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UNIVERSITÀ DEGLI STUDI DI TORINO

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Keywords: molecular imprinting, Pickering emulsion, hierarchical imprinting, mimic template, penicillins

Abbreviations: AIBN: 2,2′-azo-bis-(2-methylpropionitrile), APA: 6-aminopenicillanic acid, amp: ampicillin, clox: cloxacillin, CPTMS: 3-cyanopropyltrimethoxysilane, dcl: dicloxacillin, DIPCD: N,N'-diisopropylcarbodiimide, DMF: N,N-dimethylformamide, NHS: N-hydroxysuccinimide, MAA: methacrylic acid, MIP: molecularly imprinted polymer, NIP: non imprinted polymer, oxa: oxacillin, penG: penicillin G, penV: penicillin, PTFE: polytetrafluoroethylene, TEOS: tetraethoxysilane, TRIM: trimethylolpropane trimethacrylate

Abstract: the aim of this paper was the evaluation of the binding performances and selectivity of molecularly imprinted beads prepared towards several penicillins (i) by hierarchical bulk polymerization in the pores of template-grafted silica microbeads (hMIPs) and (ii) by Pickering emulsion polymerization in the presence of template-decorated silica nanobeads (pMIPs). 6-aminopenicillanic acid was chosen as common fragmental mimic template. Both approaches produced micron-sized polymeric beads with good recognition properties towards the target ligands

whereas the selectivity pattern appeared quite different. The polymer prepared by Pickering emulsion approach showed binding properties similar to imprinted beads prepared by hierarchical approach. Equilibrium binding constants changed their values from 0.1-0.2 x 10^6 M^{-1} (hMIPs) to 0.2-0.6 x 10^6 M^{-1} (pMIPs), while the binding site densities changed from 3.7-4.8 μmol/g (hMIPs) to 0.3-0.55 μmol/g (pMIPs). Compared to the hierarchical polymerization, Pickering emulsion polymerization represents a more practical approach when a template mimic needs to be used.

Lay abstract: penicilling-binding molecularly imprinted beads were prepared by using 6-aminopenicillanic acid as mimic template through hierarchical bulk polymerization in the pores of template-grafted silica microbeads (hMIPs) and by Pickering emulsion polymerization in the presence of template-decorated silica nanobeads (pMIPs). Both approaches produced micron-sized polymeric beads with good recognition properties towards the target ligands, whereas the selectivity pattern appeared quite different. The polymer prepared by Pickering emulsion approach showed binding properties similar to imprinted beads prepared by hierarchical approach. In fact, equilibrium binding constant values doubled only, while binding site densities decreased of one order of magnitude. Anyway, Pickering emulsion polymerization resulted a more practical approach when a template mimic needed to be used.

1. Introduction

Molecular imprinting represents a popular approach to prepare synthetic materials with molecular recognition properties towards given targets [1-3]. Applications in the field of solid phase extraction requires that such materials show good binding properties and selectivity towards target molecules, but also that the polymeric matrix does not contain residual template molecules able to leach out during the SPE process to contaminate the analytical sample in the so-called “bleeding effect”. Beside efforts to individuate efficient strategies for a complete clean-up of the imprinted materials [4-5], the use of mimic templates represents a valid practical alternative [6]. In this approach, the template is not represented by the target molecule, but it is substituted by an analogue, differing minimally from the template structure but easily distinguishable during the analytical process. A variant of this approach called “hierarchical imprinting” consists in the covalent grafting of the mimic template molecule onto the pores of macroporous silica [7-9]. Then, pores are filled with a monomer/cross-linker mixture and, after the polymerization process, silica is dissolved leaving imprinted beads. This approach shows the advantage to couple the mimic template strategy (to be grafted, the template should be modified with a spacer arm, thus becoming a mimic molecule) with the direct synthesis of polymeric beads, so avoiding the cumbersome step of polymer grinding and sieving. Moreover, an additional advantage is represented by the possibility to use as grafted template a molecular substructure common to a class of analytes, obtaining an imprinted polymer with group selectivity toward molecules sharing this common structural motif. As recently shown for the aminopenicillanic acid-based antibiotics [10], this approach is particularly convenient when the main goal of the analytical process is the simultaneous isolation and subsequent quantification of related molecules similar for structure and occurrence in analytical samples.

Recently, a novel approach to mimic template imprinting has been described for β-blocker drugs which share the N-isopropylaminopropandiol molecular fragment [11]. In this approach, the template represented by that fragment is covalently grafted onto silica nanoparticles, which are then used as stabilizer of organic droplets in a Pickering-type oil-in-water emulsion. As the dispersed organic phase contains monomer, cross-linker and radical initiator molecules, the polymerization process produces imprinted beads able to bind β-blocker drugs. As stated by the authors, this approach seems to be particularly convenient as it produces micron-size imprinted beads having highly accessible binding sites and group-selectivity towards target analytes sharing a common structural motif.

The aim of the paper is to compare the binding performance and selectivity of molecularly

imprinted beads prepared towards several penicillins (i) by hierarchical bulk polymerization in the pores of template-grafted silica microbeads (hMIPs) and (ii) by Pickering emulsion polymerization in the presence of template-decorated silica nanobeads (pMIPs), in according with the approaches illustrated in figure 1. As common fragmental template for penicillins, 6-aminopenicillanic acid was chosen because it is easy to graft onto carboxylated silica surface as well as rather stable in the polymerization conditions used.

2. Materials and methods.

2.1. Materials. 6-Aminopenicillanic acid and penicillins, 3-cyanopropyltrimethoxysilane (CPTMS), N,N'-diisopropylcarbodiimide, ethanolamine, glycine, N-hydroxysuccinimide, methacrylic acid, tetraethoxysilane, tetrabultylammonium hydrogensulphate, 1,2,4-triazole, trimethylolpropane trimethacrylate were from Sigma–Aldrich–Fluka (Milan, Italy). Acetic acid, acetone, acetonitrile, ammonium hydrogen fluoride, 2,2′-azo-bis-(2-methylpropionitrile), N,N-dimethylformamide, ethanolamine, lysine hydrochloride, mercury(II) chloride, methanol, tetrahydrofurane, toluene, Tween 20 and spherical porous silica beads (PharmPrep 60CC, 25–40 μm mean diameter, mean pore size 6 nm, specific pore volume: 0.80 ml/g, specific surface area: 500 m^2/g) were from VWR International (Milan, Italy). All the solvents were of HPLC grade, other chemicals were of analytical grade.

Ultrapure water was obtained with a Purelab Prima System from Elga (Bucks, UK). Polymerization inhibitors in vinyl monomers were removed by clean up on activated alumina columns. Stock solutions of antibiotics were prepared by dissolving 25.0 mg of substance in 10.0 ml of ultrapure water and stored in the dark at −20 °C.

The derivatizing reagent for penicillins was prepared by mixing 60 ml of 230 g/l aqueous 1,2,4-triazole with 10 ml of 27.2 g/l of mercury (II) chloride, adjusting to pH 9.0 with 2M aqueous sodium hydroxide and to 100 ml with ultrapure water. The solution became rapidly turbid and was stored as is at 4 °C in the dark. Immediately before its use, it was accurately shaken and filtered on 0.22 μm membrane.

The high-performance liquid chromatography apparatus (G1312A constant-flow binary pump, G1315A UV–Vis detector, Rheodyne 7100 six-port injection valve provided with 100 μl injection loop, data acquisition and signal processing with Agilent ChemStation) was a 1100 model from Agilent Technologies (Milan, Italy).

Scanning electron micrographs were acquired with a Phenom Pro (Phenomworld, Eindhoven, The

Netherlands) scanning electron microscope operating at 15 kV in charge reduction mode and with backscattered electron imaging.

2.2. Synthesis of silica nanospheres. The synthesis of silica nanospheres was performed in according with literature with minor modifications [12]. Briefly, 326 mg of lysine hydrochloride were dissolved under sonication in an accurately cleaned 500-ml Erlenmayer flask provided of Liebig condenser and containing 260 ml of ultrapure water. The solution was heated to 60 °C, and 20 ml of TEOS were added in a single step under magnetic stirring at 550 rpm. The biphasic solution with molar composition of the reagents TEOS / lysine / water 1+0.02+162 was stirred for 24 hours. The resulting white-opalescent suspension was brought to room temperature and centrifuged in polypropylene Falcon tubes at 10000 g for 30 min with multiple volumes of 1M aqueous hydrogen chloride, ultrapure water and finally acetone. Then, silica nanobeads were dried in an oven to 50°C till constant weight and carefully transferred in PTFE vials to be stored. Particle size distribution was determined in ultrapure water, as 10 mg/ml dispersion, by using a ALV/NIBS-HPPS particle sizer equipped with an ALV-5000 multiple tau digital correlator (ALV, Langen, Germany). The measured autocorrelation function of the scattered light was processed with the CONTIN regularization algorithm [13] by using the ALV Correlator software (ver.3.0), obtaining the number weighed size distribution of the nanoparticles.

2.3. Grafting of mimic template onto silica. The grafting of 6-aminopenicillanic acid onto the surface of carboxylated silica (nanospheres or porous microbeads) was performed according to a modified protocol earlier reported in literature [10]. In a 500-ml round-bottomed flask, 10 g of silica nanospheres or silica porous beads were suspended in 100 ml of 6 M aqueous hydrogen chloride solution under sonication. The homogeneous dispersion was refluxed overnight, diluted with 400 ml of cold ultrapure water and washed by repeated centrifugation (nanospheres, 10000g for 30 min) or filtration (porous microbeads, 0.22 μm nylon membrane) with ultrapure water and toluene. Then, silica was transferred in a 250-ml round-bottomed flask containing 100 ml of toluene and dried by azeotropic distillation. 1.75 ml (10 mmoles) of CPTMS were added and the dispersion was refluxed overnight. Silanized silica was washed with toluene, acetone and water, transferred in a 250-ml round-bottomed flask containing 100 ml of 1 M aqueous sulphuric acid and refluxed overnight. The flask was cooled to room temperature and silica was washed with water and acetone. Finally, nanobeads were dried under vacuum at room temperature and stored in the dark at −15 °C.

Grafting with APA was performed on 3 g-aliquots of carboxylated silica. It was suspended in 15 ml of ice-cold anhydrous DMF, and 38 mg (0.33 mmoles) of NHS and 51 μl (0.33 mmoles) of DIPCD were rapidly added. Then, the reaction mixture was gently stirred at 4 °C for 1 h. The activated silica was washed with DMF and transferred in a 50-ml Erlenmayer flask containing 30 ml of a freshly prepared solution of APA (84 mg, 0.405 mmoles) in cold 1+1 (v/v) DMF – 0.15M bicarbonate buffer, pH 8.5. The reaction mixture was incubated at 4°C overnight. The grafted silica was washed by repeated centrifugation with DMF, ultrapure water and acetone, dried under vacuum at room temperature and stored in the dark at −15 °C.

Silica nanospheres and porous microbeads for the preparation of the non-imprinted polymers (NIPs) were treated with the same protocol, substituting APA with glycine.

2.4. Synthesis of molecularly imprinted beads by hierarchical imprinting (hMIP). A prepolymerization mixture was prepared as previously reported [10] by dissolving AIBN (1% of the vinyl groups present in the final mixture) in dried acetonitrile (10 % of the resulting total volume) adding MAA and TRIM in molar ratio 2+3, and purging with a gentle stream of nitrogen under sonication in a water bath for 5 min. In order to obtain discrete silica–polymer composite beads and to prevent particle agglomeration the amount of prepolymerization mixture added to the silica beads was slightly lower (about 5 %) than the nominal pore volume of the silica. Thus, in a 10 ml thick wall glass vial maintained under continuous sonication to remove any entrapped air bubbles, an adequate amount of the prepolymerization mixture was slowly added to 3 g of silica porous microbeads grafted with APA. Then, the mixture was homogenized with a steel spatula obtaining a free flowing powder, sparged with nitrogen, sealed and allowed to polymerize in a heating block at 60 °C for 3 days. After polymerization was completed, the silica–polymer composite was transferred into a 50 ml screw-capped polypropylene tube and 30 ml of 1+2 (v/v) acetone - 3M aqueous ammonium hydrogen fluoride solution was added. Suspension was gently stirred overnight to completely dissolve the silica matrix of the composite. After dissolution of the silica, the suspension of imprinted polymeric beads was diluted with 100 ml ultrapure water, filtered on a polycarbonate filter funnel equipped with a 0.22 μm nylon membrane and washed extensively with 9+1 (v/) methanol - acetic acid to remove any soluble components and residual template. NIP beads were prepared in the same way, except for the use of silica nanospheres blocked with ethanolamine. Complete elimination of sacrificial silica was veryfied by gravimetry after exhaustive combustion of a sample of beads in a furnace at 600 °C for 24 h.

2.5. Synthesis of molecularly imprinted beads by Pickering emulsion (pMIP). In a 25-ml thick wall glass vial 80 mg of 6-aminopenicillanic acid-grafted silica nanoparticles were suspended in 5 ml of ultrapure water containing 138 μl (1.57 mmoles) of MAA and 0.05% (v/v) of Tween 20, and sonicated for 10 min to produce a stable suspension of colloidal particles. Then, a mixture of 758 μl (2.36 mmoles) of TRIM and 14 mg (0.0865 mmoles, ~1% of the vinyl groups present in the final mixture) of AIBN were added, and the suspension was sonicated again for 10 min and mechanically shaken for 15 min (50 Hz) to give a stable Pickering emulsion (no coalescence within 3 h). After purging the emulsion with nitrogen, the vial was sealed and allowed to polymerize in a heating block at 60°C overnight. After polymerization was completed, the suspension was filtered on a 0.22 μm nylon membrane, washed with tetrahydrofurane and the resulting solid treated with ammonium hydrogen fluoride as reported in section 2.4. NIP beads were prepared in the same way, except for the use of silica nanospheres blocked with ethanolamine.

2.6. Nitrogen sorption measurements. Nitrogen adsorption/desorption isotherms were measured at 77.2K on an porosimetry analyzer ASAP 2020 (Micromeritics Instruments Corporation, Norcross, GA, USA). Before measurements, 100 mg of the samples were degassed by heating at 378 K under high vacuum (10^{-5} Pa) for 3 h. The specific mesopore surface area (S) was calculated in the p/p_0 range of 0.05–0.30 of the nitrogen adsorption isotherm using the BET theory. The volume of mesopores in the range 1.7–300 nm (V_p) was calculated from the amount of nitrogen vapor adsorbed at a relative pressure close to unity, on the assumption that all the pores are filled with liquid nitrogen. The average mesopore diameter (d_p) was calculated as $d_p = 4V_p/S$, assuming cylindrical geometry.

2.7. Equilibrium batch rebinding. About 10 mg of polymers were exactly weighed in 4-ml flat bottom amber glass vials, suspended in 0.500 ml of acetonitrile and sonicated for 10 min. Then, 500 μl of penicillin solutions (1, 1.5, 2, 6, 8, 10, 12, 14, 16, 18 and 20 μg/ml) in acetonitrile were added into the vials and incubated overnight at room temperature under continuous agitation on a horizontal rocking table. Therefore, the solutions were filtered on 0.22 μm nylon membranes, and the free amount of penicillin was measured by HPLC analysis (see section 2.7). Each experimental point of the binding isotherm was assessed as the average of three repeated measures.

The binding isotherms were calculated by using Table Curve 2D 5.0 (Systat Software Inc., Richmond, CA, USA). Non-linear least square fitting was applied to the averaged experimental data by using the Freundlich-Langmuir isotherm equation:

$$B = B_{max}(K_{eq}F)^n/(1+(K_{eq}F)^n)$$

where B is the ligand bound to the polymer, F is the ligand not bound to the polymer, K_{eq} is the equilibrium binding constant, B_{max} the binding site density and n the heterogeneity parameter. To assure robust results, Pearson VII limit minimization was chosen as minimization method. To avoid being trapped in local minima which would give incorrect results, the fitting was carried out several times by using different initial guess values for the isotherm parameters. The imprinting factors (IFs) were calculated as K_{eq}(MIP)/K_{eq}(NIP) for the penicillins used as ligands.

2.8. Ion-pair liquid chromatography. An ion-pair HPLC with pre-column sample derivatization and UV detection protocol taken from literature was modified and used to quantify free penicillins [14-15]. 100-μl aliquots of acetonitrile samples containing penicillins were added in 4-ml flat bottom amber glass vials containing 400 μl of derivatizing reagent (2M 1,2,4-triazole, 10mM $HgCl_2$). After vortexing, the mixture was incubated at 65°C for 10 min, chilled at 4°C for 20 min and injected into the HPLC column. Chromatographic quantification was made onto a 250 x 3.2 mm, 5 μm, Nucleosil C-8 column from Alltech (Milano, Italy) by injecting 100 μl of derivatized sample. Isocratic elution was performed at a flow rate of 1 ml/min with a mobile phase 0.1M phosphate buffer pH 6.5 - acetonitrile (50+50 v/v for dicloxacillin; 55+45 v/v for penicillin V, penicillin G, oxacillin, cloxacillin; 60+40 v/v for ampicillin) containing 60 mM tetrabultylammonium hydrogensulphate as ion-pair reagent. UV detection was set at 320 nm. Reference standard solutions of penicillins at concentrations of 0.10, 0.25, 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00 and 10.00 μg/ml were analysed three times consecutively and peak areas were plotted against concentration. A calibration plot was drawn by using weighted linear regression (weight = 1/conc).

3. Results and discussion.

3.1. Synthesis of imprinted microbeads. Direct targeting of the penicillin class requires imprinting approaches involving molecular structures common to the whole class as templates. Thus, such

approaches are quite different from what successfully reported in literature for single penicillins [16,17]. The use of 6-aminopenicillanic acid as common template to prepare molecularly imprinted polymers with selectivity towards the antibiotic class of penicillins has been described previously by our group [10]. In that paper, we describe the use of the hierarchical approach suitable to prepare beads fit for solid phase extraction applications without needs of grinding and sieving steps typical of bulk polymerization. By using silica nanoparticles decorated with 6-aminopenicillanic acid as dispersant agent, the Pickering emulsion polymerization approach involves the same principles, potentially producing imprinted microbeads with the same binding properties. It should be noted that this approach is greatly appealing, as the use of decorated nanobeads simplifies the MIP synthesis protocol, it is quicker, and there is no need of a careful control for the amount of pre-polymerization mixture impregnating silica beads typical of the hierarchical approach.

Preliminary experiments showed that mean size and dispersion of silica nanobeads are crucial to prepare stable Pickering emulsion. In fact, when large (>200 nm) or small but polydispersed particles were used, the stability of emulsions showed erratic behaviour. On the contrary, when we used a protocol involving lysine as co-reagent during the silica synthesis, the resulting nanobeads were small and nearly monodispersed (mean diameter 21.2±0.4 nm), and emulsion showed to be stable. As the subsequent carboxylation step did not change the nanobeads behaviour, such nanobeads were chosen to be decorated with 6-aminopenicillanic acid.

The considered synthetic methods produced polymeric beads whose morphology and size was studied by SEM. Beads prepared by the hierarchical approach (h-beads, figure 2) were spherical and polydispersed, with mean diameters comprised between 15 and 40 μm. The absence of irregular polymeric materials confirms that polymerization was confined inside the pores of the silica mold, reproducing shape and dimension of parent silica beads. Anyway, some crushed beads are visible, denouncing apparent mechanical damage during the processes of silanization or polymerization. Beads prepared by ~~the~~ Pickering emulsion approach (p-beads, figure 2) showed an apparent bimodal distribution composed of particles of about 2 μm of diameter and a minor presence of much smaller particles (>0.5-1 μm diameter), very similar to beads prepared by precipitation polymerization [18]. Imprinted and non-imprinted beads were indistinguishable, showing that, on the micrometer scale, the imprinting process has no effect on the visible polymer morphology.

Nitrogen adsorption isotherms were measured on hMIPs and pMIPs. The nitrogen adsorption plots

reported in figure 3 exhibit a typical Brunaurer's Type IV isotherm for hMIPs, confirming experimental data previouly reported for analogous materials [19]. On the contrary, pMIPs exhibit a isotherm characterized by a reduced nitrogen adsorption in comparison with hMIPs and an hysteresis loop in the adsorption–desorption process. This isotherm can be identified with a material with a limited mesoporosity. This is confirmed by the calculated differential pore distributions in the 1.7–300 nm range, where it is possible to see that hMIPs show a broad pore size distribution with a mean porosity of about 6 nm, well in the range of mesoporosity, while pMIPs show a monotonically decreasing porosity distribution, with predominating micropores and small mesopores. Comparing the specific surface area (hMIPs = 289 m^2/g; pMIPs = 55 m^2/g) and the cumulative pore volume (hMIPs = 0.61 cm^3/g ; pMIPs = 0.04 cm^3/g) it is possible to conclude that while hMIPs show the typical morphology related to the parent silica beads, pMIPs show a completely different morphology, with small surface area and limited porosity.

3.2. Binding behaviour of imprinted microbeads. As clearly shown in literature [20], the use of single-point bound-to-free data is not an affordable method to correctly characterize the binding properties of an imprinted polymer, so binding parameters calculated from appropriate isotherm models should be used instead. On this assumption, experimental binding isotherms for imprinted and non-imprinted beads were measured in acetonitrile for six different penicillins reported in figure 4 (penicillin G, penicillin V, ampicillin, oxacillin, cloxacillin, and dicloxacillin). Assuming also that such materials show a continuous affinity distribution typical of imprinted polymers prepared by a non-covalent approach [21,22], the well-known Freundlich-Langmuir isotherm model was used to calculate the equilibrium binding constant, K_{eq}, and the binding site density, B_{max}.

We hypothesized that the use of 6-aminopenicillanic acid as mimic template grafted onto the silica surface through a short spacer arm could generate imprinted binding site with molecular recognition properties directed mainly towards the [3.2.0] bicyclic structure unique to all the penicillins considered, and that different experimental approaches to the imprinting polymerization (Pickering emulsion or hierarchical imprinting) have a limited impact on binding properties.

From table 1 it is possible to see that our initial hypothesis was partially correct. In fact, by considering the equilibrium binding constants for imprinted beads, it is possible to see that the affinity values are in the 0.1-0.2 x 10^6 M^{-1} range for hMIPs whereas a net increase to 0.2-0.6 x 10^6 M^{-1} range for pMIPs was observed. Moreover, imprinted beads bind penicillins in a more pronounced way than the corresponding non imprinted beads regardless of the imprinting

approach used. As a consequence of this, the imprinting factors reported in figure 5 show that the molecular recognition for the penicillins increases from about 3- to 7-fold for h-beads and from 3- to 10-fold for p-beads with respect to the non-imprinted beads. Anyway, comparing hMIPs *vs* pMIPs, there are different selectivity patterns in the binding behaviour: hMIPs recognize better penicillin V and the more hydrophobic of the penicillins (oxacillin, cloxacillin and dicloxacillin), while pMIPs recognize better the more hydrophilic penicillins (penicillin G, penicillin V and ampicillin).

As regards the binding site densities reported in table 2, the experimental results show that there is not a significant difference between imprinted and non-imprinted beads, both for h- and p-beads, confirming what previously reported for several different imprinted systems prepared by bulk polymerization [23]. Moreover, all the penicillins bind the polymers in a relatively narrow range of values for B_{max}, thereby indicating that the ligands bind presumably the same class of binding sites directed towards the [3.2.0] bicyclic structure common to all the penicillins. As in the case of the equilibrium binding constants, the binding site densities calculated for hMIPs are higher than about one order of magnitude if compared to pMIPs.

Finally, increased equilibrium binding constants and decreased binding site densities for pMIPs respect to hMIPs can be explained by considering the differences in the imprinting approaches. The hierarchical approach is based on the use of template molecules grafted onto the inner surfaces of the parent silica pores. The resulting mesoporous imprinted beads show a high specific surface area (289 m^2/g) but a relatively low binding site density in the order of magnitude of 10 $nmol/m^2$. Also, binding kinetics are slow because of the necessity for the ligands to diffuse into the mesopores before to have any interaction with the binding sites. On the contrary, in the Pickering emulsion approach the template molecules are grafted onto the surface of the silica nanoparticles which act as emulsion stabilizer. As a consequence, the binding sites will form only on the outer surface of the imprinted beads. Anyway, considering the limited specific surface area available (55 m^2/g), the mean binding site densities will result comparable than those measured for hMIPs, in the order of magnitude of 5-9 $nmol/m^2$, though the equilibrium binding constants result higher as effect of the better binding site accessibility for the ligand molecules.

It should be observed that the results obtained with hMIPs and pMIPs differs dramatically from previous results obtained by Urraca *et al.* [16,17]. In fact, even if binding site densities result of 2 (hMIPs) and 3 (pMIS) orders of magnitude lower, the affinity constants are much more higher and the selectivity patterns are completely different. Anyway, these differences are not unexpected, as the imprinting approach considered by Urraca *et al.* and based on the combination of a traditional

non convalent imprinting with a designed functional monomer is deeply divergent in aims and methodology from the approaches considered in this work. Thus, lower binding site densities can be explained by taking in account the template confinement onto the oil-in-water droplets (pMIPs) or at the surface of the silica pores (hMIPs), while higher affinity constants could depends from a better accessibility of the binding sites as effect of their surface confinement, and different selectivity is a direct consequence of the fragmental imprinting vs. template target approaches.

4. Conclusions.

In this work we prepared two different 6-aminopenicillanic acid-imprinted beads by hierarchical bulk polymerization and by Pickering emulsion polymerization. Both the approaches produced micron-sized polymeric beads with good recognition properties towards the target ligands with respect to the corresponding non imprinted beads. Considering the possible practical application in the field of solid phase extraction, the beads prepared by the Pickering emulsion polymerization show binding properties essentially preserved if compared to imprinted beads prepared by the hierarchical approach and before described as solid phase extraction materials for penicillins. In fact, the equilibrium binding constants changed their values from 0.1-0.2 x 10^6 M^{-1} to 0.2-0.6 x 10^6 M^{-1}, while the binding site densities decreased only from 3.7-4.8 μmol/g to 0.3-0.55 μmol/g. Anyway, the Pickering emulsion polymerization can represent a more practical approach when a template mimic needs to be used. In fact, the experimental protocols to prepare the template-decorated silica nanobeads and, subsequently, the oil-in-water emulsion are more simple than the lengthy protocols which imply the grafting of the same template into the pores of the parent silica microbeads, the pore filling with the pre-polymerization mixture and the synthesis of the imprinted silica-polymer composite. Thus, it is possible to envisage the use of Pickering emulsion approach to the straightforward preparation of template-mimic imprinted beads for analytical and separative applications.

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Tables.

Table 1: equilibrium binding constant (K_{eq}, x 10^{-6} M^{-1}) measured for MIPs and NIPs prepared by hierarchical approach and Pickering emulsion

	hMIP	hNIP	pMIP	pNIP
penG	0.12 ± 0.03	0.03 ± 0.02	0.56 ± 0.02	0.053 ± 0.04
penV	0.16 ± 0.09	0.02 ± 0.01	0.33 ± 0.06	0.055 ± 0.03
amp	0.15 ± 0.03	0.05 ± 0.01	0.57 ± 0.1	0.060 ± 0.02
oxa	0.20 ± 0.08	0.03 ± 0.02	0.32 ± 0.05	0.069 ± 0.04
clx	0.11 ± 0.03	0.02 ± 0.01	0.21 ± 0.07	0.067 ± 0.07
dcl	0.12 ± 0.07	0.02 ± 0.01	0.27 ± 0.03	0.093 ± 0.06

Table 2: binding site density (B_{max}, μmol/g) measured for MIPs and NIPs prepared by hierarchical approach (h) and Pickering emulsion (p)

	hMIP	hNIP	pMIP	pNIP
penG	4.5 ± 0.4	3.8 ± 0.8	0.51 ± 0.01	0.56 ± 0.2
penV	4.8 ± 1	4.8 ± 1	0.55 ± 0.03	0.59 ± 0.1
amp	4.4 ± 0.4	3.9 ± 0.5	0.31 ± 0.02	0.57 ± 0.09
oxa	3.7 ± 0.6	4.1 ± 0.9	0.29 ± 0.02	0.29 ± 0.07
clx	4.2 ± 0.6	4.5 ± 1	0.38 ± 0.04	0.29 ± 0.1
dcl	4.1 ± 1	4.8 ± 2	0.33 ± 0.02	0.28 ± 0.07

Legend of figures.

Figure 1. Schematic illustration of the experimental protocols used to prepared imprinted beads by hierarchical (h-beads) and Pickering emulsion (p-beads) approaches.

Figure 2. Scanning electron micrograph of imprinted polymer microbeads prepared by hierarchical polymerization (hMIP) and Pickering emulsion polymerization (pMIP).

Figure 3. Low temperature (77 K) nitrogen adsorption (open symbols) and desorption (gray symbols) isotherms for hMIP (circles) and pMIP (squares). In the insert: pore size distribution in the 1.7–300 nm range computed from the desorption branch of the isotherm by the BJH method for hMIP (circles) and pMIP (squares).

Figure 4. Chemical structures of penicillins used as ligands in the determination of experimental binding isotherms. In red colour the common 6-aminopenicillanic acid sub-structure.

Figure 5. Imprinting factors (IFs) calculated as K_{eq}(MIP)/K_{eq}(NIP) for the penicillins used as ligands. Red bars: IFs for hMIPs; green bars: IFs for pMIPs.