Noncovalent Imprinted Microspheres: Preparation, Evaluation and Selectivity of DBU Template

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ABSTRACT: 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) imprinted polymer was prepared as microspheres by precipitation polymerization method to obtain molecular recognition systems based on the noncovalent interactions between DBU template, methacrylic acid (MAA), and ethylene .glycol dimethacrylate (EDMA) in acetonitrile. ^{1}H NMR analysis of DBU/MAA mixture has been performed and hydrogen bonding interactions have been established. Microspheres have been characterized by FTIR studies with evidence of DBU linkage in polymer particles and by Scanning Electron Microscopy (SEM) to study their morphological properties. How pH values affect the binding capacity of imprinted polymer during the binding stage has been also discussed and results suggest that imprinted poly-(MAA-EDMA) behavior is related to the influence of DBU basicity during rebinding processes and the optimum pH value for binding has been found around neutral range. Binding ability of the imprinted polymer towards different concentration of DBU buffered solutions has been evaluated and compared with binding ability of the non-imprinted polymer. A more sensitive response to the template in the imprinted system suggests that a reasonable number of specific binding sites is formed. Finally, differential selectivity towards other less strong than DBU nitrogen bases, such as pyridine, imidazole, and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) has been also discussed. Our results indicate that both specific sites and basic properties are involved in the rebinding process. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 2190–2197, 2007

Key words: molecular imprinting; 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU); templates; precipitation polymerization; electron microscopy

INTRODUCTION

Molecularly imprinted polymers (MIPs) are synthetic polymers usually obtained by polymerization of functional and cross-linking monomers in the presence of a target molecule (template) capable of forming complexes with the monomer (Fig. 1). Thus, during the polymerization process the template is incorporated in the polymer, forming in this way MIPs. Then, the template is removed from the polymer by washing procedure, so that definite cavities are left, the shape and size of which are similar to the template molecules. The resultant polymer can exhibit high affinity towards the target molecule which can be selectively re-bound (Fig. 1). MIPs have gained increasing research interest during the past years, since they have been considered a useful approach for molecular recognition applications in various analytical areas,[1–4] such as solid-phase extraction,[5–8] chromatography,[1,9,10] capillary electrophoresis,[11,12] assays and sensors,[13,14] catalysis,[15,16] and so on.

One of the most important features in the preparation of MIPs is the interaction between template and monomer. Literature data point out three different ways of interaction: covalent, noncovalent, and semicovalent. In the first one, lead to covalent imprinting systems, templates and monomers are covalently bound so that a chemical process is needed during extraction and re-binding steps.[17,18] Although this interaction permits a high degree of specificity, the rebinding process is slow and complex. The second one, the noncovalent approach, was first introduced by Mosbach and coworkers.[19,20] In this case a physical process occurs, involving hydrogen-bonding, electrostatic or π–π interactions. Although this is considered the most straightforward and flexible method it may generate heterogeneous binding sites due to weak interactions involved. The last one, the semicovalent approach, consists in covalent imprinting with noncovalent re-binding.

Traditionally, MIPs have been synthesised using bulk polymerization which lead to macroporous monolith polymers. Unfortunately this procedure is time-consuming and provides only moderate amounts of useful imprinted polymer, since bulk polymer must be ground and sieved to obtain particles of a suitable

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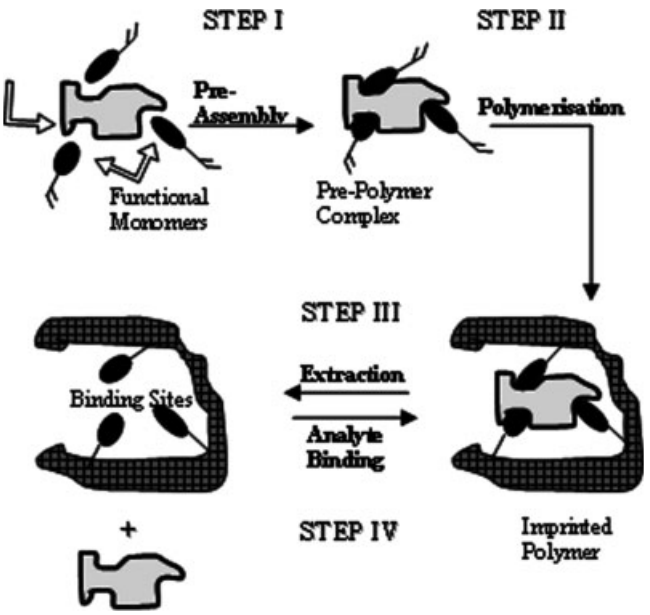

Figure 1 Scheme of molecular imprinting process.

size. Other procedures with the aim of regulating MIPs particle morphology have been developed, including suspension polymerization,[21] multistep swelling polymerization, sol–gel imprinting,[22] and precipitation polymerization.[23,24] Precipitation polymerization is an economical and labor-saving method to obtain homogeneously sized MIP microspheres.[25–27] Moreover it is not affected by the addition of surfactants or stabilizers needed in dispersion or suspension polymerization methods. In the precipitation technique polymerization is carried out using higher amount of a porogenic solvent than in the bulk polymerization procedure. In this diluted system a dispersion of microgel particles is formed, then the polymer is easily recovered by washing and centrifugation since no grinding or fractioning operations are needed. Usually methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EDMA) or trimethylol-propane trimethacrylate (TRIM) as cross-linking monomer are employed.

For most molecular imprinting approaches templates are usually small molecules, such as amino acids, sugars, oligo-peptides or steroids.[28]

It is well known that DBU belongs to the class of amidine compounds having in their structure an amino and an imino group bound to the same carbon atom. Amidines are strong bases used as drugs and they have interesting role in biological research.[29,30]

DBU as hindered non-nucleophilic strong base is employed in many different organic reactions such as base-induced intra and inter molecular dehydrohalogenations or eliminations,[31,32] introduction and removal of certain protecting groups,[33,34] and phthalocyanines and related macrocyclic compounds formation by cyclotetramerization of aromatic 1,2-dinitriles precursors.[35]

More recently, some researchers have focused their studies on DBU ability to create nucleophilic interactions with organic molecules.[36,37] In this context we have recently characterized novel Zinc phthalocyanine complexes, in which DBU[38] acts as bulky axial ligand.

In the present study, we have chosen DBU molecule as model ligand for understanding the behavior of a strong organic base within imprinting systems and therefore to investigate potential applicability of MIP systems in amidine recognition. This would represent the first attempt of using DBU as template in molecular imprinting system. So we report here an example of noncovalent imprinting technique for molecular recognition system, using precipitation polymerization of methacrylic acid (MAA) employed as functional monomer and ethylene glycol dimethacrylate (EDMA) as cross-linker, in a diluted acetonitrile solution and in the presence of DBU as template. Polymeric microspheres obtained in this way have been characterized by FT IR and SEM studies and ^{1}H NMR analysis of DBU/MAA mixture has been also performed in order to investigate DBU behavior within imprinting systems,. In addition to this, how the pH values affect the binding capacity of the imprinted polymer during the binding stage has been discussed.

The binding ability of MIP system towards different concentration of DBU buffered solutions has been evaluated by spectrophotometric analysis. As a control, binding capacity of DBU imprinted poly -(MAA-EDMA) and non-imprinted poly-(MAA-EDMA) have been compared. Finally, the differential selectivity towards other nitrogen bases, such as pyridine, imidazole and DBN was also discussed.

EXPERIMENTAL

Reagents

DBU (>98%), pyridine (>99.9%, HPLC grade), imidazole (>99%), DBN (>98%), methacrylic acid (MMA, >99%), ethylene glycol dimethacrylate (EDMA, >98%), and azobis(isobutyronitrile) (AIBN, >98%), were purchased from Aldrich and used as received. Buffer solutions were prepared from sodium dihydrogen phosphate monohydrate (Fluka, >99%, ACS grade) and phosphoric acid (85% wt solution in water, Aldrich A.C.S. reagent) using hydrochloric acid (Baker, 36–38%, analyzed grade) or sodium hydroxide (Fluka, >98%, pellets) to adjust the pH to the desired value. Distilled water was used after purification by an ultrapure water system model EASYpure II from Barnstead International. Acetonitrile (MeCN) (Baker, analyzed grade) was dried by leaving overnight under

molecular sieves and then distilled on calcium hydride before use. All other solvents (Baker, analyzed grade) were used without further purification.

Apparatus

Sonication was carried out using a Sonorex RK 102H ultrasonic water bath from Bandelin Electronic. Centrifugation was achieved with a PK121 multispeed centrifuge from Thermo Electron Corporation. A rocking table Type Rotamax 120 from Heidolph Instrument was used for shaking incubated mixtures. Absorbances were measured by UV Visible spectrophotometer type Cary 100 scan (Varian). FTIR spectra were recorded on a JASCO IRT 30 infrared microscope spectrometer equipped with a MCT detector. Scanning Electron Microscopy observations were carried out on a JEOL JSM 6500 F microscope, equipped with a field emission source.

Polymers preparation and template removal[26]

Synthesis of DBU imprinted microspheres (imprinted poly-(MAA-EDMA)) was carried out following the method previously described by Jiang and Tong[39] and slightly modified by us. 6.2 mmol EDMA, 0.43 mmol DBU, 1.55 mmol MAA, and 0.15 mmol AIBN were added to 40 mL acetonitrile in a 100 mL three necks round bottom flask. The solution was first sonicated for 5 min, saturated with nitrogen, and then kept at 60°C for 22 h to allow polymerization. After cooling at room temperature, the reaction mixture was sonicated for further 5 min and the microspheres formed were separated by centrifugation at 8000 rpm for 10 min. The template included in the microspheres was removed by washing several times with 90 mL of methanol/2.47 pH phosphate buffer (95/5, v/v) solution until DBU signal at 217 nm was no more detected. Microspheres were finally rinsed twice with acetone and then dried under vacuum for 48 h. Poly-(MAA-EDMA) was stored under vacuum to avoid any contamination.

As a control, non-imprinted microspheres (non-imprinted poly-(MAA-EDMA)), following the same procedure described earlier except for the template, were also prepared.

Calibration curves and Binding experiments

To evaluate the amount of template extracted during the washing step and the amount of template bound in the binding stages, calibration curves reporting absorbance versus template concentration were prepared.

In a polypropilene tube, 30 mg of imprinted poly-(MAA-EDMA) were suspended in 4.0 mL of MeCN/phosphate buffer 2.2, 7.2, or 11.0 pH solution (60/40, v/v) containing DBU at well known concentration (concentration range was from 8×10^{-4} to 2×10^{-2} mol/L). The mixture was sonicated for 3 min to promote polymer dispersion and incubated for 20 h using a rocking table working at room temperature and 75 rpm. After centrifugation at 8000 rpm for 10 min and separation of polymer microspheres, the mixture was filtered through a 0.22 μm porosity PTFE filter. DBU concentration in the solution after the binding process was determined by measuring the absorbance at 217 nm and the result was compared with concentration before incubation. The same procedure was followed for non-imprinted poly-(MAA-EDMA) as a blank reference. Analogously to DBU, solutions of pyridine, imidazole, or DBN at the concentration of 1.8×10^{-3} M in MeCN/7.22 pH phosphate buffer solution (60/40, v/v) were incubated with microsphere polymer and treated as already reported. Pyridine, imidazole, and DBN concentrations in the solution after the binding process were determined by measuring the absorbance at 200 nm, 205 nm, and 212 nm, respectively and compared with the initial concentration. Binding processes and measurements were performed in triplicates and their average binding percentage were calculated.

RESULTS AND DISCUSSION

MIPs performances depend on many parameters such as cross-linking density, monomer/template ratio,[40] temperature, type and concentration of monomers and solvent. Recently many attempts have been made to improve the comprehension of the mechanisms of template/monomer interactions by monitoring these parameters. In this study we have focused our attention on binding capacity evaluation considering pH values and influence of template concentration.

Polymer synthesis

To study DBU behavior in imprinted systems, we have chosen precipitation polymerization method, recently introduced by Mosbach,[26] and successively optimized by Jiang and coworkers. So that, DBU as template, methacrylic acid (MAA) as functional monomer, and ethylene glycol dimethacrylate (EDMA) as cross-linker in a diluted solution of acetonitrile, as porogenic solvent, has been used for the preparation of the MIPs. Acetonitrile, which is commonly used in imprinting polymerization, is also able to solve DBU. In the first step of the process DBU and MAA has been mixed together and a noncovalent interaction between the nitrogen centre of DBU and the carboxylic group of methacrylic acid occurs. Once all the other reactants had been added to the reaction mixture the polymerization has been started up by heating at 60°C. It is worth noting that all the reaction

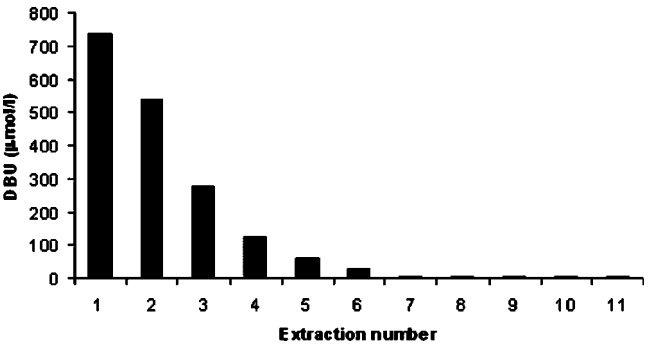

Figure 2 Extraction profile of DBU from imprinted poly-(MAA-EDMA) during washing procedure.

parameters should be accurately defined to grant a good polymerization process. In our experience, no coalescent product is observed working at temperatures lower than 60°C; nevertheless higher working temperatures lead to a bulk polymer.

At the end of the reaction the solvent has been removed from the polymer by centrifugation obtaining imprinted poly-(MAA-EDMA) microspheres. Then, the template has been taken out by washing the microspheres for several times with methanol/phosphate buffer solution. The microspheres has been washed until no more template was detected at 217 nm. As an example, Figure 2 reports DBU concentration (mmol/L) in solution after each extraction. Histograms show a typical extraction trend: high values of DBU extracted at the beginning, low values after several extractions. Usually after 8–9 extractions DBU amount is no more significantly detectable.

As a control, non-imprinted microspheres (non-imprinted poly-(MAA-EDMA)) have been also treated

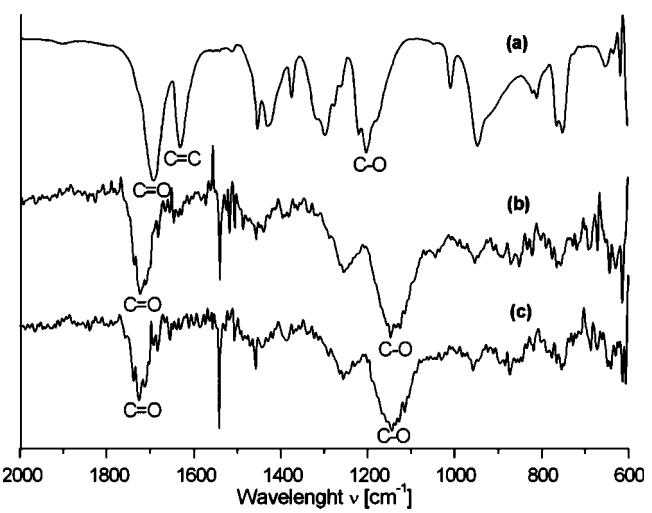

Figure 3 FTIR spectra of methacrylic acid (MAA) (a); non imprinted poly-(MAA-EDMA) (b) and imprinted poly-(MAA-EDMA) after DBU extraction (c).

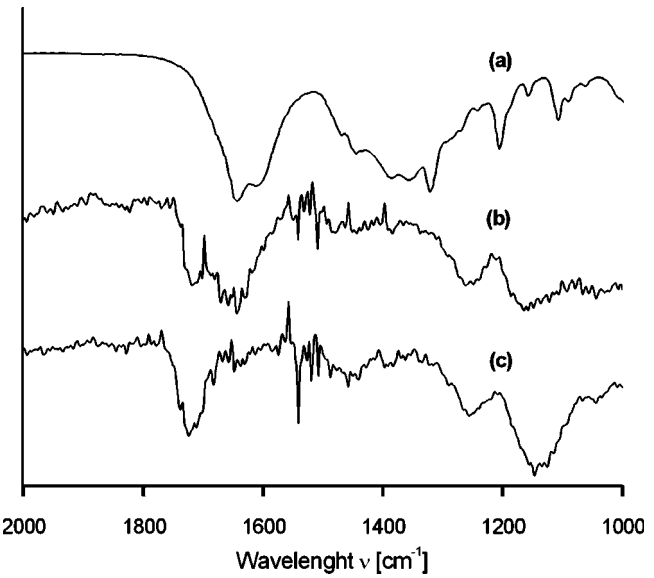

Figure 4 FTIR spectra of DBU (a); dried imprinted poly-(MAA-EDMA) after binding of DBU (b) and dried imprinted poly-(MAA-EDMA) after DBU extraction (c).

in the same way as imprinted poly-(MAA-EDMA) except for the template.

Polymer characterizations

Polymer characterization has been made by FTIR and SEM analysis. Spectra have been recorded directly on dried powder without any treatment. Figure 3 shows FTIR spectra of methacrylic acid (MAA) (a), non imprinted polymer (b), and imprinted polymer after DBU extraction (c). In MAA spectrum it is possible to

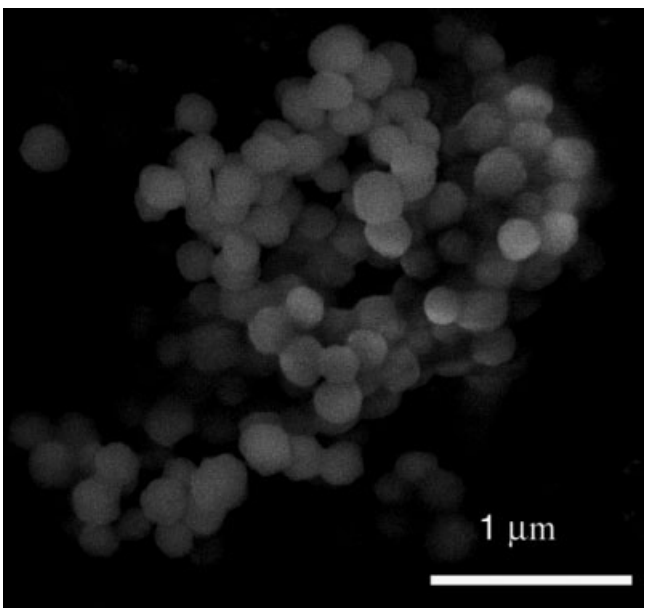

Figure 5 Scanning electron microscopy (SEM) image of non-imprinted poly-(MAA-EDMA).

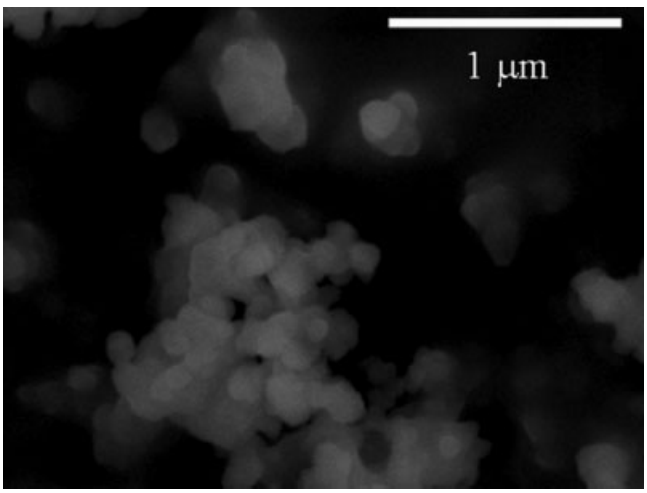

Figure 6 Scanning electron microscopy (SEM) image of imprinted poly-(MAA-EDMA).

observe a strong C=O band centred at 1694 cm^{-1} (typical of conjugated carboxylic acid), a C=C band centred at 1633 cm^{-1} (due to carbon double bond stretching) and an other band at 1203 cm^{-1} (due to C—O stretching).[41]

Polymer spectra showed in Figure 3(b,c) are similar to each other but different from monomer spectrum (Fig. 3a). In agreement with loss of conjugation, peak relative to the C=O stretching is shifted to 1725 cm^{-1}. A loss of conjugation is ascribable to cross-linking reactions as also confirmed by a decrease of carbon double bond stretching peak at 1633 cm^{-1}.

In Figure 4 FTIR spectra of DBU (a), dried imprinted poly-(MAA-EDMA) after binding of DBU, (b) and dried imprinted poly-(MAA-EDMA) after removing DBU (c) are reported. It is possible to observe that in imprinted poly-(MAA-EDMA) after incubation with DBU solution a strong band appears [Fig. 4(b)] and this could be an evidence of DBU linkage in the polymer since DBU spectrum shows its stronger absorbance in the same region.

In Figure 5 a secondary electron SEM image of non-imprinted poly-(MAA-EDMA) is reported. The image shows spherical particles, which exhibit a very narrow size distribution peaked at about (18 ± 3) cm^{-2} μm. Figure 6 is the relevant image from imprinted poly-(MAA-EDMA). Particle size distribution is wider when compared with non-imprinted poly-(MAA-EDMA) and peaked at about (10 ± 43) cm^{-2} μm.

Studies on the interactions between template and functional monomer

To evaluate interactions between DBU template and MAA functional monomer we have performed ^1H NMR measurements and rebinding studies at different pH values.

Firstly ^1H NMR spectrum of DBU/MAA mixture, in the same ratio utilized for polymer synthesis, has been performed in $CDCl_3$ and a comparison of MAA, DBU, and DBU/MAA mixture signals is reported in Table I. As evidence of complex formation, all DBU signals in the mixture are shifted downfield, while almost all protons of MAA appear to be shielded; no additional proton signals have been revealed. Wiench et al.[42] in 1999 reported that protonation of imine site of DBU occurs in the case of DBU/CF_3COOH (TFA) mixture observing in ^1H NMR spectrum an additional signal at around 8.6–8.0 ppm assigned to protonation of the DBU imine nitrogen site. Considering these data, we have performed ^1H NMR analysis of DBU/TFA mixture in the conditions that we have used for DBU/MAA spectrum. In the case of DBU/TFA mixture, in good agreement with Wiench et al. observations, we have found a new signal at 8.7 ppm, ascribable to protonation of DBU imine site. On the contrary, in the case of DBU/MAA mixture, the absence of new proton signals suggests that N protonation does not occur then the interaction between acid and base could be mainly based on hydrogen-bonding.

Secondly how the pH values affect the binding capacity of imprinted polymer during the binding stage has been studied. We have carried out binding tests using 1.8 × 10^{-3}M of DBU solution at different pH values. To ensure that pH value do not change during incubation processes, we have used aqueous phosphate buffered solution and MeCN. It is worth noting that, as reported in the literature,[43] addition of MeCN in buffered water solution increases pH values. For example when MeCN is mixed with buffered water (7.2 pH Phosphate buffer) in the mixture ratio

TABLE I
^1H NMR Signals of MAA, DBU, and DBU/MAA Mixture

Compound	NMR signals (ppm) assigned to MAA protons	NMR signals (ppm) assigned to DBU protons
MAA	12.29 (b, 1H), 6.34–6.12 (m, 1H), 5.79–5.48 (m 1H), 1.87 (m, 3H)	/
DBU	/	3.31–3.23 (m, 2H), 3.23–3.14 (m, 4H), 2.43–2.33 (m, 2H), 1.85–1.74 (m,2H), 1.71–1.61 (m,4H) 1.61–1.51 (m,2H)
DBU/MAA mixture	11.99 (b,1H), 6.24–5.99 (m, 1H), 5.63–5.47 (m, 1H), 1.92 (m, 3H)	3.55–3.30 (m, 6H), 2.91–2.75 (m,2H), 2.045–1.97 (m, 2H), 1.79–1.60 (m, 6H)

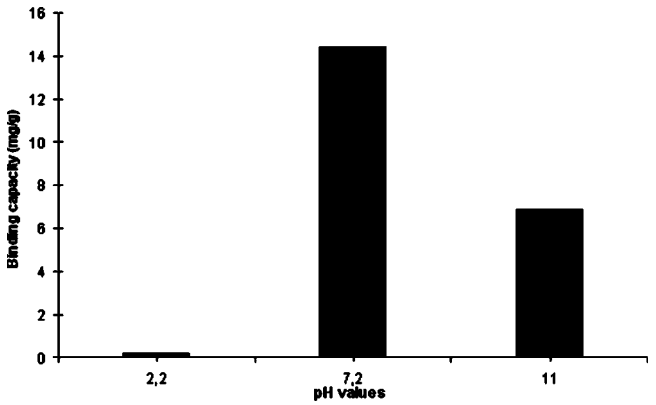

Figure 7 Binding capacity versus pH of DBU solution in the binding stage.

of 60/40 (v/v), final pH reaches 8.3 value. Taking this into account, we have chosen aqueous buffers at three different pH values (2.2, 7.2, and 11.0) mixed with MeCN in 60/40 v/v ratio following the procedure described in the experimental section in order to compare binding capacities.

The results have been resumed in Figure 7. It is possible to note that MIP exhibit the best binding capacity around neutral values while it decreases at high pH values and no binding capacity is shown at low pH values. These results suggest that imprinted poly-(MAA-EDMA) behavior at different pH values is related to the influence of DBU basicity during the rebinding process. This is in accord with literature data reported for other templates[44] in which the optimum of pH is around neutral value where the H^+ or OH^- concentration is minimal.

It is well known that DBU is a very strong base with 24.13 pKa value in acetonitrile.[45] Even if we cannot calculate its exact value in our system, the behavior observed at different pH lead to the following conclusions: around neutral pH values the base (DBU) and the acid (MAA) could be in the optimum conditions to form hydrogen bond; at higher pH values MAA probably become deprotonated and the hydrogen bond can not be formed while at lower pH DBU could be protonated.

Binding capacity evaluation

Considering the results achieved, we have chosen to work at around neutral pH values using MeCN/7.22 pH buffer (60/40, v/v) as binding solution and following the usual procedure to study binding capacity.

In Figure 8 binding capacity versus DBU concentration is reported. Solid line is referred to imprinted poly-(MAA-EDMA) binding capacity whereas dash line is referred to non-imprinted poly-(MAA-EDMA) binding capacity.

Non-imprinted poly-(MAA-EDMA) shows reasonable binding ability, probably due to non-specific binding sites, in agreement with previous results reported in the literature. In fact, it has been observed that non-specific binding sites are still formed where noncovalent approach is used and this represents one of the limits of this procedure. However, imprinted poly-(MAA-EDMA) shows a more sensitive response to the template. This suggests that during the polymer synthesis a reasonable number of specific binding sites are also formed in addition to non-specific binding sites.

A comparison between the amount of DBU entrapped in the polymer and saturation data has been done. 24 mg as DBU amount extracted for each gram of polymer during washing procedure has been calculated from Figure 2. A specific binding capacity of 35 mg of DBU for each gram of polymer, ascribable

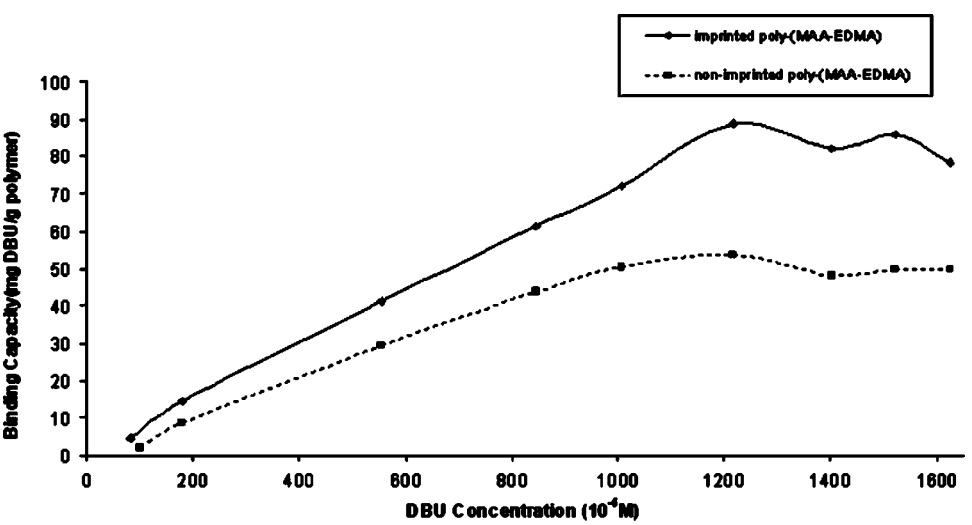

Figure 8 Binding capacity in MeCN/7.22 pH buffer (60/40, v/v)for imprinted poly-(MAA-EDMA) (◆) and non-imprinted poly-(MAA-EDMA) (■) versus DBU concentration.

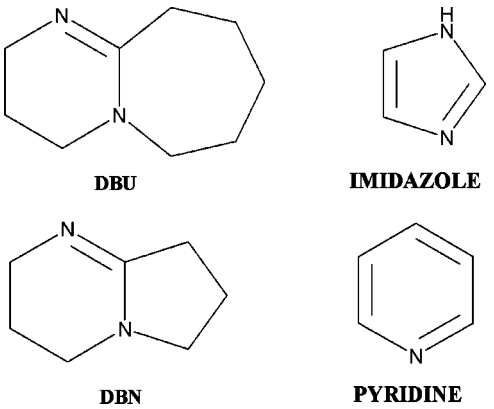

Figure 9 Chemical structure of bases used for selectivity studies.

to the presence of specific binding sites in the polymer, has been calculated as the difference between maximum MIP binding capacity and maximum NIP binding capacity arise from Figure 8. These results are comparable with the value calculated from washing procedure.

Moreover, to confirm the specificity of DBU binding we have also considered studies on the selectivity (see next paragraph).

Selectivity evaluation

Imprinted poly-(MAA-EDMA) selectivity towards DBU molecule in comparison with other three nitrogen bases, such as DBN, pyridine, and imidazole, has been investigated.

It is worth noting that DBN structure is similar to DBU (Fig. 9), but different from imidazole and pyridine structure which are also weaker bases when compared with DBU and DBN. So 1.8×10^{-3} M solution of DBN, pyridine, and imidazole have been incubated with imprinted poly-(MAA-EDMA) using the same procedure employed for DBU and resulting binding abilities are shown in Figure 10.

It is clear that the best binding results are obtained for DBU. DBN shows a significant affinity towards imprinted poly-(MAA-EDMA), but lower than DBU. Imidazole and Pyridine did not show any affinity towards imprinted poly-(MAA-EDMA).

Considering that DBN, imidazole, and pyridine are smaller than DBU, the results obtained suggest also that re-binding process depends on specific interaction and cavity shape more than cavity size.

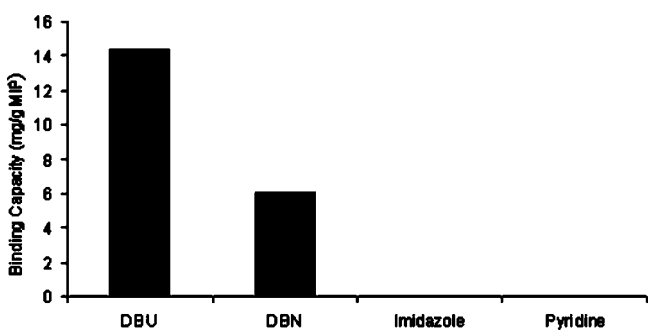

Figure 10 Binding capacity of imprinted poly-(MAA-EDMA) versus different nitrogen bases.

CONCLUSIONS

Noncovalent imprinted microspheres with DBU template have been prepared and their activity as imprinted systems has been demonstrated. ^{1}H NMR analysis of DBU/MAA mixture has been performed and hydrogen bonding interactions have been established.

We have found that the behavior of imprinted poly-(MAA-EDMA) at different pH values is related to the influence of the DBU basicity during the rebinding process. The optimum pH value has been observed around neutral values, which grant the optimal conditions for hydrogen bonding.

Higher binding capacity of imprinted poly-(MAA-EDMA) in comparison with non-imprinted poly-(MAA-EDMA) has been observed. Thus, a reasonable number of specific binding sites are formed during the polymer synthesis of imprinted poly-(MAA-EDMA) in addition to non-specific binding sites.

Moreover, the specificity of imprinted polymer towards DBU has been also confirmed by considering its binding capacity towards other similar bases such as DBN, imidazole, and pyridine. Finally, it is possible to conclude that specific sites in the imprinted poly-(MAA-EDMA) and also basic DBU property are both involved in the rebinding process.

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