Notes

Ionization State Selective Modification of Carboxyl Groups in Molecularly Imprinted Polymers: Supporting Evidence for a Binding Site Model

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Introduction

Artificial receptors in the form of molecularly imprinted polymers (MIPs) are rapidly gaining acceptance in a range of applications, notably in solid-phase extraction, affinity chromatography, chemical sensors, preparative scale separations, and drug delivery.[1−3] This approach to molecular recognition is attractive; because it is based on a simple self-assembly concept to generate the receptor, it uses mainly simple constituents and it allows fine-tuning of the binding site chemistry in a controlled manner. The result is commonly a robust network material capable of effectively recognizing a target molecule in a complex mixture.

One important drawback of most MIPs is their binding site heterogeneity.[4,5] Because of the nonstoichiometric complexation of the template by the functional monomers, only a fraction of the template added to the prepolymerization mixture gives rise to high-energy discriminative binding sites. To improve these binding properties, a number of approaches have been reported. These can generally be divided into, on one hand, those involving changes of the polymerization procedure or prepolymerization mixture constituents and, on the other hand, those focusing on postmodifying an existing material.[6] The latter approaches allow, by simple means, i.e., thermal posttreatments or chemical modifications, to improve the existing affinity and selectivity of a MIP.[7] For instance, with the aim of permanently blocking sites of low selectivity, binding sites may be blocked by chemical coupling based on the size exclusion principle[8] or in the presence of template for protection of the high-affinity sites.[9]

One can here draw a parallel between these approaches to site-selective modifications and those commonly used to chemically modify proteins.[10] Along this line, affinity labels for side chain or site-selective modification of essentially all polar functional groups in proteins have been developed. This has resulted in important structural or mechanistic information concerning the binding or active sites as well as their microenvironment. Such reagents could in principle be used for similar purposes to modify imprinted polymers. We are interested in labels for carboxylic acids because this is the functional group found in model MIPs (Figure 1) exhibiting particularly impressive recognition properties.[4,11−13]

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On the basis of potentiometric titrations and studies of the binding thermodynamics at different pH values, we previously postulated that imprinted sites in such MIPs carry more acidic carboxyl groups than nonimprinted sites.[11,14] Hence, we reasoned that selective modification of the carboxylic groups at a pH where the polymers exhibit optimum selectivity could lead to site-selective blocking of the polymer functional groups. Such reactions are commonly employed for site-selective blocking of carboxylic groups in proteins.[10] Thus, carbodiimide-catalyzed coupling of amines is known to lead to complete conversion of side chain and terminal carboxylic acid groups in proteins to amides,[15] whereas reactions with propyleneoxide preferentially convert anionic carboxylate groups to esters (Figure 2).[16] Here, we have used this protein modification chemistry to modify MIPs imprinted with different templates (Figure 1) and investigated the effect of the modifications on chromatographic retentivity, selectivity, and carboxylic acid content.

Experimental Details

General Procedures. All monomers and solvents used for the polymerization were purified before use. MAA and EDMA were obtained from Sigma-Aldrich Chemie. EDMA was purified by extraction with 10% sodium hydroxide, washing with brine and water, drying over magnesium sulfate, and followed by distillation. MAA was purified by drying over anhydrous magnesium sulfate followed by distillation under reduced pressure. The porogen dichloromethane was purchased by Sigma-Aldrich Chemie and was distilled under a positive nitrogen atmosphere prior to use. AIBN was purchased from Acros Chemicals and recrystallized from methanol before use. Glycine ethyl ester (GlyOEt), propyleneoxide, and *N*-cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimid-methyl-*p*-toluenesulfonate (CME) and the ion pair reagent phenyltrimethylammonium chloride (PTA) were purchased from Sigma-Aldrich Chemie and used as received. L-Phenylalanine anilide (L-PA) and D-phenylalanine anilide (D-PA) were synthesized as described elsewhere,[17] whereas terbutylazine (TER) and ametryn (AME) were obtained from Riedel-de Haen (Seelze, Germany).

The UV lamp used for the photopolymerizations was a high-pressure mercury vapor lamp (Philips, HPK 125 W). All chromatographic evaluations were performed using an Agilent 1100 instrument equipped with a binary pump, an autosampler, a variable wavelength detector, and a work station.

Polymerizations. Imprinted polymers were prepared according to a previously reported procedure.[18] EDMA (40 mmol, 7.6 mL), MAA (8 mmol, 0.68 mL), the template terbutylazine (**P**TER) or L-phenylalanine anilide (**P**LPA) (2 mmol) and AIBN (0.48 mmol, 80 mg) were dissolved in 11.2 mL of dichloromethane, and the solutions were then transferred to glass tubes. The polymerization mixture was degassed with nitrogen for 5 min while cooled on ice, and then the tubes were sealed and placed in a cryostat bath at ca. 10 cm distance from a UV light source. Polymerization was then initiated photochemically and allowed to proceed for 24 h at 15 °C. A nonimprinted control polymer (**P**N) was prepared in an identical manner but in the absence of the template. The tubes were then crushed, the polymers were ground and sieved under water, and the particle size fraction 25−36 μm was collected. This fraction was then repeatedly washed with 50 mL aliquots of CH_3OH/H_2O 1/1, CH_3OH, CH_3OH/CH_3COOH 80/20, and CH_3OH, dried in a

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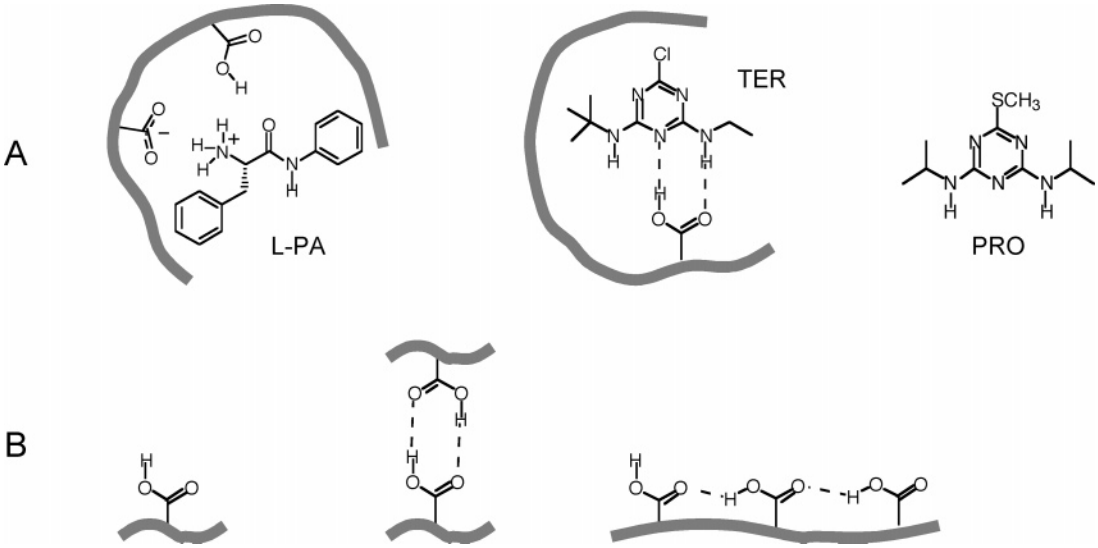

Figure 1. Examples of different local environments of carboxylic acid groups in molecularly imprinted polymers. Depending on the local environment, the groups exhibit different acidities and reactivities vis à vis esterification or condensation reactions. A: Highly discriminative imprinted sites. B: Nonimprinted sites.

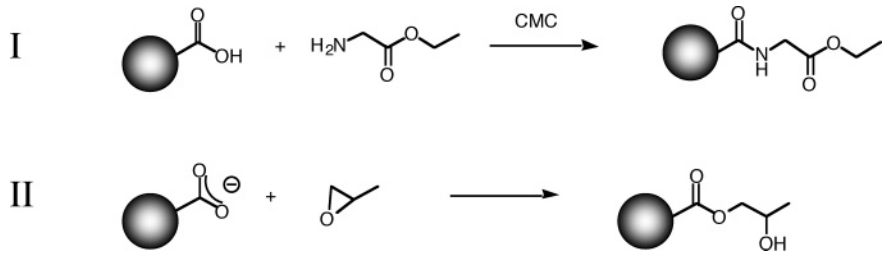

Figure 2. Reactions used to modify the carboxylic acid groups quantitatively (A) or the carboxylate groups selectively (B). The solvent in both cases were MeCN/H_2O 50/50 (v/v) with pH adjusted to 4.6.

vacuum oven at a temperature below 30 °C, and then used for the chromatographic evaluation or for the further postmodifications.

Postmodifications. *Reaction of Carboxylic Acid Groups with GlyOEt (PLPAI and PTERI).* The carboxylic acid groups of the imprinted polymers were reacted with GlyOEt as described by Hoare and Koshland for the modification of carboxyl groups in proteins.[15] The polymer (**P**LPA or **P**TER) (0.66 g (≈0.5 mmol COOH)) was suspended in a solution of GlyOEt (1.88 g, 15 mmol) in MeCN/H_2O 50/50 (v/v) (15 mL) in screwcap vials. To monitor the reaction progress by TLC ($CHCl_3$/MeOH 9/1) a separate vial was prepared where the polymer sample was replaced by cinnamic acid (222 mg, 1.5 mmol). After pH adjustment to 4.6, the reaction was started by addition of the water-soluble carbodiimide CME (0.64 g, 1.5 mmol). During the first 5 h of reaction, the pH was repeatedly adjusted to 4.6 by the addition of 0.5 M HCl or 0.5 M NaOH. Thereafter, the reaction was allowed to proceed for 24 h at 35 °C and an additional 24 h at 50 °C under continuous agitation in a thermostated shaking bath. TLC test of the cinnamic acid reaction indicated at this point near full conversion of the acid groups. The polymers were thereafter washed with MeCN/H_2O 50/50 (3 × 15 mL) and MeCN/10% HOAc 50/50 (2 × 15 mL) and were thereafter packed in columns. A dried sample was submitted to elemental microanalysis for establishing the nitrogen content. This showed that the modified polymer contained 0.14% N, whereas the nonmodified polymer contained less than 0.05% N. On the basis of a maximum content of 0.7% corresponding to full conversion of the acid groups, it was estimated that ca. 20% of the carboxylic acid groups had been converted to amides in the reaction.

Reaction of Carboxylate Groups with Propyleneoxide (PLPAII and PTERII). The carboxylate groups of the imprinted polymers were reacted with propyleneoxide as described by Frankel-Conrat for the modification of carboxylate in proteins.[16] The polymer (**P**LPA or **P**TER) (0.66 g (≈0.5 mmol COOH)) was suspended in MeCN/H_2O (4.5/7.5 mL) in screwcap vials. To monitor the reaction progress by TLC ($CHCl_3$/MeOH, 9/1), a separate vial was prepared where the polymer sample was replaced by cinnamic acid (222 mg, 1.5 mmol). After pH adjustment to 4.6, the reaction was started by addition of propylenoxide (3 mL, 45 mmol). During the first 5 h of reaction, the pH was repeatedly adjusted to 4.6 by the addition of 0.5 M HCl or 0.5 M NaOH. Thereafter, the reaction was allowed to proceed for 24 h at 35 °C and an additional 24 h at 50 °C under continuous agitation in a thermostated shaking bath. TLC indicated at this point full conversion of the acid groups. The polymers were thereafter washed with MeCN/H_2O 50/50 (3 × 15 mL) and MeCN/10% HOAc 50/50 (2 × 15 mL) and were thereafter packed in columns.

Selectivity Tests by Chromatography. The 25−36 μm particle fraction of each polymer batch was slurry packed into stainless steel HPLC columns (40 × 4 mm) using MeOH/H_2O 80/20 as the pushing solvent at pressures up to 300 bar. The columns were then evaluated using CH_3CN/H_2O/CH_3COOH 92.5/2.5/5 (v/v/v) (**P**LPA polymers) or CH_3CN (**P**TER polymers) as mobile phases at a flow rate of 0.1 or 1 mL/min, respectively. The wavelength of detection was 254 nm, and the injection volume was 10 μL. Solutions (1 mM) of the analytes in the mobile phase were injected unless otherwise stated. The capacity factor k was calculated as $(t - t_0)/t_0$ where t is the retention time of the analyte and t_0 the retention time of a void marker (acetone).

Effect of pH on Ion Pair Retention. The retention of the ion pair reagent phenyltrimethylammonium chloride on the imprinted polymer columns was investigated as a function of mobile phase pH, as described by Haginaka and Kagawa.[19] Two mobile phases, A and B, were prepared: A: Phosphoric acid (0.02 M)/MeCN 70/30; B: K_3PO_4 (0.02 M)/MeCN 70/30. A solution of PTA (10 mM) in MeCN/H_2O (70/30) was then injected (10 μL) on the columns equilibrated in mobile phases made up of A and B in different proportions using a binary pump. The investigation started using the most basic mobile phase (10% A). Thereafter, A was increased

Table 1. Results from the Chromatographic Evaluation of the L-PA Imprinted Polymers[a]

polymer	sample load	k_D	k_L	α
PLPA	10 nmol	3.6	27	7.5
PLPAI		0.8	5.7	7.1
PLPAII		0.43	1.7	4.0
PLPA	100 nmol	2.5	11	4.4
PLPAI		0.49	1.3	2.7
PLPAII		0.49	1.7	3.5

[a] Retention factor of different injected amounts of D-PA (k_D) and L-PA (k_L) and the corresponding separation factor ($\alpha = k_L/k_D$) measured by chromatography using the different L-PA-imprinted polymers as stationary phases. The mobile phase was $CH_3CN/H_2O/CH_3COOH$ 92.5/2.5/5 (v/v/v) and the flow rate was 0.1 mL/min.

in steps of 10% to finally arrive at the most acidic phase tested (90% A). Accuracy was checked by subsequent back-titration to the basic mobile phase. The apparent pH of each composition was checked manually. The flow rate was 1 mL/min and the wavelength of detection was 260 nm.

Results and Discussion

Polymer samples of the two model MIPs (PLPA and PTER) were subjected to the two modifications, as shown in Figure 2, at or near the optimum pH (4.6) found from previous investigations.[11] This resulted in modified MIP variants I and II with the numbers referring to the type of chemical postmodification. Reaction conversion monitored by TLC of a homogeneous carbodiimide-catalyzed reaction of cinnamic acid showed that nearly complete conversion had been reached after 2 days reaction at elevated temperature.

From the nitrogen content of a dried polymer sample modified by reaction I, it was concluded that only ca. 20% of the carboxylic acid groups had been converted to amides in the reaction. This should, however, be seen as a low estimate due to the hygroscopic nature of the materials. Conversion in the case of reaction II could not be established by this technique but could be inferred from the retention of positively charged solutes in chromatography.

The modified polymers were packed in chromatographic columns and assessed for their affinity for their templates or structural analogues in terms of retention factors and separation factors at different sample loads (Table 1 and Figure 3). Thus, the **P**LPA polymers were assessed by comparing the retention of the two enantiomers of PA, whereas the **P**TER polymers were tested for their ability to separate terbutylazine (TER) from prometryn (PRO) and L-PA. First of all, Table 1 and Figure 3 show that the modified polymers exhibited lower selectivity and retentivity than the nonmodified polymers. However, among the former polymers, those modified by reaction I exhibited higher selectivity and retentivity compared with that of the polymers modified by reaction II (with the exception of the L-PA imprinted polymers at high sample load). Particularly for the PTER system, the latter reaction resulted in a complete loss of imprinting effects (Figure 3). Obviously these different effects may be the simple result of reaction II leading to higher conversion than reaction I. Given that the retention under these conditions is due, almost exclusively, to electrostatic interactions,[11] the similar retention of L-PA on **P**TERI and **P**TERII suggests this factor to be of minor importance. This is further supported by the similar retention factors observed for D-PA on PLPAI and PLPAII at the high sample load. Under these conditions, D-PA should sample the low- and high-affinity sites more equally, with a corresponding retention factor reflecting the total number of carboxylic groups in the polymers.

Under the presumption that reaction II affects mainly the high-energy binding sites, this modification may offer a means of

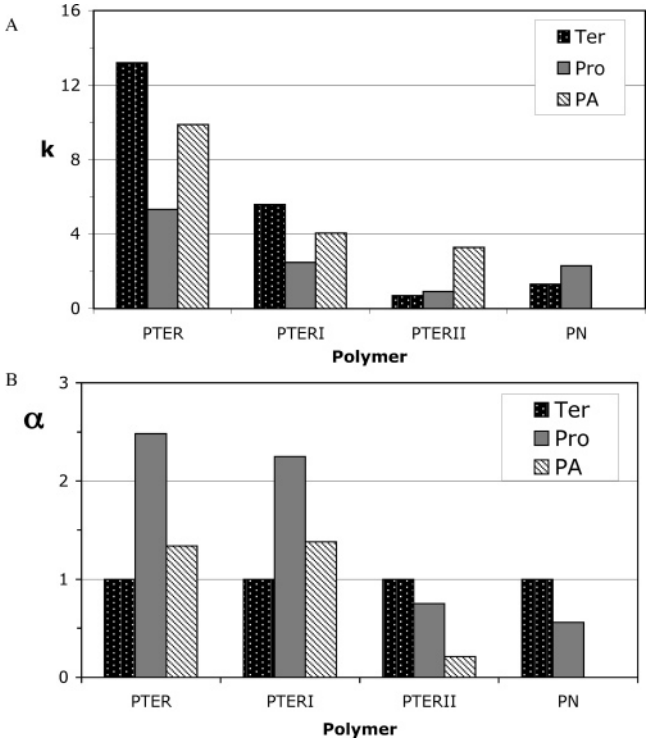

Figure 3. (A) Retention factor of TER (black bars), PRO (grey bars), and L-PA (striped bars) and (B) the corresponding selectivity factor ($= k_{TER}/k_X$) where k_X = retention factor of analyte X and k_{TER} = retention factor of the template measured by chromatography using the different TER-imprinted polymers and a nonimprinted polymer (PN) as stationary phases. Black bars: X = TER, grey bars: X = PRO, and striped bars: X = L-PA. The mobile phase was CH_3CN, the sample load 10 nmol, and the flow rate 1 mL/min.

reducing the binding site heterogeneity of MIPs. One indication that this may be possible is seen in the comparably small reduction in the separation factor (α) of PLPAII in response to a 10-fold increase in sample load (from 4.0 to 3.5). This contrasts with the large drop in α for PLPAI from 7.1 to 2.7 in the same sample load interval.

To gain further insight into the extent of acid group modification, the pH-dependent retention of a positively charged ion pair reagent (phenyltrimethylammonium chloride, PTA) was studied. This technique was previously used to estimate the average pK_a of MIPs and should reflect the extent of ionized acid groups present at each investigated mobile phase pH.[19] The retention of PTA was lower on the modified materials than on the native MIPs at all mobile-phase pH values (Figure 4).

Only small differences were seen in the pH-dependent retention profiles of materials subjected to the two modifications, which indicates that they have been substituted to similar degrees (vide supra). At a high mobile-phase pH (pH_{app} = 11), the retention factor was ca. 20−30% higher on the nonmodified polymers versus that of the modified counterparts. This value is in rough agreement with the conversion determined after reaction I based on elemental analysis (ca 20%). Attempts to further determine the conversion of the carboxylic groups by IR spectroscopy failed due to the absence of bands uniquely attributable to the reacting partners.

One source of error in these titrations is the susceptibility of pendent ester groups resulting from reaction II and reaction I (in the latter case, probably to a lesser extent) to hydrolysis under basic conditions. Partial hydrolysis of these pendants during the chromatographic run could thus lead to artificially high retention factors in some of the polymers. This effect was

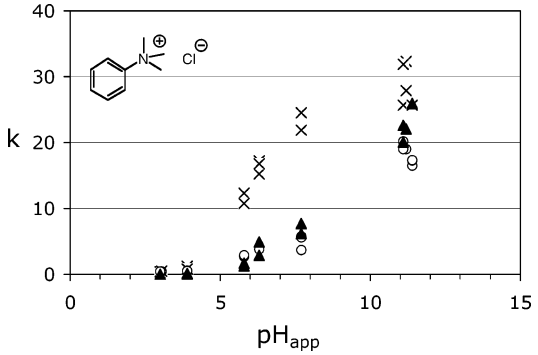

Figure 4. Effect of mobile phase pH on the retention of PTA (see inserted structure) on PTER (black crosses), PTERI (black triangles), and PTERII (open circles). Mobile phase: (A) Phosphoric acid (0.02 M)/MeCN 70/30; (B) K_3PO_4 (0.02 M)/MeCN 70/30 with varying mixing ratio as given in the Experimental Section. Flow rate: 1 mL/min. Detection wavelength: 260 nm.

observed for PLPAII, which by accident was equilibrated at high pH values for a prolonged period. After this equilibration, this polymer exhibited a similar pH retention profile, as observed for PLPA, indicating hydrolysis of most of the modified groups.

Nevertheless, the reproducible data obtained for the other polymers by back-titration indicated that, within the time window of the analysis, this side reaction was not influencing the results.

The two model MIPs studied here have previously been the subject of a number of investigations. From these, it is known that binding isotherms measured for these MIPs can be fitted with Langmuir binary site or Langmuir−Freundlich isotherm models with a low abundance of high-energy sites (a few μmol/g) and a high abundance of low-energy sites.[4,14] Furthermore, it is known that the retention behavior of ionic solutes can be described by an electrostatic retention model stating a proportional dependence of the retention on the product of the degree of ionization of the solute and that of the stationary phase.[11] Thus, the MIPs exhibit high selectivity at low pH values and loss of selectivity at pHs exceeding the pK_a value of the template. On the other hand the retentivity is low at low pH values and increases with pH to reach a maximum at a pH near the apparent pK_a of the solute. Above this pH, the charge of the solute is lost. In the case of L-phenylalanine anilide (L-PA) and terbutylazine (TER), this maximum is found at an apparent pH between 4 and 5 (acetonitrile/buffer 70/30). Still, from potentiometric titrations, it is known that only a fraction of the carboxylic acid groups are ionized at this pH, whereas up to 85% of the nominal number of acid groups are titrated in the apparent pH range 4−12.[11] In this study, we postulated, as schematically depicted in Figure 1, that the carboxylic acid groups of the imprinted sites are more acidic than those of the nonimprinted sites. Support for this postulate was later on obtained from a study of the thermodynamics at different pH values.[14] Thus, at a pH were optimum retention and selectivity are observed, epoxides would react preferentially with the carboxylate groups of the imprinted sites. This agrees with the results obtained.

The following concluding remarks can be made. The extent of accessible acid groups in the polymers remaining after reaction II appeared to be similar to those remaining after reaction I. These modifications had completely different effects on the retentivity toward the template and their analogues. Taken as a whole, the results indicate that these reactions selectively can target different carboxyl groups in the polymers. In particular, the epoxide modification of carboxylate groups may allow a selective poisoning of the high-energy sites of carboxylic acid containing imprinted polymers.

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Supporting Information Available: Transmission IR spectra (KBr) recorded for PTER, PTERI, and PTERII after modification and workup of the polymers. Effect of mobile phase pH on the retention of PTA on PLPA (black crosses), PLPAI, and PLPAII (open circles). This material is available free of charge via the Internet at http://pubs.acs.org.

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