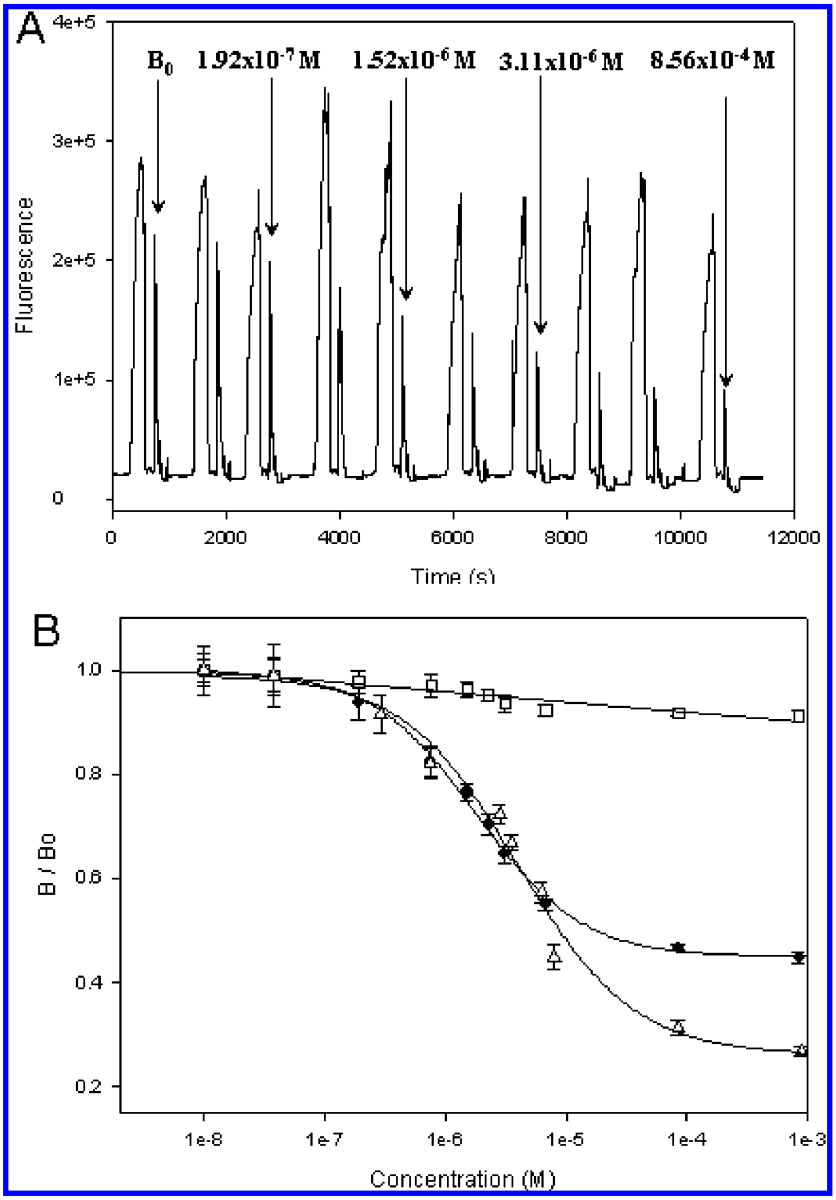

Figure 5. (a) Dose−response curve measured with the MIA over long measuring times. **(b)** Calibration curves ($n = 3$) for PENG (from 0.0013 to 890 μM) in acetonitrile:aqueous HEPES (0.1 M, pH 7) (40/60, v/v) for the MIP (▼), the NIP (□), and in urine samples for the MIP(△). PAAP tracer 25 nM, column packed with 19 mg of polymer in all cases. The experimental points have been fitted to eq 2.

polymer, in the presence and in the absence of the analyte, corresponds to a MIP amount of 19 mg, which was thus selected for further experiments.

The amount of tracer (Ag*) used in the assay should be kept low enough to achieve good sensitivity but high enough to provide an acceptable signal. Figure 4b shows the change in the fraction of PAAP bound (%) to a constant amount of MIP/ NIP (19 mg) as function of the fluorescent probe concentration after 24 h incubation. Saturation is achieved sooner for the MIP than the NIP, and there are significant differences between the amounts of PAAP bound to the polymers, especially for concentrations lower than 50 nM, where the nonspecific retention is minimized. As described in the previous experiment, when the assay was performed in the presence of a constant amount of 1 mL of PENG (400 μM), PAAP was displaced from the MIP binding sites and the fraction bound to the polymer was equivalent to that obtained for the NIP. The largest differences between the MIP and the NIP were obtained when using 25 nM PAAP.

The imprinted polymer (19 mg) was packed in a stainless steel reactor connected to the automatic flow-through system for the subsequent measurements (see scheme in Supporting Information). As has been reported for immunoassays, the contact time between the labeled compound and the MIP may have a direct effect on the sensitivity of competitive MIAs. A PAAP solution (20 nM, 1 mL) was injected into the system at flow rates ranging from 0.125 to 1.5 mL min^{-1}. The amount of fluorescent antibiotic bound to the sorbent and the sensitivity of the assay increased as the sample flow rate decreased, but the measuring times were also longer. Thus, a flow rate of 0.25 mL min^{-1} was selected as a compromise between optimal response and minimum analysis time.

Methanol was chosen as eluting solvent, as it allowed both quantitative elution of the labeled BLA and reuse of the reactor for more than 150 cycles. The flow rate of the eluting solvent also has an important effect on the sensitivity of the assay. A strong increase in the analytical signal was observed with decreasing

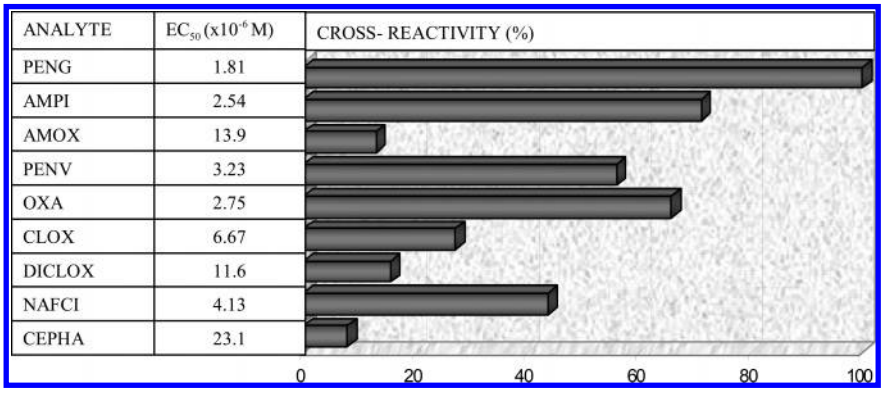

Figure 6. Cross-reactivities related to PenG when the MIP assay is conducted for an antibiotic concentration ranging from 2×10^{-8} a 9×10^{-3} M in acetonitrile:aqueous HEPES (0.1 M, pH 7) (60/40, v/v). Fluorescent competitor: 25 nM PAAP ($n = 3$).

methanol flow rates (from 1.5 to 0.125 mL min^{-1}), as the interaction with the polymer is favored and allows the disruption of binding between the PAAP, PENG, and the polymer. Finally, a flow rate of 0.75 mL min^{-1} was selected for the elution step.

Figure 5a shows a typical dose−response curve measured with the immunosensor over long measuring times. The normalized competition curves obtained for PENG standards, in acetonitrile: aqueous HEPES (0.1 M, pH 7) at concentrations ranging from 10^{-8} to 10^{-3} M, are depicted in Figure 5b. The EC_{50} value corresponds to 1.81×10^{-6} M PENG, and the detection limit was 1.97×10^{-7} M. The sensor showed a dynamic range (normalized signal in the 20−80% range) from 6.80×10^{-7} to 7.21×10^{-6} M PENG. The LOD for the analysis of PENG is much lower than that obtained using the same labels and a methacrylic acid-based molecularly imprinted polymer,[17] and only 30 times higher than that obtained using anti-PENG antibodies as the recognition element.[5] Nevertheless, the MIA provides shorter analysis times, and the cost per analysis is drastically reduced in comparison to immunoassays.

The intraday relative standard deviation ($n = 3$) was below 11% in the calibration range. Interday reproducibility was evaluated by measuring the calibration standards on different days. The IC_{50} values ranged from 1.52×10^{-6} to 2.02×10^{-6} ng mL^{-1} (RSD 10%, $n = 3$). Typical RSD values reported in the literature for immunoassays[23] are between 10−25%, and the MIA results reported here are within this range.

3. Cross-Reactivity. The specificity of the developed fluorescence MIA was evaluated in the presence of other β-lactam antibiotics. Cross-reactivity was calculated as the percentage between the EC_{50} value for PENG and the EC_{50} for the interfering compound, and the results are depicted in Figure 6. The polymer showed group specificity, and the relative cross-reactivity was most pronounced for other penicillins, such and AMPI (71%) and OXA (66%). Medium values were obtained for PENV (56%) and NAFCI (46%), and lower cross-reactivities were obtained for CLOX (27%), DICLOX (16%), and AMOX (13%). The observed cross-reactivity of penicillins to compete with PAAP for the polymer binding sites seems to be related predominantly to the hydrophobicity of the antibiotic. For instance, DICLOX is more hydrophobic than PENG (log P values 2.89 and 1.21, respectively[24]) while AMOX is much more hydrophilic (log $P = -0.12$[24]) so that both penicillins show little cross-reactivity. However, AMPI with a log P value closer to the template (1.35) displays the highest cross-reactivity. Nevertheless, shape selectivity also plays a role in the competition with PAAP. For instance NAFCI that has a log P 3.37 but still shows a cross-reactivity of 46%. Cephapirin, a cephalosporin-type β-lactam antibiotic, shows very low cross-reactivity (8%). Hence the size, geometry, and polarity of the molecules all play an important role in the recognition properties of the polymer and in the competition for the binding sites with PAAP.

Table 1. Recovery Results for the MIA and HPLC Urine Samples Analysis, Spiked with PENG at Several Concentration Levels ($n = 3$)

analyte	spiked, 10^{-6} M	MIA-FLD, 10^{-6} M[a]	HPLC-DAD, 10^{-6} M[a]
PENG	8	7 (±2)	8 (±1)
PENG	4	3.7 (±0.8)	3.9 (±0.6)

[a] $\pm ts/\sqrt{n}$ ($t_{95\%,\ n\ =\ 3}$: 4.303).

4. Sample Analysis. As a proof of concept, the optimized fluorescent MIA has been applied to the analysis of PENG in human urine samples. With this aim, a calibration curve was prepared by spiking the urine samples with increasing concentrations of PENG potassium salt in the range of 10^{-8} to 10^{-3} M. As shown in Figure 5b, there is a slight matrix effect in the analyses of urine, which interestingly and for unknown reasons consists of an increase in the dynamic range of the assay. Hence, matrix-matched calibration curves were used for the analysis of these samples. The analytical characteristics of the assay were: dynamic range 7.87×10^{-7} to 1.71×10^{-5} M, detection limit 2.97×10^{-7} M and EC_{50} 4.00×10^{-6} M.

Urine samples were fortified with increasing concentrations (4×10^{-6} and 8×10^{-6} M) of PenG and analyzed using the optimized method and HPLC-DAD as an alternative technique for validation purposes. As shown in Table 1, no significant differences at a 95% confidence limit were observed between the results obtained by both methods.

CONCLUSIONS

In conclusion an automated MIA compatible with aqueous samples has been developed. Targeting BLAs, the flow-through

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solid-phase competitive assay exhibited excellent robustness and performance when applied to biological samples. This unique performance was enabled through the use of a recently reported stoichiometrically imprinted polymer showing good target binding in aqueous media and should pave the way for the use of MIPs in routine analytical applications.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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