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Recognition of oxytocin and oxytocin-related peptides in aqueous media using a molecularly imprinted polymer synthesized by the epitope approach

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Abstract

An artificial polymeric receptor prepared by the epitope approach of molecular imprinting was shown to recognize the peptide hormone, oxytocin, in aqueous media. The proposed approach is based on using (as a template) a compound, whose structure represents a small exposed fragment of a larger molecule (as an epitope represents an antigen). A HPLC study has demonstrated the important role of ionic interactions and the N-terminal amino group of oxytocin and oxytocin-related peptides in the process of their recognition by the molecularly imprinted polymer in the aqueous-rich media. However, the specificity of the process is considered to be defined by hydrophobic interactions and hydrogen bonding. Moreover, it was shown that the selectivity of the molecularly imprinted polymer can be attenuated by water content, ionic strength and pH of the chromatographic mobile phase: depending on these factors the template, Tyr–Pro–Leu–Gly–NH_2, or, for example, oxytocin, a larger peptide, which possesses the same three amino-acid C-terminal parts of the structure, can be preferentially retained by the molecularly imprinted polymer.

Keywords: Molecular imprinting; Oxytocin; Peptides; Hormones

1. Introduction

Stable and selective artificial receptors for a wide variety of chemical substances can be prepared by molecular imprinting. This method consists of a formation of labile complexes in solution between target molecule (template) and so-called functional monomers (it occurs due to reversible covalent or noncovalent interactions of their functional groups). Polymerization in the presence of a large quantity of cross-linking agent results in the fixing of these complexes inside a stable polymeric material. The removal of the template by a washing procedure reveals a polymer containing free sites able to recognize the template [1–4]. Molecularly imprinted polymers (MIPs) are able to recognize definite substances and may successfully replace selective but unstable biological structures in both fundamental investigations of molecular recognition and various practical applications [5–7].

Until the mid-1990s the processing and handling of MIPs was performed in organic solvents and water-soluble compounds (including macromolecules) were not used as templates. These circumstances are considered serious drawbacks in MIP technology. Therefore, during the past few years

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different methods to overcome this problem have been under intensive investigation. Mixtures of methanol and water are used as the porogen (polymerization solvent) for the preparation of MIPs selective to relatively low-molecular-mass compounds, such as adenine and 2,4-dichlorophenoxyacetic acid [8,9]. Indeed, water or aqueous buffer solutions are only used during attempts to involve the whole protein molecules in the polymerization process by entrapping them in polyacrylamide polymers [10–12]. Recently, an amphiphilic, polymerizable β-cyclodextrin was used as a functional monomer to utilize molecular imprinting in aqueous solution [13]. A method of derivatization of the template, increasing its solubility in apolar solvents, can provide more impressive results: Leu^5-enkephalin anilide was shown to be a good substitute for the free peptide since the resulting polymers showed efficient recognition of the parent enkephalin structure [14]. It was also shown that MIPs synthesized in nonaqueous porogens can be successfully used in aqueous-based media [9,15–17].

To date, some success in the preparation imprints to macromolecules such as proteins, was mainly achieved using the so-called surface-imprinting procedure. Proteins containing exposed histidine can be recognized using a metal (Cu^{2+}) chelating monomer [18,19]. Thermodynamic considerations [20] indicate that only rigid template structures with limited numbers of conformations lead to more defined recognition sites of MIPs, and result in a higher efficiency of the rebinding procedure due to a small loss of entropy on binding. By this means, the synthesis of MIPs selective to proteins is a very difficult task. However, we hope that it is possible to solve this problem by following how nature does it. In recognizing an antigen, an antibody interacts only with a small part of it, the epitope (the antigenic site of macromolecule). It should be noted that epitopes may be described as surface domains composed of three to six amino acid residues [21]. Hence, if a short peptide representing only the small exposed fragment of a protein structure is used as a template, then the resulting macroporous, MIP will also be able to retain the whole protein molecule.

The proposed ''epitope'' approach [22] of the synthesis of MIPs selective to macromolecules (polypeptides and proteins) is the essential extension of the applicability of molecular imprinting. The tetrapeptide, Tyr–Pro–Leu–Gly–NH_2 (YPLG), was used as a template for the preparation of the MIPs by the epitope approach. It was shown that in organic (acetonitrile-based) chromatographic mobile phases the MIP can recognize not only the template but some other peptides possessing Pro–Leu–Gly–NH_2 sequence at the C-terminus (including the neurohypophyseal hormone oxytocin) as well. The aim of the present investigation is a chromatographic study of molecular recognition by MIPs, prepared by the epitope approach, in aqueous media.

2. Experimental

2.1. Materials

Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA) and 2,2′-azobis(2,4-dimethylvaleronitrile) were obtained from Wako (Osaka, Japan). YPLG, oxytocin and Gly–Leu–Tyr (GLY) were obtained from Sigma (St. Louis, MO, USA); mesotocin or (Ile^8)-oxytocin and tocinoic acid were from Bachem (Bubendorf, Switzerland); desaminooxytocin (Desamin-Oxy) was from Biogenesis (Poole, UK). [$Asu^{1,6}$]-oxytocin (Asu: L-α-aminosuberic acid), acetyl-L-tyrosine amide (Ac–Tyr–NH_2), acetyl-L-tyrosine ethyl ester (Ac–Tyr–OEt) and Pro–Leu–Gly amide (PLG) were purchased from the Peptide Institute (Osaka, Japan). The acetonitrile used was of HPLC grade. Water was of Milli-Q grade.

2.2. Polymerization

The polymers were prepared by the following procedure. To 22 μmol of template (10 mg of YPLG), and 220 μmol of the functional monomer (MAA) in 1.2 ml of acetonitrile was added 6.6 mmol of cross-linking agent (EGDMA). 25 mg 2,2′-azobis-(2,4-dimethylvaleronitrile) was used as an initiator. To the final polymerization mixture containing equal volumes of acetonitrile and monomers (MAA+EGDMA), a small quantity of water (about 3%, v/v) was added to ensure complete solubilization of the YPLG. The mixture was placed in a 10-ml glass vial, degassed in a sonicating bath, and purged with

nitrogen for 2 min. The polymerization was performed at 40°C for 16 h in an oven. The polymers obtained were ground in a mortar and sieved to collect the 20–45-μm fraction. The polymers were washed several times with acetonitrile and water containing 5% (v/v) acetic acid, until the template could no longer be detected in the supernatant. Finally, the polymers were dried in a vacuum oven at 40°C. The control (nonimprinted) polymer is synthesized under the same conditions, except for the addition of template.

2.3. Chromatographic evaluation

Chromatographic analyses were performed using a Tosoh 8010 high-performance liquid chromatography (HPLC) system (Tosoh, Tokyo, Japan) including a system controller, UV–Vis and fluorescence detectors. Polymers were slurry packed into 100×4.6 mm stainless steel columns. Volumes (20 μl) of the 0.1 m*M* samples were analyzed at a flow-rate of 1 ml min^{-1}, monitored simultaneously with a UV detector set at 275 nm or 225 nm (using $NaNO_3$ or acetone as a void marker) and a fluorescence detector (λ_{ex}=275 nm, λ_{em}=305 nm). The capacity factor (k') was calculated as $(t-t_0)/t_0$, where t is the retention time of the solute and t_0 is the retention time of the void marker [23].

To check the role of ionic strength, different concentrations of citrate–phosphate buffer, pH 6 with ionic strengths ca. 0.5, 2.5 and 20 m*M* were used. To obtain higher values of the ionic strength, NaCl was added to a 20 m*M* buffer solution to give concentrations of 50, 100 and 200 m*M*, respectively. The dependence of analyte–polymer binding on pH was investigated over the range from 3.5 to 8.1 using a citrate–phosphate buffer with constant ionic strength ca. 2.5 m*M* [24].

3. Results and discussion

Usually molecular imprinting creates specific recognition sites that complement a given molecule in size, shape and arrangement of functional groups by using the given molecule as a template. Here, to prepare a MIP selective to macromolecules (polypeptides or proteins) employing the epitope approach, we have chosen the neurohypophyseal hormone, oxytocin (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2 with disulfide bond between Cys residues) as a final target for recognition. The tetrapeptide, YPLG has been chosen as the template for the preparation of the MIP. Both compounds possess the same three-amino acid C-terminal sequence, Pro–Leu–Gly–NH_2. The Tyr residue in the peptide YPLG was chosen to facilitate UV and fluorescence detection.

Many of the peptides used in this study are only partially soluble in pure (100%) acetonitrile, therefore, various mixtures of acetonitrile and water have been investigated to obtain the optimum chromatographic mobile phase. However, use of such mobile phases gave poor reproducibility. To ensure a more stable level of analyte–polymer interactions, the following modifications to the composition of mobile phase were performed: the organic or aqueous-poor mobile phases (5–20% water) were modified by the addition of acetic acid, and the aqueous-rich phases (70–90% water) by the addition of a citrate–sodium phosphate buffer.

The study of the role of the chromatographic mobile phase allows us not only to achieve the optimal separation conditions, but also to gain insight into the terms of the retention mechanism. Depending on the solvent's properties, the strength of interaction between all three counterparts (polymer, solvent and solute) can vary significantly. For example, a mobile phase with polarity lower than pure acetonitrile and poor hydrogen bonding propensity, reducing nonspecific hydrophobic interactions of all tested analytes efficiently increased the selectivity of the MIP imprinted with β-estradiol [25]. On the contrary, a partial substitution of acetonitrile in the mobile phase for a compound with higher propensity to hydrogen bonding (e.g., water or methanol) strongly suppressed the specific binding of the template via hydrogen bonds [26,27].

Indeed, in Table 1 one can see that the increase in the water content up to 20% of the mobile phase, results in a drastic reduction in the retention of the peptides, whereas the level of interaction between both polymers and Ac–Tyr–NH_2 is almost the same at the very low level. The further increase in water content from 70 to 90% again leads to an increase in retention of the analytes, especially for oxytocin.

Table 1
Influence of the water content of the mobile phase on the retention of the analytes by the MIP (capacity factor, k')

Analyte	Water content of the mobile phase (%)						
	5[a]	10[a]	20[a]	70[b]	80[b]	85[b]	90[b]
YPLG	33.7	12.2	5.00	0.47	1.08	2.09	5.83
Oxytocin	30.3	5.32	4.09	0.44	2.05	6.50	31.8
Ac–Tyr–NH_2	0.05	0.17	0.25	0.32	0.47	0.80	1.36
Ac–Tyr–OEt				1.14	3.02	6.27	15.0

[a] Mobile phase contains 1 m*M* acetic acid and indicated water contents in MeCN.
[b] Mobile phase contains 2.5 m*M* citrate–phosphate buffer, pH 5.2, and from 10 to 30% MeCN in water.

These results coincide with those obtained for MIPs selective to triazine herbicides and for MIPs selective to *N-tert*.-butoxycarbonyl (t-Boc)-L-Trp: when the water content in the mobile phase was increased from 0 to 30%, disruption of hydrogen bonds between the solute and recognition sites of the MIP resulted in a strong decrease in both retention and selectivity. The further increase in water content strengthens the analyte binding, which can be explained by the role of ionic and hydrophobic interactions [28,29].

Our previous investigations of YPLG-imprinted polymers in aqueous-poor mobile phases have clearly demonstrated that the synthesized MIPs recognize not only the template, but oxytocin too. Furthermore, the retention of YPLG is stronger than that of oxytocin [22]. However, under the given conditions in aqueous-rich mobile phases, the reverse is seen.

To explain this phenomenon and to elucidate the contribution of electrostatic interactions, a study of the influence of ionic strength and pH has been undertaken. An increase in the ionic strength from 0.5 to 20 m*M* sharply reduces the retention time of YPLG and oxytocin, but has practically no effect on that of the derivatives of tyrosine, Ac–Tyr–OEt and Ac–Tyr–NH_2 (Table 2). This means that ionic interactions make essential contributions to the process of recognition of YPLG or oxytocin, but their influence is only apparent for ionic strengths no higher than 20 m*M*. It should be noted also that, under the given conditions, the reduction in retention time of oxytocin occurs slower than that for YPLG, resulted in a stronger retention of the nonapeptide at ionic strength above 2.5 m*M*.

For the study of pH dependency the citrate–sodium phosphate buffer system of constant ionic strength, which itself covers a vast pH range, was chosen to avoid a possible uncertainty of employing chemically different compositions of a set of particular buffer solutions. To reveal the selectivity of the synthesized MIP, a set of oxytocin-related peptides and compounds with structural properties closely resembling the template was used (Fig. 1).

Firstly, the template YPLG (and PLG also) is retained by the MIP more strongly than the other test compounds (except at low pH).

Secondly, the peptides which possess the C-terminal sequence Pro–Leu–Gly–NH_2 (including YPLG and oxytocin), interact with the MIP ca. 2–3-times more strongly than with the control polymer. The other test analytes fail to show any significant difference (Fig. 2). It should be noted that tocinoic acid (the cyclic part of oxytocin without the C-terminal tail) is not recognized by the MIP. The same is true for GLY possessing amino acid contents similar to that of the template, but adopting a different sequence. All these facts are clear evidence for the true identity of the imprinted character of the MIP.

Table 2
Influence of mobile phase ionic strength on the retention of the analytes by the MIP (capacity factor, k')

	Ionic strength (m*M*)[a]					
	0.5	2.5	20	70	120	220
YPLG	8.90	3.42	1.10	0.79	0.94	0.78
Oxytocin	7.52	4.84	2.07	1.56	1.60	1.37
Ac–Tyr–NH_2	0.74	0.80	0.76	0.75	0.74	0.73
Ac–Tyr–OEt	4.23	4.34	3.82	3.89	3.93	3.98

[a] Mobile phase contains citrate–phosphate buffer, pH 6, with ionic strength 0.5, 2.5 or 20 m*M* and 20% MeCN in water. To obtain higher ionic strengths, NaCl was added to an 20 m*M* buffer solution to give concentrations of 50, 100 and 200 m*M*, respectively.

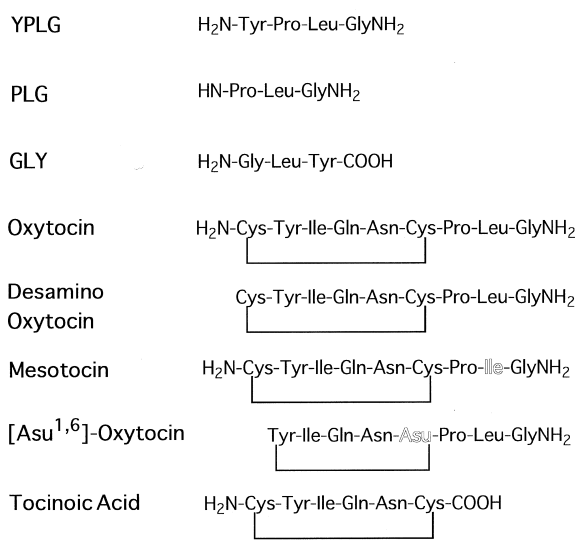

Fig. 1. Amino acid sequences of the tested peptides. Amino acid substitutions (compared with oxytocin) are designated in outline. [Asu^{1,6}]-oxytocin – cyclic form between Asu ω-carboxyl group and Tyr α-amino group. Asu: L-α-Aminosuberic acid.

Thirdly, those peptides containing an N-terminal amino group (or imino group) demonstrate a very pronounced pH-dependence, more so than those without. The peak position for the oxytocin pH-dependencies seems very reasonable, because the isoeletric point (p*I*) for oxytocin, mainly caused by the pK_a value of the N-terminal amino group of cysteine is 7.7. At pH values greater than 6, the level of ionization of the positively charged amino group falls off. The pK_a value of the imino group of proline is much higher than that of the α-amino group of the remaining natural amino acids; it also exceeds the pK_a values for tyrosine and cysteine by 1.5 and 2.3 units, respectively. The pH-dependencies of PLG, YPLG, oxytocin and mesotocin are consistent with these data. Consequently, the selectivity of the MIP can be attenuated by pH; depending on the pH of the mobile phase the YPLG template or, for example, oxytocin, can be preferentially retained by the MIP.

Three-dimensional models of YPLG and oxytocin demonstrate that their N-terminal amino groups can be situated at a similar distance from the C-terminus of the peptides. Therefore the contribution of this amino group in overall recognition can be reasonably explained. Desaminooxytocin, [Asu^{1,6}]-oxytocin, Ac–Tyr–OEt and Ac–Tyr–NH_2, have no free N-terminal amino group. As a result, all these compounds interact with the polymers almost independently of pH. Former two possess the C-terminal Pro–Leu–Gly–NH_2 sequence and, consequently, demonstrate a stronger interaction with the MIP than with the control polymer, whereas latter two demonstrate no difference between the polymers. Relatively high levels of retention of Ac–Tyr–OEt by both polymers can be explained by its hydrophobicity.

In spite of the presence of free N-terminal amino group GLY and tocinoic acid have neither pH-dependence nor an essential retention by any of the polymers, probably, owing to the absence of the C-terminal Pro–Leu–Gly–NH_2 sequence and electrostatic repulsion between the C-terminal carboxy group of the peptides and MAA residues within the polymers.

A comparison of the chromatographic results obtained using aqueous-poor and aqueous-rich mobile phases is presented in Table 3. The imprinting effect can be clearly seen when using both types of mobile phase. However, in an aqueous-rich mobile phase, the selectivity of the MIP can be attenuated by pH, as it is mentioned above. Regarding this phenomenon, it is possible to say that compounds containing the C-terminal Pro–Leu–Gly–NH_2 sequence and amino group situated like the N-terminal amino group of the template YPLG, show a similar pattern of molecular recognition by the MIP when using both types of the mobile phase.

In contrast, some other tested analytes interacting with the polymers demonstrated a drastic difference depending on the composition of the mobile phase. In the aqueous-rich media, hydrophobic Ac–Tyr–OEt interacts with both polymers (nonspecifically) much stronger than in the aqueous-poor mobile phase. A small tripeptide GLY possessing some similarity in conformation comparing to that of the template, tetrapeptide YPLG, is strongly retained in the aqueous-poor mobile phase. However, in the aqueous-rich mobile phases, a more intensive ionization of the C-terminal carboxy group leads to electrostatic repulsion and results in a sharply decreased retention of the peptide by MAA-containing polymers. The absence of the N-terminal amino group decreases polarity of desaminooxytocin and [Asu^{1,6}]-oxytocin and increases their solubility in acetonitrile. As a result, the presence of the C-

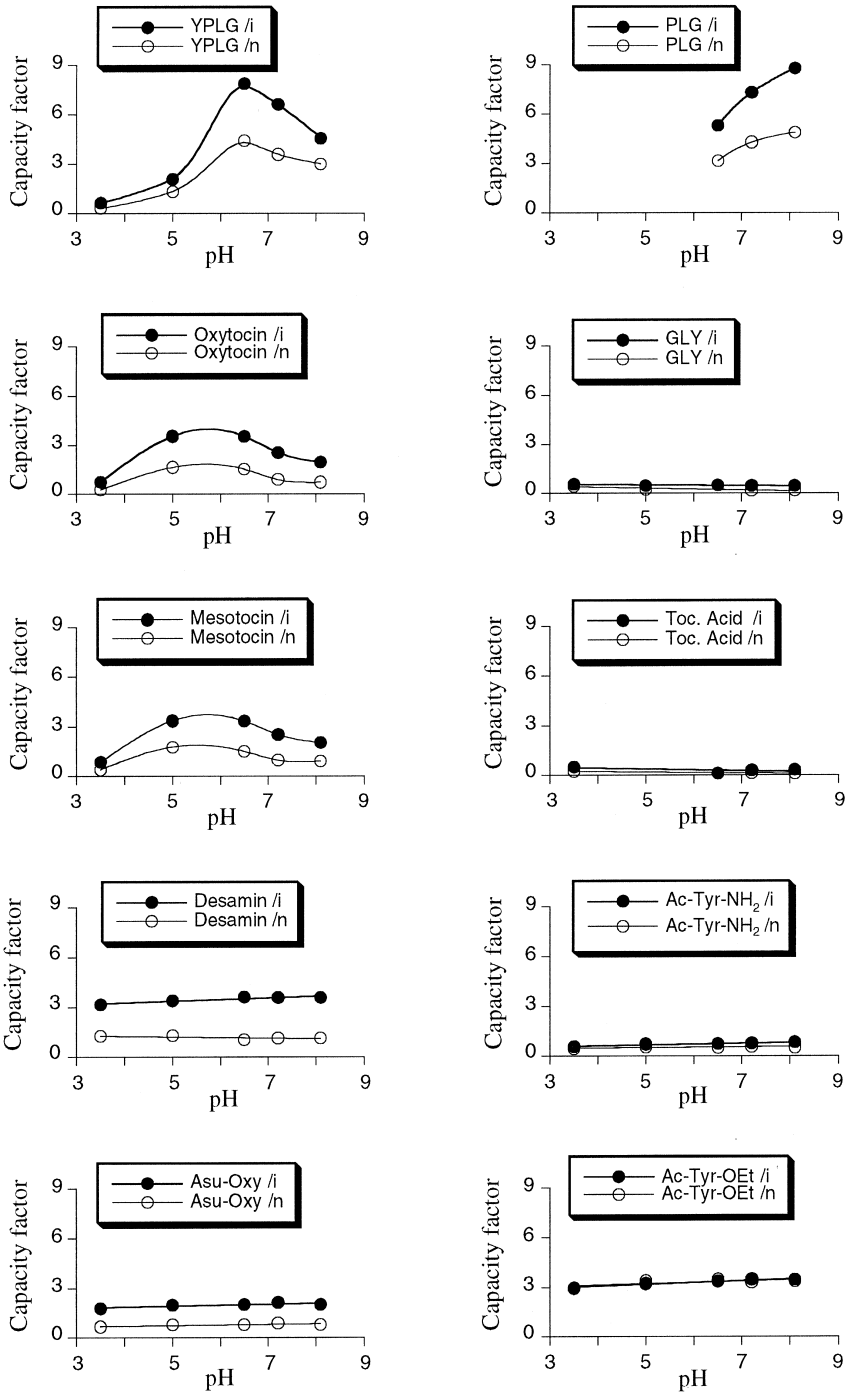

Fig. 2. Influence of mobile phase pH on the degree of interaction between the analytes and the MIP (analyte/*i*) or the control polymer (analyte/*n*). The flow-rate is 1 ml min^{-1}. The mobile phase contains a citrate–sodium phosphate buffer with ionic strength 2.5 m*M* in water–acetonitrile (80:20). UV detection at 225 nm.

Table 3
The comparison of the level of interactions between tested analytes and the MIP (k_i') or the control polymer (k_n') using aqueous-poor and aqueous-rich mobile phases

	Aqueous-poor mobile phase[a]		Aqueous-rich mobile phase[b]			
			pH 5.0		pH 6.5	
	k_n'	k_i'	k_n'	k_i'	k_n'	k_i'
YPLG	5.40	13.8	1.32	2.06	4.39	7.90
PLG	6.06	14.0			3.16	5.28
Oxytocin	2.85	6.83	1.63	3.53	1.52	3.52
Mesotocin	3.00	6.85	1.79	3.37	1.51	3.34
Desamin-Oxy	0.11	0.45	1.29	3.38	1.04	3.60
Asu-Oxy	0.11	0.43	0.80	1.97	0.81	2.02
Ac–Tyr–NH_2	0.21	0.49	0.54	0.72	0.51	0.74
Ac–Tyr–OEt	0.19	0.47	3.67	3.20	3.46	3.36
GLY	5.70	11.4	0.24	0.48		0.32

[a] Mobile phase contains 5 m*M* acetic acid and 7% water in MeCN.
[b] Mobile phase contains 2.5 m*M* citrate–phosphate buffer and 20% MeCN in water.

terminal sequence Pro–Leu–Gly–NH_2 can assure the recognition of the peptides by the MIP only in a polar aqueous-rich medium. It seems that the study of thermodynamic parameters of the peptide recognition by the MIP, which is in progress in our laboratory, can support this conjecture.

Generally, our experimental results are in good agreement with the ion-exchange mechanism for the retention of analyte by MIP in aqueous medium [30]. However, it is clear that in the case of peptides other types of intermolecular interaction should be also taken into consideration. It is possible that a protonated amino group may promote initial approach of the peptide to the MAA-containing recognition site, and subsequently more specific interactions within the recognition site can be occur. While electrostatic interactions provide the primary binding force (especially in an aqueous-rich mobile phase), other factors such as hydrogen bonds and hydrophobic interactions are thought to be involved in binding within the specific recognition sites of MIPs. A similar notion was developed on the basis of both molecular–mechanic simulations as well as nuclear magnetic resonance (NMR) measurements of complex formation between cyclodextrin and nucleotides in water: Coulomb forces alone lead to strong accumulative binding, which can, however, actually diminish the contribution of other forces responsible for fine-tuned molecular recognition. Moreover, in aqueous medium, the hydrogen bond and hydrophobic interactions, while important for the selectivity, do not contribute significantly to the overall binding ΔG [31]. Direct evidence for the hydrogen bonding in water between the ligand and its polymeric receptor should also be mentioned [32,33]. It was assumed that the polymeric receptor provides a rather apolar micro-domain, in which hydrogen bonds between guest and host are formed.

The present study has revealed the particular manner of the influence of water content, ionic strength and pH of the chromatographic mobile phase on the retention of the analytes using the MIP, synthesized by the epitope approach. The level of analyte retention by MIPs selective to relatively low-molecular-mass compounds was also reported to be sensitive to the mobile phase composition [26,27,30,34,35]. When transferring MIPs from organic to aqueous phase, the interaction mechanism changes from relying mainly on ionic and hydrogen bonds to depend more on ionic and hydrophobic interactions [36]. However, a very limited variety of points of interactions between the template and the MIP does not usually afford a switching over of the MIP selectivity. Using a somewhat larger template (e.g., Leu^5-enkephalin), the total molecular recognition involves several distinct chemical functionalities and the modification of a part of them is not essential for the cooperative binding of the whole molecule

[14]. Here, the increased diversity of points of interactions between the MIP synthesized by the epitope approach and relatively high-molecular-mass peptides, gives a unique opportunity to change the contribution for each kind of interactions.

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