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ORIGINAL ARTICLE

Molecularly imprinted polymers for histamine recognition in aqueous environment

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Abstract Molecularly imprinted polymers (MIP) for histamine using methacrylic acid were developed and recognition mechanisms were thoroughly characterized for the first time in this study. The binding affinity of imprinted polymer with structurally related compounds was studied in organic and aqueous media, at various conditions. In organic media, MIP was found to bind histamine two and six times more than ranitidine and fluoxetine, respectively, whereas higher selectivity was observed in the case of dimentidene or disodium cromoglycate. The specific binding sites of MIP recognized histamine over L-histidine in aqueous conditions, while higher affinity for histamine compared to ranitidine, disodium cromoglycate, putrescine and to a putrescine analogue was observed. A combination of NMR and UV spectroscopy analyses for investigation of imprinting and recognition properties revealed that strong specific interactions between the functional monomer and histamine in the prepolymerization and in the aqueous solutions were probably responsible for histamine recognition. The preparation of histamine MIPs and elucidation of imprinting and recognition mechanism may serve as useful insight for future application of MIPs.

Keywords Aqueous assay · Histamine · Molecular imprinting · Molecular recognition

Abbreviations

L-His	L-Histidine
MIP	Molecularly imprinted polymer
REF	Reference polymer
PUT	Putrescine
HI	Histamine
SPMD	Spermidine
SPM	Spermine
MAA	Methacrylic acid
EDMA	Ethylene glycol dimethacrylate
TRIM	Trimethylolpropane trimethacrylate
SPE	Solid phase extraction
MISPE	Molecularly imprinted solid phase extraction

Introduction

Molecular imprinting is a technique widely used for the preparation of polymeric materials containing recognition sites of predetermined specificity. Imprinted binding sites are generated by co-polymerization of a functional monomer with a cross-linking monomer in the presence of a template molecule. The functional monomer interacts with the template by non-covalent or covalent bonding, forming a stable complex during polymerization. Removal of the template results in cavities, within the polymer, which are complementary to the template. Consequently, the polymer is able to rebind the template with high affinity and specificity, in a manner comparable to antibody–antigen or receptor–ligand binding (Mosbach and Ramström 1996; Haupt and Mosbach 2000; Ye and Haupt 2004).

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The chemical and physical stability of molecularly imprinted polymers in both aqueous and organic solvents, their capability to remain intact at extreme pH and temperature conditions and their reusability revealed their promising applications in the field of biocatalysis, diagnostics (Hilt and Byrne 2004), bioseparations (Wei et al. 2006; Greene and Shimizu 2005), biomimetic sensors (Kempe and Mosbach 1995; Watabe et al. 2005), affinity chromatography (Sellergren 1994; Yu and Mosbach 1998), drug delivery systems (Moral and Mayes 2006), and as artificial antibodies in immunoassays (Vlatakis et al. 1993).

In non-covalent approaches, multiple interactions between templates and functional monomers are essential for the formation of ligand-selective high affinity binding sites. Non-covalent interactions such as hydrogen bonding, π–π and electrostatic interactions, hydrophobic effect and metal–ion coordination, can be exploited to organize the functional monomers around the template (Xu et al. 2007). In most instances, the binding of ligands is more efficient using organic solvents, which can be beneficial in situations where the analyte is poorly soluble in aqueous buffers (Papaioannou et al. 2007). The need of artificial receptors that recognize biologically important molecules has directed research towards polymer synthesis in more aqueous systems (Rachkov et al. 2004) and enhancement of recognition ability in water (Xu et al. 2007; Andersson 1996).

The present study describes the synthesis of MIP against histamine, a biogenic amine of biological significance, eliciting its effects through four identified receptors (Ohtsu 2008). Histamine is also a critical marker of food quality, being an indicator of bacteria contamination (Santos 1996). Molecular imprinting of histamine has been reported earlier (Allender et al. 2000; Hart and Shea 2001; Tong et al. 2002; Dmitrienko et al. 2006) and MIP-sensors has been generated (Pietrzyk et al. 2009; Horemans et al. 2010; Bongaers et al. 2010; Broeders et al. 2011). The influence of pH on the affinity of a MIP-sensor has been tested (Bongaers et al. 2010) but without clarifying the nature of binding forces (Tong et al. 2002; Dmitrienko et al. 2006; Pietrzyk et al. 2009; Horemans et al. 2010; Bongaers et al. 2010; Broeders et al. 2011). In the present study, the resulting polymers were analyzed in aqueous media and the effects of pH and ionic strength upon ligand-binding affinity were investigated. Furthermore, this is the first time that MIPs' affinity is tested against other biogenic amines. Finally, previous study was conducted with histamine antagonists (Allender et al. 2000) and histidine (Bongaers et al. 2010) but affinity was never thoroughly tested in complete aqueous environment. The major goals of this work were as follows: (1) to prepare and study MIPs as adsorption materials against the template molecule of histamine; (2) to elucidate the imprinting and recognition mechanism between histamine and the corresponding MIPs in organic and aqueous solutions, (3) to explore MIPs affinity over structurally and functionally related to histamine compounds (Scheme 1) both in organic and aqueous environment making feasible the potential application of polymers as recognition elements in binding assays.

Experimental

Materials

Methacrylic acid (MAA, 98.5 %) was purchased from ACROSS (Geel, Belgium). Ethylene glycol dimethacrylate (EDMA), Tween 20, putrescine·2HCl, putrescine freebase, spermidine·3HCl, taurine and ninhydrin were purchased from Sigma-Aldrich (St. Lewis, USA). Histamine freebase and 2-methoxyethanol were purchased from Fluka (Buchs, Schweiz). Spermine was purchased from Applichem GmbH (Darmstadt, Germany). Azobisisobutyronitrile (AIBN, 98 %) and chloroform were purchased from Merck (Darmstadt, Germany). AIBN was recrystallized from methanol before use. Ranitidine and dimetindene were purchased from Sigma Chem Co., (MO, USA). Fluoxetine hydrochloride was kindly provided by Remedina (Athens, Greece) and disodium cromoglycate from Cooper (Athens, Greece).

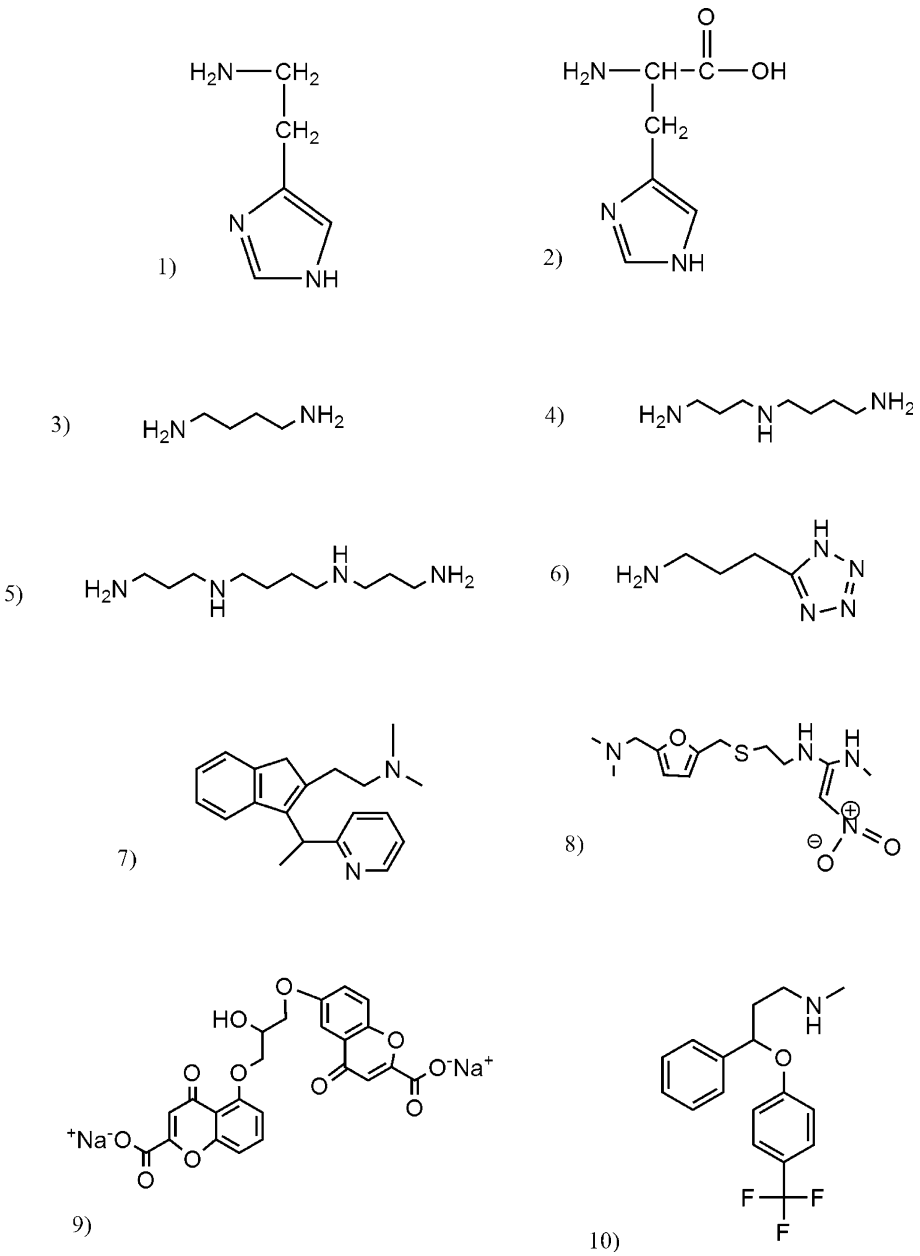
Instrumental

Polymer monoliths were milled using a Planetary Micro Mill Pulverisette 7 (Fritsch GmbH, Germany). Spectroscopic measurements were performed with a Beckman Coulter DU 800 Spectrometer. Analysis of porosity was performed with nitrogen sorption on a Quantachrome autosorb automated gas sorption system and the data fitted to Brunauer, Emmet and Teller (BET) equation for the calculation of the specific surface area.

Polymer synthesis

MIPs were synthesized using bulk and precipitation polymerization techniques (Table 1). The template molecule was dissolved in the porogen ($CHCl_3$ or MeCN) in a borosilicate glass tube equipped with a screw cap. The functional monomer (MAA), the cross-linking monomer (EDMA or TRIM,) and the initiator (AIBN) were added. The solution was purged with a gentle flow of N_2 for 5 min and sealed under N_2. Polymerization was carried out in a water bath at 60 °C for 20 h. In the case of bulk polymerization (MIP 1–2; Table 1) the resultant monolith was ground with a milling machine to give fine particles. Molecularly imprinted microspheres were synthesized

Scheme 1 Structures of **1** histamine, **2** L-histidine, **3** putrescine, **4** spermidine, **5** spermine, **6** putrescine analogue, **7** dimentidene, **8** ranitidine, **9** disodium cromoglycate, **10** fluoxetine



Scheme 2 Structures of **a** MAA and **b** HI

based on a previously reported protocol (Yoshimatsu et al. 2007) by varying the amount of the template (MIP 3–5; Table 1). Histamine was removed using batch mode solvent extraction with methanol containing 10 % v/v acetic acid, until no template was detectable in the washing solvent by ninhydrin assay. The polymer particles were finally washed with acetone and dried in a vacuum chamber. Non-imprinted reference polymers were synthesized under the same conditions in the absence of the template.

Determination of histamine

The quantitative determination of free histamine was performed either by ninhydrin colorimetric assay as described by Rosen (1957) and modified by Winters et al. (2002) or spectrometrically at 210 nm, depending on the nature of the solution and the concentration of the analyte. The amount of bound template was estimated by subtracting the

Table 1 Compositions of histamine imprinted polymers by bulk and precipitation polymerization

Polymer	Template (mmol)	MAA (mmol)	EDMA (mmol)	TRIM (mmol)	Solvent
MIP1	1.00	4.00	20	–	$CHCl_3$
MIP2	0.85	4.00	20	–	$CHCl_3$
MIP3	0.53	1.31	–	2.02	MeCN
MIP4	0.26	1.31	–	2.02	MeCN
MIP5	0.13	1.31	–	2.02	MeCN

amount of free histamine from the total amount added. Data are mean values of triplicate measurements.

Binding analysis in organic media

Binding capacity of molecularly imprinted particles was estimated with batch-wise rebinding experiments. Increasing amounts of polymer particles were suspended in a mixture of 4:1 (v/v) $CHCl_3$:MeCN. Histamine was added at a final concentration of 1 mM and the total volume of the incubation solution was 1 ml. After addition of histamine the mixture was incubated at room temperature overnight. A rocking table was used to provide gentle mixing. Samples were subsequently centrifuged at $8{,}000\times g$ for 10 min and the supernatant (900 µl) was dried in a vacuum chamber. After evaporation, 900 µl of water was added and free histamine was determined. Binding is expressed as the ratio of bound histamine (B) to the total amount added (T) and specific binding is expressed as the difference of B/T on the imprinted polymer minus B/T on the non-imprinted polymer. For the determination of selectivity, two different selectivity factors were calculated (Silvestry et al. 2005) as indicated in Eqs. (1a) and (1b): the selectivity factor (α) as ratio between template and structural or functional template analogue rebinding (1a) and specific selectivity factor (α') were calculated considering the specific HI and analogue binding:

$$\alpha = \frac{(\text{template bound})}{(\text{analogue bound})} \tag{1a}$$

$$\alpha\prime = \frac{(\text{template bound by MIP}) - (\text{template bound by REF})}{(\text{analogue bound by MIP}) - (\text{analogue bound by REF})}. \tag{1b}$$

Binding analysis in aqueous media

Increasing amount of polymer particles was suspended in aqueous solution. The imprinting efficiency of polymers was tested in the following buffers: 25 mM sodium citrate buffer pH 3–6, 25 mM Na-phosphate buffer pH 6–8 and 25 mM Na-carbonate buffer pH 9.0. After addition of 1 ml of 1 mM histamine the mixture was incubated at room temperature overnight under gentle agitation. Following incubation, samples were centrifuged at $8{,}000\times g$ for 10 min and the free histamine was determined.

Determination of binding kinetics

The kinetics of the binding for histamine to MIP and REF were investigated as follows: 5 mg of the polymer particles was incubated with 0.05 mM histamine standard solution, dissolved in 25 mM Na-phosphate buffer pH 7.4. The mixture was incubated at room temperature under gentle agitation. Samples were taken at different times (0–150 min) and free template (F) was determined.

^{1}H NMR study

The ^{1}H NMR spectra were recorded at room temperature on a Bruker 300 MHz spectrometer (Avance 300) and the software Bruker topsin 2.1 was used for the analysis. The samples for the titration study with a constant volume of 1 ml were prepared with a fixed concentration of histamine at 85 mmol/l and varying concentrations of MAA from 0 to 400 mmol/l in deuterated chloroform ($CDCl_3$). TMS was used as an internal standard.

UV–vis spectrophotometric analysis

A series of solutions were prepared including a fixed concentration of histamine (0.85 mmol/l) and various amounts of MAA (0–4 mM) in 25 mM Na-phosphate buffer pH 6.0 and 7.4. The absorption spectra of these solutions were determined with corresponding MAA solutions as references. A UV–vis Beckman Coulter DU 800 Spectrometer was used.

Results and discussion

A histamine-binding polymer was prepared by co-polymerization of MAA and EDMA or TRIM using the technique of non-covalent molecular imprinting. By varying the amount of template in the pre-polymerization mixture, different polymers were obtained by bulk and precipitation methods (Table 1). Compared to precipitation polymerization in acetonitrile, bulk polymerization with chloroform as the porogen yielded a higher titer of high affinity imprinted sites per polymer unit weight (data not shown). Both solvents are widely used as porogens in non-covalent approach because of their non-polarity and non-hydrogen-bonding properties. However, due to different polarity (chloroform is less polar than acetonitrile) a larger concentration of pre-polymer complex would be expected

using chloroform as the porogen (Yan and Ramström 2005a). From preliminary binding experiments, it was shown that highest affinity for the template was obtained with bulk MIP2, having the highest amount of dissolved histamine in the pre-polymerization solution and this was chosen for further experiments. The template molecule was removed from the polymer by more than 98 %. Porosity analysis showed that imprinted and control polymers were mesoporous materials with comparable surface areas of 312.8 and 317.8 m^2/g and average pore diameter of 86.54 and 91.92 Å, respectively. The average imprinted and control polymer particle size was 16.21 ± 6.5 and 17.12 ± 5.35 μm, respectively, as estimated from SEM images using the digital image analysis program SemAfore demo Ver 4.0 (JEOL).

Binding analysis in organic environment

The binding of histamine as a function of polymer concentration was tested by adding increasing amounts of imprinted and control polymer in a 1 mM histamine solution in a mixture of 4:1 $CHCl_3$:MeCN. To achieve effective particle sedimentation, 20 % v/v acetonitrile was added in the incubation solvent. At low polymer concentrations, binding of histamine by the imprinted and control polymers was comparable (Fig. 1), while specific binding increased with increasing polymer concentrations reaching the highest value when 5 mg/ml polymer was used. At this concentration, imprinted polymers bound 50 % of histamine. This was chosen as the optimal polymer concentration for the rest guest batch-wise experiments.

The high specific binding of histamine to the imprinted polymer in organic media (Fig. 1) was indicative of the presence of ligand-selective binding sites. At the highest polymer concentration specific binding was reduced due to the non-specific adsorption caused by the increased polymer surface area (Andersson 2000) as it has already been reported before (Karlsson et al. 2001). In addition, the morphological characteristics of imprinted and control polymers were comparable, in contrast to previous work (Dmitrienko et al. 2006), indicating that histamine binding by MIP resulted from imprinting and was not due to sorption phenomena.

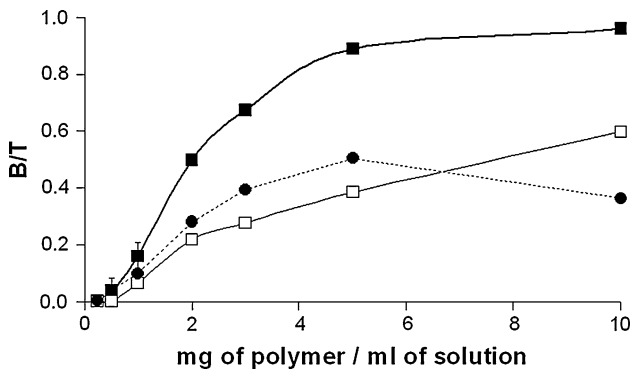


Fig. 1 Binding of histamine to MIP (*filled square*) and reference polymer (*open square*) in a mixture of $CHCl_3$:MeCN (4:1, v/v) as a function of mg of polymer/ml of solution. Specific binding of histamine is presented by *filed circle*

Selectivity analysis with histamine receptor antagonists and compounds related to histamine in organic environment

The affinity of the imprinted polymer for different antagonists of histamine and other related compounds was tested (Scheme 1). The substances of interest were the H_2 receptor antagonist ranitidine, the H_1 receptor antagonist dimetindene, and the selective serotonin re-uptake inhibitor fluoxetine, the latter bearing structural similarities with some histamine receptor antagonists. Disodium cromoglycate was considered as a negative control compound, since evidence does not imply any obvious direct interaction of this agent with histamine receptors, yet it stabilizes the membrane of the histamine releasing and histamine receptor-expressing mast cells and basophils, thereby preventing histamine release.

As it is demonstrated in Table 2, binding of histamine was almost twice higher than that of ranitidine, and six times higher than fluoxetine. For dimentidene and disodium cromoglycate, the imprinted polymer showed weak binding, indicating potential selectivity of the imprinted polymer for histamine compared to the other histaminergic compounds in the organic environment. MIPs' selectivity for histamine is further supported by the high specific selectivity factors. As it is shown in Table 2, selectivity is high for all the tested compounds having the highest values for dimentidene and disodium cromoglycate.

In previous work (Allender et al. 2000), where MIPs' affinity was tested with the H_2 receptor antagonist ranitidine, poor selectivity was obtained. In the present study, it was demonstrated that imprinted polymers exhibited higher specific selectivity factor for histamine than ranitidine,

Table 2 Specific binding and specific selectivity factor (α') of imprinted and control polymers (5 mg/ml) of histamine against histamine antagonists and structurally related compounds in a mixture of $CHCl_3$:MeCN (4:1 v/v)

	Specific binding	α'
Histamine	44.85	–
Ranitidine	20.25	2.21
Dimentidene	1.23	36.46
Fluoxetine	7.53	5.93
Disodium cromoglycate	0.87	51.55

while regarding the rest histaminergic compounds high selectivity was observed as well, pointing to template's specificity.

Binding analysis in aqueous environment

The biological significance of histamine and its related compounds directed the present study towards the assessment of polymer binding in aqueous environment. Both imprinted and control polymers showed higher affinity for histamine under basic conditions, while in more acidic pH a gradual decrease of binding was observed (Fig. 2). The maximal difference between specific and non-specific binding was observed at pH 7.0. At pH 6.0, non-specific binding was low, yet histamine was bound to the imprinted polymer by approximately 20 %. At more acidic conditions no binding was observed (Fig. 2). Saturation experiments performed at pH 6.0 showed specific binding for histamine, since uptake increased with polymer concentration only for the imprinted but not for the non-imprinted polymer (Fig. 2 inset) indicating that imprinting was present.

Potentiometric pH titration of imprinted and blank polymers in aqueous and organic-aqueous systems has shown that the ionization states of polymers and template influence template binding (Sellergren and Shea 1993a). The binding of histamine to both imprinted and control polymers were shown to be strongly influenced by the pH of the incubation medium which is in agreement with previous studies (Bongaers et al. 2010). In aqueous solution and at pH 7.4, histamine is protonated at the amino side, while at pH $>$ 7.4 it becomes neutral and at more extreme conditions, anionic; imidazole moiety is deprotonated (Raczyńska et al. 2003). At pH $<$ 7.4 the imidazole moiety is also protonated and histamine exists in the dicationic form. The variation of binding within this pH range can be explained by the different interactions that take place between the carboxyl group of the polymer and the amino group of histamine. At pH higher than the pKa of MAA, the carboxyl groups are increasingly ionized and may interact with histamine mainly through an ion exchange mechanism.

Polymer-ligand-binding analysis was carried on with the investigation of ionic strength using NaCl concentrations up to 1 M in 25 mM phosphate buffer pH 6.0 (Fig. 3) and up to 200 in 25 mM carbonate buffer pH 9.0 (Fig. 4). As it is shown in Fig. 3, increasing NaCl concentrations resulted in gradual decrease of histamine binding for both imprinted and control polymer. At 100 mM NaCl the non-specific binding was reduced leaving the specific binding relatively high, while at lower NaCl concentrations, the decrease in binding was equal for both polymers (Fig. 3, inset). On the other hand, at higher pH (Fig. 4), addition of low concentrations of NaCl (below 100 mM) resulted in gradual decrease of histamine binding to both polymers, that remained constant at higher concentrations of NaCl.

Attenuation of non-specific electrostatic interactions can be achieved with increasing ionic strength in the incubation media. At pH 6.0, the presence of low concentrations of NaCl resulted in a parallel reduction of non-specific binding to both polymers (Fig. 3, inset), with the optimal concentration of 100 mM, where specific binding was relatively high. If imprinting was absent, salt would have the same impact on both polymers binding. In addition, if histamine binding was of hydrophobic nature, with increase of solvent polarity (due to NaCl), the hydrophobic

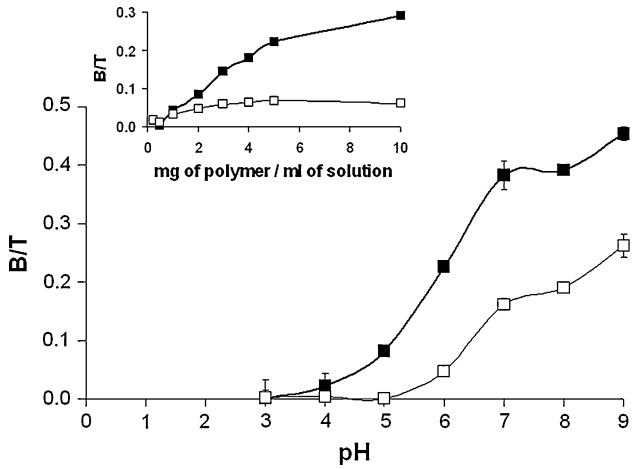


Fig. 2 Binding of histamine to MIP (*filled square*) and reference polymer (*open square*) at various pH values. The buffers (25 mM) were citrate (pH 3.0–5.0), phosphate (pH 6.0–8.0) and carbonate (pH 9.0). *Inset* Binding of histamine to MIP (*filled square*) and reference polymer (*open square*) in 25 mM phosphate buffer pH 6.0 as a function of mg of polymer/ml of solution

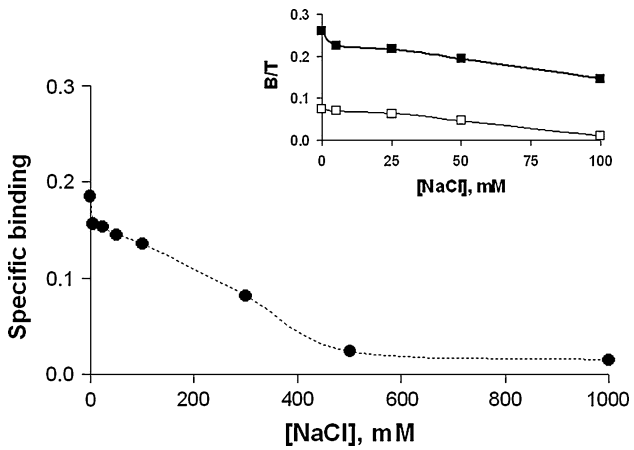


Fig. 3 Specific binding of histamine in 25 mM Na-phosphate buffer pH 6.0 as a function of ionic strength expressed as the difference in B/T between the imprinted and reference polymers (*filled circle*). *Inset* Binding of histamine to imprinted (*filled square*) and reference polymers (*open square*) in 25 mM Na-phosphate buffer pH 6.0 as a function of low ionic strength

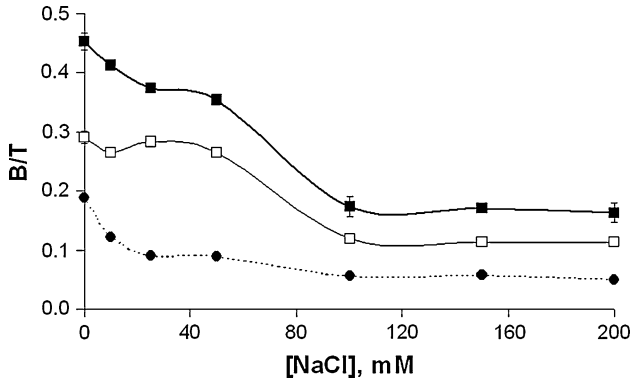


Fig. 4 Binding of histamine to imprinted (*filled square*) and reference polymers (*open square*) in 25 mM Na-carbonate buffer pH 9.0 as a function of low ionic strength. Specific binding of histamine is presented by *filled circle*

repulsion between HI and solvent would become stronger and more template would preferentially be absorbed by polymer matrix (Cai and Gupta 2004). At pH 9.0, the effect of NaCl revealed that histamine binding to both imprinted and control polymers was of dual nature. At pH > 7.4, histamine is neutral while MAA is ionized. The reduction of histamine binding caused by the addition of NaCl lower than 100 mM indicated that recognition is due to electrostatic interactions. In molecular imprinting, interactions between ions and dipoles are important and very common when MAA is used as functional monomer (Yan and Ramström 2005b). Furthermore, the fact that specific binding of histamine was not further affected at higher concentrations of NaCl, possibly ascribes to hydrophobic adsorption of the template on the polymeric backbone.

The kinetic study of histamine adsorption to MIP and REF particles (Fig. 5) revealed a rapid increase during the

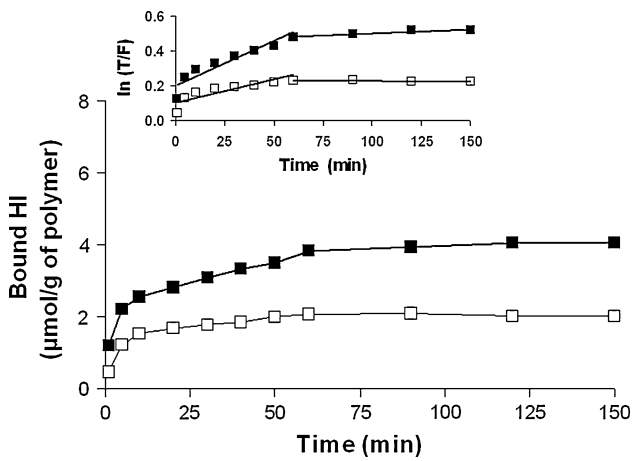


Fig. 5 Adsorption study of histamine to MIP (*filled square*) and REF (*open square*) in 25 mM phosphate buffer pH 7.4 as a function of time. *Inset* The relevant graph of ln(*T*/*F*) versus time

first 60 min reaching a maximum value that remained almost constant with the time extension. A relevant graph of ln(*T*/*F*) versus *t* (Fig. 5, inset) gave a biphasic plot bisecting around the first hour. From the first part of the kinetic plot it is shown that histamine adsorption on the polymer network is fast in the beginning, while the second almost linear part indicates that the adsorption process has reached equilibrium. The kinetic study revealed that histamine mass transfer kinetic in aqueous environment is fast at the beginning but after 1 h, the mass transfer is slowed and the process has reached equilibrium (Fig. 5, inset). This mode of histamine adsorption to MIP resembles the molecule's release after an allergic event, which is high in the beginning and reaches a constant value after a time (Fukui 2008).

Most MIP applications in aqueous media require the addition of an organic modifier or surfactant in the incubation buffer (Andersson 1996; Karlsson et al. 2001). Binding of histamine in aqueous media has been reported earlier (Tong et al. 2002; Dmitrienko et al. 2006; Pietrzyk et al. 2009; Horemans et al. 2010; Bongaers et al. 2010; Broeders et al. 2011). However, this is the first time that a more thorough study of histamine affinity is conducted in order to elucidate binding phenomena. In the present work, ligand recognition was performed successfully in aqueous media without the addition of any organic modifier or surfactant, thus simplifying the assay method. The fact that the incubation of polymer particles and analyte can be performed equally well using an organic solvent (Fig. 1) or an aqueous buffer (Fig. 2, inset) adds flexibility to the development of an assay method.

Binding analysis with histamine receptor antagonists and compounds related to histamine in aqueous environment

Since histamine is the product of histidine decarboxylation in vivo, the specificity of polymers was tested against the amino acid L-His. Rebinding analysis showed that imprinted polymers selectively bound histamine, but they were unable to recognize L-His at acidic pH. At neutral environment histamine binding was double but remained $\sim$5 % for L-His (Fig. 6). The higher affinity of imprinted particles for histamine compared to L-His, the most structurally related compound, pointed to their selective binding. These findings are in agreement with previous work (Horemans et al. 2010; Bongaers et al. 2010).

For the first time, MIPs' selectivity was also tested against the polyamines putrescine, spermidine, spermine and an analogue of putrescine. At pH 7.0, higher affinity of the imprinted polymers for the polyamines compared to histamine was observed. However, at pH 6.0, the imprinted polymer exhibited stronger binding for histamine

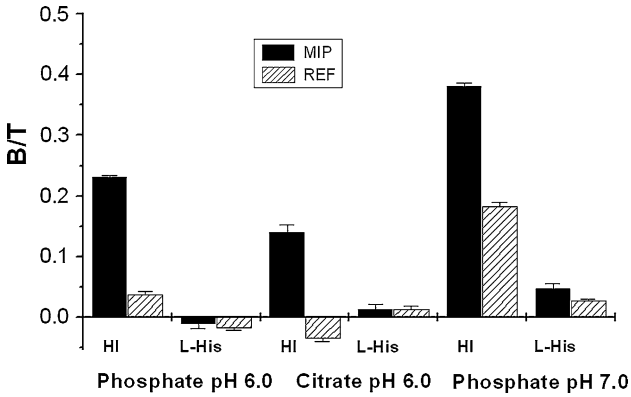


Fig. 6 Binding of histamine (HI) and L-His to imprinted and reference polymers in aqueous buffers. The buffers (25 mM) were citrate, or phosphate pH 6.0, or phosphate pH 7.0

compared to putrescine while spermine and spermidine continued to have affinity for the imprinted polymer (Table 3). Reduction of non-specific binding with 50 mM NaCl improved the selectivity of MIPs for histamine compared to putrescine alone, while binding of spermine and spermidine to the imprinted polymer was not affected. Binding of the putrescine analogue was around 1–2 % at all conditions (Table 3).

Regarding MIPs' selectivity for histamine against the biogenic amines, specific selectivity was observed for histamine against putrescine and putrescine's analogue while in the case of spermine and spermidine, even in the presence of NaCl, some affinity for the imprinted polymer was observed. This could be attributed to their greater number of aliphatic chain amino groups that may form additional electrostatic interactions with the MAA resulting in non-specific binding compared to L-histidine and putrescine. Scheme 1 shows that putrescine and its analogue may form such electrostatic interactions, while MIP showed low affinity. At the corresponding pH, the ionization of amino and imino groups is favored resulting in additional non-specific electrostatic interactions with the functional monomer. At pH 6.0, these interactions are minimized thus non-specific binding is decreased.

Regarding histamine receptor antagonists, higher adsorption for both imprinted and control polymers was obtained in aqueous than in organic media. Only disodium cromoglycate constantly exhibited low affinity for the polymers as in the organic solvent. Attenuation of non-specific hydrophobic interactions between the antagonists and the backbone of the polymer can be achieved through the use of detergents such as Tween 20 and Triton X-100 (Andersson et al. 2002). Among the detergents tested, reduction of hydrophobic interactions was achieved with Tween 20 for ranitidine (Fig. 7), while in the case of fluoxetine and dimentidene, adsorption was not affected by the addition of the surfactant. Regarding ranitidine, binding to both imprinted and control polymers decreased with increasing concentrations of Tween 20 (Fig. 7) and adsorption was reduced up to 50 % when 0.3 % v/v of the detergent was used. Higher concentration of the detergent did not affect further the non-specific adsorption. The effect of surfactant is better illustrated with the selectivity factor (Table 4). The values of selectivity factor ($\alpha_{HI/ranitidine}$) were increased with increasing surfactant concentration, depicting MIPs' selectivity for histamine.

Concerning histamine, increased concentrations of Tween 20 induced binding strength in both imprinted and control polymers (Fig. 8a). The effect of surfactant on polymer affinity is better demonstrated with the recognition factor (Table 4). It was shown that the surfactant increment caused a reduction on the recognition factor. To evaluate the influence of the surfactant on histamine affinity, different concentrations of NaCl were added in the incubation medium (25 mM phosphate buffer, pH 7.4 containing 0.3 % v/v of Tween 20). The increment of ionic strength caused a high reduction of histamine binding to imprinted polymers (Fig. 8b).

The interactions of histamine receptor antagonists with the polymer in aqueous environment are primarily hydrophobic, being strong in the presence of water and weak in organic solvents. It is well known that imprinted polymers exhibit different swelling properties in different matrices thereby changing the morphology of the polymer network, the size, shape and relative position of the functional

Table 3 Specific binding and specific selectivity factor (α') of histamine and other diamines to imprinted polymers in aqueous environment

Specific binding					
Incubation media	HI	PUT	SPMD	SPM	PUT analogue
Phosphate pH 7.0	21.42	25.42	25.84	20.55	0.86
Citrate pH 6.0	13.00	6.81	16.92	19.63	2.40
50 mM NaCl in citrate pH 6.0	11.94	4.30	17.20	16.95	1.03
α' of MIP in 50 mM NaCl in Citrate pH 6.0	–	2.77	0.69	0.70	11.59

The buffers were citrate (25 mM) pH 6.0 and phosphate (25 mM) pH 7.0

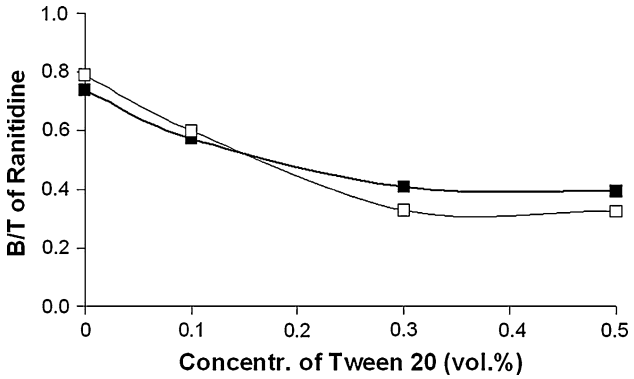


Fig. 7 Binding of ranitidine to imprinted (*filled square*) and reference polymers (*open square*) in 25 mM Na-phosphate buffer pH 7.4 at various Tween 20 concentrations

Table 4 Selectivity and recognition factor of HI-MIP in 25 mM Na-phosphate pH 7.4 at various concentrations of Tween 20

Concentration of Tween 20 (%v/v) in 25 mM Na-phosphate pH 7.4	Selectivity factor ($\alpha_{HI/ranitidine}$)	Recognition factor of HI (MIP/REF)
0.0	0.45	3.03
0.1	1.09	2.01
0.3	2.43	1.52
0.5	2.51	1.12

groups of the sites, which are essential for recognition (Sellergren and Shea 1993b). In previous studies, addition of Tween 20 increased dramatically the recognition and the retention times of naproxen on imprinted polymers in aqueous buffers (Xu et al. 2005), which is similar to our observation. In the present study, it was demonstrated that highest selectivity and recognition can obtained when 0.3 % v/v Tween 20 are used. These findings can be explained either by the surfactant adsorbing and changing the polymer surface of the MIP or by the formation of intermolecular interactions between the analyte and the surfactant. The observed increase of histamine binding when Tween 20 was added implies the formation of intermolecular interactions between histamine and the surfactant. The neutral surfactant possesses a hydrogen bond acceptor characteristic in the polyether chain and a hydrogen bond donator characteristic in the hydroxyl groups (Wang et al. 2004), thus resulting in enhanced binding of the template to the polymer. When the ionic strength of the incubation medium was increased, binding of histamine was reduced implying that the interactions between the surfactant and the template were of electrostatic nature. Although the underlying interactions are yet unresolved, these results are indicative of an effect between the polymer morphology modification and intermolecular histamine–surfactant interactions.

Recognition mechanism of MIPs

UV–vis spectroscopic analysis and ^{1}H NMR study are commonly applied to characterize the nature of interactions and the extent of complex formation between functional monomers and template molecule in solution (Karim et al. 2005). The change of chemical shift and UV–vis spectra could be used for demonstration of the types and strength of the interactions (Xu et al. 2006). Since the cross-linker and initiator had much less effect on the specific interactions between the template and functional monomers (Zhu et al. 2002), the spectra were determined with systematically varied ratios of HI to MAA in the $CDCl_3$.

Since the nature of interactions and the extent of complex formation between functional monomers and template molecule have not been studied previously, a ^{1}H NMR study of the pre-polymerization solution was conducted. As shown in Table 5, the chemical shift of the two basic hydrogen of histamine (H-4 and H-8) is downfield by the addition of MAA to the HI solution. This chemical shift is further increased with increasing MAA concentration in HI solution reaching a maximum value when the ratio of

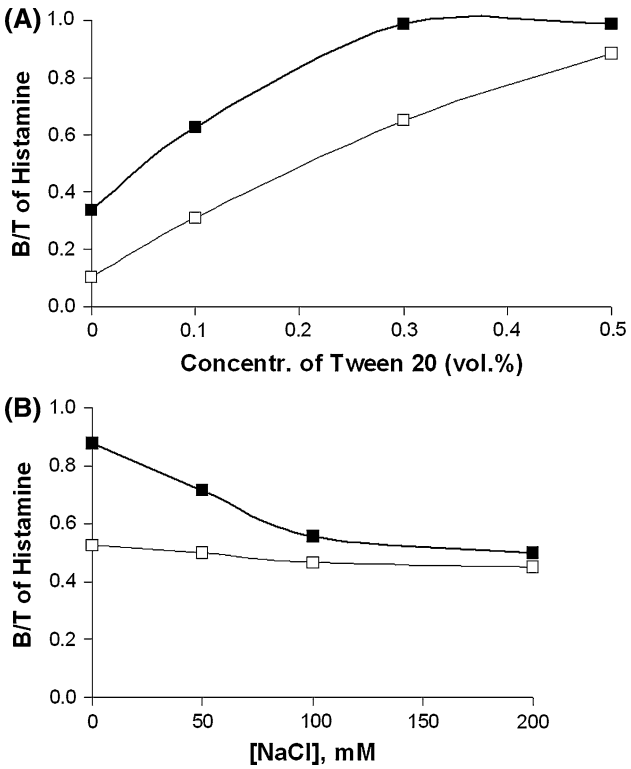


Fig. 8 Binding of histamine to imprinted (*filled square*) and reference polymers (*open square*) in 25 mM Na-phosphate buffer pH 7.4 at various Tween 20 concentrations (**a**) and binding of histamine to imprinted (*filled square*) and reference polymers (*open square*) in 25 mM Na-phosphate buffer pH 7.4 containing 0.3 % v/v of Tween 20 as a function of ionic strength (**b**)

HI:MAA was 0.85–4 (from $\delta \sim 1.5$ to $\delta \sim 11.08$). It should be noted that the acidic hydrogen of MAA (H-3) is also shifted to $\delta \sim 11.08$. In non-covalent imprinting, hydrogen bonds and ionic interactions have been shown to be of major importance for recognition in organic media (Karlsson et al. 2001). The strong chemical shifts of the two basic hydrogen of histamine (H-4 and H-8) provided strong evidence that was involved in the formation of hydrogen bonding with the functional monomer, and combined with the very large variation in chemical shift, this suggested that the interaction could be strong (Pretsch et al. 2000; Shi et al. 2007). These findings illustrate that the interactions between HI and MAA were mainly hydrogen bonding when chloroform was used as the solvent.

In aqueous systems, many of the weaker non-covalent interactions are lost, leaving ionic bonds as the main interaction mechanism. In aqueous media, the interaction mechanisms rely mainly on ionic and hydrophobic interactions (Ramström et al. 1998). However, in some cases strong hydrogen bonds may still be the leading interaction in the recognition mechanism (Yu et al. 1997).

Since biological molecular recognition of histamine occurred mainly in the aqueous phase, the interaction of HI and functional monomer in the aqueous solution was studied for the first time by the UV–vis absorption spectrum. The effect of MAA on UV-spectra of histamine was studied at neutral (Fig. 9a) and acidic environment (Fig. 9b) due to the different ionization states of histamine. As it is demonstrated in Fig. 9, the absorption spectra of HI showed a red shift of the absorption peaks with the increase of MAA in water and the maximum absorbance decreased with increasing MAA. It is also shown that at pH 6.0 the reduction of maximum absorbance was more intense than at neutral pH.

The observed hypochromic and red shift of the absorption peak with the increase of MAA in water (Fig. 9) indicated that a complex had been formed by ionic interaction in water (both at pH 6.0 and pH 7.4) between MAA and histamine (Shi et al. 2007). It was also shown that at pH 6.0, with histamine in the dicationic form, the hypochromic shift was more intense than at neutral pH where histamine is protonated at the amino side. This indicates that probably stronger interactions take place between HI-MAA resulting in low non-specific adsorption to the polymers.

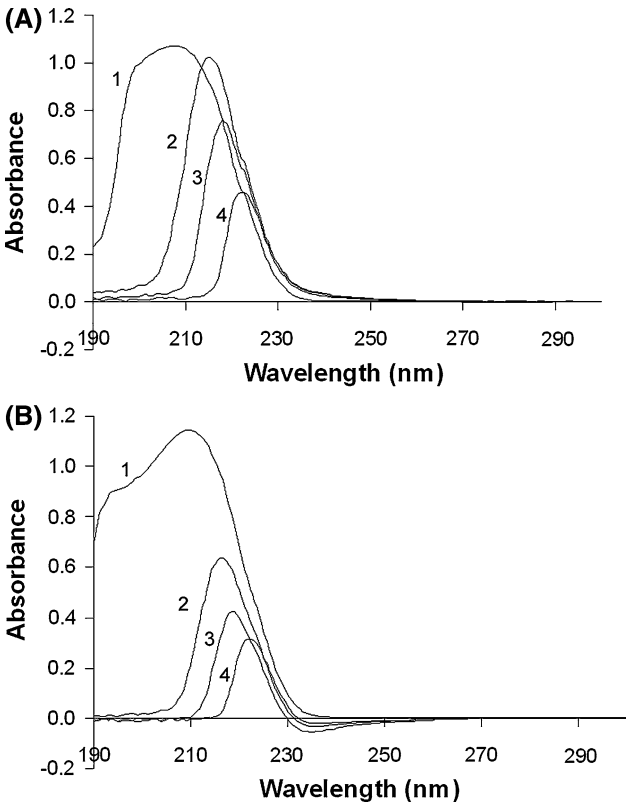


Fig. 9 Absorption spectra of HI for addition of MAA in 25 mM Na-phosphate buffer at **a** pH 7.4 and **b** pH 6.0. Corresponding pure MAA solution was used as reference. The relevant curves are: *1* HI, *2* HI:MAA (0.85:1), *3* HI:MAA (0.85:2), *4* HI:MAA (0.85:4)

Conclusion

In this work, molecularly imprinted materials have been developed that exhibited sufficient capacity and affinity for histamine both in organic and aqueous media, allowing the

Table 5 The chemical shifts of HI and MAA hydrogen from the ^{1}H NMR study of the pre-polymerization mixture

Chemical shift (δ)									
Solution	H-1	H-2 (a and b)	H-3	H-4	H-5	H-6	H-7	H-8	H-9
HI	–	–	–	~1.50[a]	3.01	2.73	6.82	~1.5[a]	7.55
MAA	1.96	6.25 & 6.78	11.50	–	–	–	–	–	–
HI:MAA 0.85:2	1.90	6.00 & 5.45	10.67	10.67	3.17	2.96	6.88	10.67	7.77
HI:MAA 0.85:4	1.92	6.08 & 5.52	11.08	11.08	3.22	3.01	6.97	11.08	7.91

The correspond hydrogen (H-1 to H-9) are presented in Scheme 2

[a] $\delta \sim 1.5$: overlapping values with sample's humidity

development of an assay method. Furthermore, MIPs' higher affinity for histamine over structurally and functionally related compounds, both in organic and aqueous media, pointed to the presence of selective binding sites. From spectroscopic studies, it was found that recognition in organic media was based on hydrogen bond interactions while in aqueous environment electrostatic interactions were the main recognition mechanism.

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