Imprinted Membranes for Sensor Technology: Opposite Behavior of Covalently and Noncovalently Imprinted Membranes

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ABSTRACT: New types of polymeric membranes with molecular recognition sites for L-phenylalanine (L-Phe), 6-amino-1-propyluracil (APU), atrazine, and sialic acid have been prepared using the molecular imprinting approach. The membrane synthesis includes radical polymerization of ethylene glycol dimethacrylate (EDMA) and functional monomers in the presence of a template. Several compounds—-(diethylamino)ethyl methacrylate (DEAEM), methacrylic acid (MAA), allylamine (AA), and (4-vinylphenyl)-boronic acid—were as functional monomers, which are able to form covalent, ionic, or hydrogen bonds with the corresponding templates. Template specific conductometric sensors, based on these polymers, were constructed and studied. An opposite response of covalently versus noncovalently imprinted membranes was demonstrated and discussed in detail. Sensors based on these materials could detect the target molecules at concentrations of 1−50 μM in solution. The high specificity and stability of these imprinted membranes render them promising alternatives to enzymes, antibodies, and other natural receptors usually used in sensor technology.

Introduction

Biosensors technology is a developing area in the quest for innovative approaches to analysis. They hold the potential to provide a powerful and often considerably less expensive alternative to traditional, well-established laboratory techniques in medicine, environmental monitoring, biotechnology, pharmaceutical and food industries.[1,2] Microorganisms, enzymes, receptors, and antibodies were used as the molecular recognition biomaterials, contributing to an extremely high selectivity.[3] However, insufficient stability of biological materials often limits their incorporation to biosensor development.

In previous papers we have described the construction of sensor systems, based on synthetic polymers, that mimic biological receptors.[4−6] The synthesis of the artificial receptors was carried out by the imprinting procedure proposed by Wulff.[7−9] The imprinting polymerization consists of cross-linking of the functional monomers in the presence of a substrate template by radical polymerization followed by removal of the target molecules. This procedure results in formation of the cavity imprints of a specific shape and a defined arrangement of functional groups on the polymer surface. On subsequent addition of the template molecules, recognition occurs by a combination of reversible binding and shape complementarity (Figure 1). This approach mimics the unique characteristics of a receptor that binds the ligand in a perfectly fitting cavity or cleft containing functional groups in the appropriate stereochemistry for binding.

The response of imprinted polymer based sensors seems to be related to a change in the polymer porous structure resulting from the interactions of the template molecules with the selective cavities.

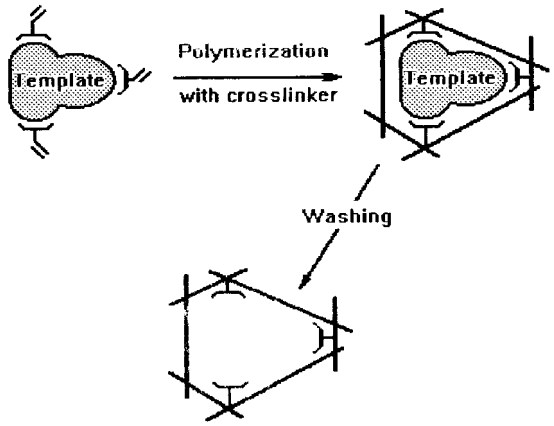

Figure 1. Scheme of the imprinting polymerization.

The advantages of sensors based on imprinted polymers are their high stability, which allows long operation under harsh conditions without loss of sensitivity,[10] and their high specificity, which is comparable to that of polyclonal antibodies.[11]

In the present article we describe the synthesis of stable polymeric membranes imprinted with templates that are able to interact through covalent, ionic, or hydrogen bonds with the corresponding functional monomers, and their applicability in sensor development.

Experimental Section

Materials. All compounds were obtained from commercial sources and used as received except for ethylene glycol dimethacrylate, which was distilled in a vacuum prior to use in order to remove stabilizers. All chemicals and solvents were of analytical or HPLC grade.

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Table 1. Polymerization Conditions of the Monomeric Mixtures[a]

polymer/ template	(vinylphenyl)boronate, mM	allylamine, mM	MAA, mM	DEAEM, mM	template, mM	EDMA, mM
P1/APU				2	1	26
K1/APU				2		26
P2/atrazine				6	0.5	22
K2/atrazine				6		22
P3/ L-Phe				1	1	26
K3/ L-Phe				1		26
P4/atrazine			3		1	26
K4/atrazine			3			26
P5/sialic acid	1	1			1	30
K5/sialic acid	1	1				30

[a] The polymerizations were performed in a surplus of EDMA as cross-linker on the glass filter surface. DMF was added as the porogen in an amount equal to that of EDMA. The initiator azobis(isobutyronitrile) was added in 1 wt % with respect to the monomeric mixtures. Polymerization was initiated by heating in a thermostat for 24 h at 353 K. Abbreviations: APU = 6-amino-4-propyluracil; MMA = methacrylic acid; DEAEM = (diethylamino)ethyl methacrylate; EDMA = ethylene glycol dimethacrylate.

Preparation of the Sugar Monomer. Sialic acid (309 mg, 1 mmol) was esterified with 148 mg (1 mmol) of tris(4-vinylphenyl)boroxine in 30 mL of dioxan by azeotropic distillation (88 °C) of the water formed during the reaction. Subsequently, the solvent was removed by vacuum evaporation and the crude product (M1) was used without further purification.

Preparation of Imprinted Polymeric Membranes. The polymeric membranes, imprinted by L-phenylalanine, 6-amino-1-propyluracil (APU), and atrazine, were prepared with methacrylic acid (MAA) or (diethylamino)ethyl methacrylate (DEAEM) as monomers and a surplus of ethylene glycol dimethacrylate (EDMA) as cross-linker on the surface of a glass filter (30 mm in diameter, 4 mm thick). The sialic acid specific polymer utilized allylamine (AA) and sialic acid−*O*-((4-vinylphenyl)boronate) (M1) as functional monomers with a surplus of EDMA as cross-linker. The compositions of the monomeric mixtures are given in Table 1.

A typical preparation of a polymeric membrane-coated glass filter specific for atrazine (P2) was performed as follows: A glass filter with the tip removed was placed in a glass tube and its surface was immersed in the monomer mixture, consisting of 5 g of DMF, 4.36 g of EDMA, 1.11 g of DEAEM, 108 mg of atrazine, and 50 mg of azobis(isobutyronitrile) (AIBN), and saturated with nitrogen. All the handling was made under a nitrogen atmosphere. The glass tube was covered and transferred into a thermostat for 24 h at 353 K. Afterward, the glass filter was carefully cleaned of the excess of polymeric particles and washed with boiling DMF and boiling water in order to remove nonreacted monomer, template, etc. Finally, the polymeric membrane was transferred into 50 mM Tris−HCl buffer solution (pH 8.0) for the electrochemical measurements. All other imprinted membranes were prepared in the same way except for control polymers, which were synthesized in the absence of template.

Electroconductivity Setup and Measurements. Electrochemical measurements were carried out at room temperature in a cell with two platinum electrodes (with a surface area is 1 cm^2) and a working volume of 50 mL divided in two by the imprinted membrane. As a supporting electrolyte, 50 mM Tris−HCl buffer solution (pH 8.0) was used with intensive stirring. The change in the membrane electroresistance, induced by interaction with the template, was recorded as a function of time. The template concentration was increased gradually by addition of aliquots from a 5 mM concentrated stock solution in deionized water.

Membrane electroconductivity measurements were performed as described earlier[6] by applying a small-amplitude alternating voltage (20 mV, 1 kHz) generated by a low-frequency waveform generator (G-112/1, Russia) to the electrodes. The output signal was detected using a selective amplifier (Unipan-232B, Poland).

Results and Discussion

To obtain highly specific imprinted polymers, the formation of stable complexes between the templates and their functional monomers in the reaction mixture is crucial and must be preserved in the resulting polymers. Both covalent and noncovalent interactions could be used for the fixation of the template molecules within the selective cavities.[7,12−14] In the case of covalent interactions during polymerization, there is a stoichiometric relation between the template and the imprinted binding site. The splitting of the template from the polymer results in the formation of uniform binding cavities. In the second case, since the association constant between the polymer and the template is relatively low, an excess of functional monomer is required to saturate the recognition sites, and the removal of the template leaves a heterogeneous population of binding sites. Therefore, polymers prepared by covalent and by noncovalent binding behave quite differently, and the results of our investigation revealed the interesting fact that this might be true with respect to electroconductivity.

Several polymers specific for templates of different chemical nature were synthesized in order to investigate the influence of the polymer composition on the nature and magnitude of the sensor response. Membranes with various types of interactions between the functional monomers and the templates, i.e., hydrogen bonds (in polymers P1, imprinted with APU and P2, imprinted with atrazine), hydrogen bonds and electrostatic interaction (in polymers P3, imprinted with L-phenylalanine and P4, imprinted with atrazine), and covalent bonds (in polymer P5 with template sialic acid), were examined. The template/functional monomer ratio was optimized in a series of experiments, and only the results obtained with the best responding polymers are presented in this paper.

The molecular recognition ability of the imprinted polymers was studied electrochemically. To avoid electrode polarization, the electrical conductivity measurements were carried out using a small amplitude alternating current (ac). From the calibration curves of the imprinted membranes presented in Figure 2, it was evident that for all imprinted polymers, described above, the presence of the template molecules resulted in a change of conductivity. Membrane conductivity increased proportionally to the template concentration up to 20 μM but tended to saturate (for polymers P1, P2, and P4) at higher concentrations. The response time for the sensors was on the order of 30 min (the required time for obtaining 80% of the signal).

The signals obtained with the nonimprinted membranes were 5−10 fold lower than those obtained with the imprinted ones (data not shown).

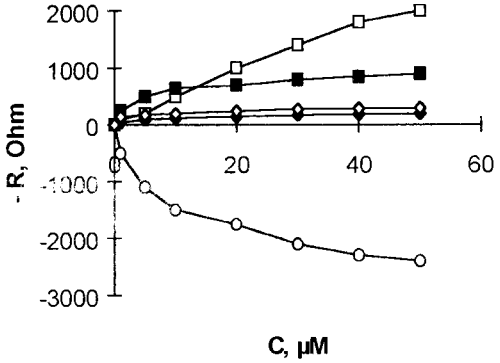

Figure 2. Dependence of the sensor responses of the imprinted polymers on template concentration: (◆) P1 (template APU); (■) P2 (template atrazine); (□) P3 (template L-Phe); (◇) P4 (template atrazine); (○) P5 (template sialic acid).

As can be seen in Figure 2, the sensors prepared with P1−P5 exhibited very diverse behavior. Sensors prepared from P4 (with template atrazine and MAA) and P1 (with template APU) showed a small positive effect, whereas sensors from P2 (prepared with atrazine and DEAEM) and P3 (prepared with L-Phe) displayed a considerable increase in membrane electroconductivity and had a different sensitivity. On the other hand, electroconductivity decreased strongly in the presence of the template for the sensors, prepared with P5. The fact that the sensor prepared from P5 showed a strong but inverse sensor response was of special interest.

A swelling of 60−100%, depending on the polymer matrix and solution, was observed following the splitting of the template for the polymer, synthesized with the template monomer prepared from phenyl α-D-mannopyranoside and (4-vinylphenyl)boronic acid (designed for covalent interactions).[9] This swelling occurred in the empty cavities by solvation of the boronic acid binding sites. Most of the functional groups that originated from the functional monomers are probably concentrated inside the selective cavities. Upon rebinding, the selectivity remained, yet the volume of the polymer was reduced nearly to the original volume. By analogy to enzymes, this effect has been called "induced fit".[9] A different behavior was observed with polymers prepared using noncovalent binding.[15] In this case, only 15% of the cavities could be reoccupied. A substantial portion of the functional groups were distributed all over the polymer, and after removal of the template, the swelling by solvation of the binding sites takes place not only inside the polymeric domains but also throughout the polymer surface. Therefore, rebinding of the template molecules can affect differently the polymer's structure of covalently and noncovalently imprinted polymers. With respect to the results obtained in the present study, the above observations implied that the polymers imprinted with sialic acid using boronic acid (covalent bonds) were highly swollen after removal of the templates. When the sialic acid was added again, the polymeric domains shrank and became more compact, and therefore membrane electroconductivity, which depends on ion transfer through the polymer's micropores is decreased.

The situation is more complicated regarding the polymers that were prepared using noncovalent interactions with the templates. The overall swelling after washing off the template seemed to be small, as is the overall shrinking that followed reloading. Shrinking and swelling could occur all over the polymer since the binding sites are more or less uniformly distributed.

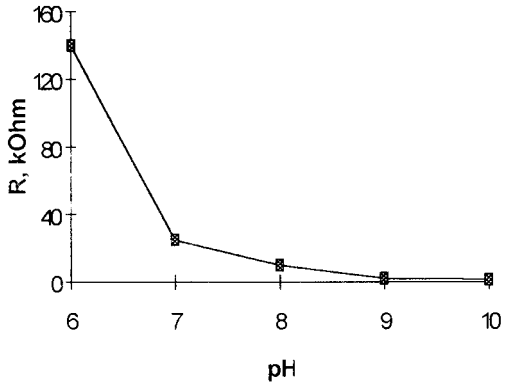

Figure 3. pH dependence of the polymeric membrane P5 electroresistance.

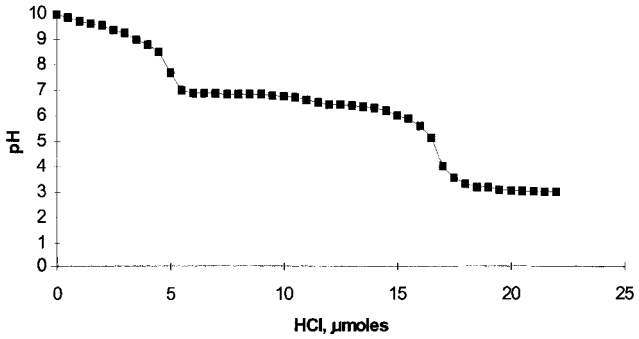

Figure 4. Potentiometric titration of P5 suspension.

Depending on the polymer morphology, partial shrinking of the polymers might also increase the size of the micro- and macropores and thus lead to an increase in electroconductivity.

The responses observed with polymers P1−P5 might be explained also by alteration of the surface charge. In the case of P5 during the interaction with the template, a change in the concentration of charged groups on the polymer surface occurs due to esterification of the boronic acid by the sialic acid. This process changes surface conductivity and could lead also to conformational reorganization in the polymer network because of a change in repulsive forces between the polymeric chains. To some degree, adsorbed template could change also the surface charge of P1−P4 and thus lead to conformational changes in the polymer network.

To check the influence of the charges within the polymer on the membrane conductivity, a series of buffer solutions with different pHs but with equal electroconductivities were prepared and adjusted by saturated KCl solution. A membrane made of P5 showed low conductivity at pH values less than 7 (Figure 3) where most of the boronic acid functional groups existed as neutral, trigonal boronic acids.

Potentiometric titration of a suspension of polymer P5 confirmed that the pK_a value of the boronic acid functional groups of this polymer was around 8, a value that is in agreement with data in the literature (phenylboronic acid has a pK_a = 8.86)[16] (Figure 4). The results of these experiments demonstrated that the presence of charged ionized boronic acid groups was essential for high membrane conductivity.

An analysis of the dependence of the membrane electroresistance on the voltage applied was carried out in order to optimize the performances of the sensors based on P5 (Figure 5). It was found that changes in the membrane electroresistance, induced by interaction

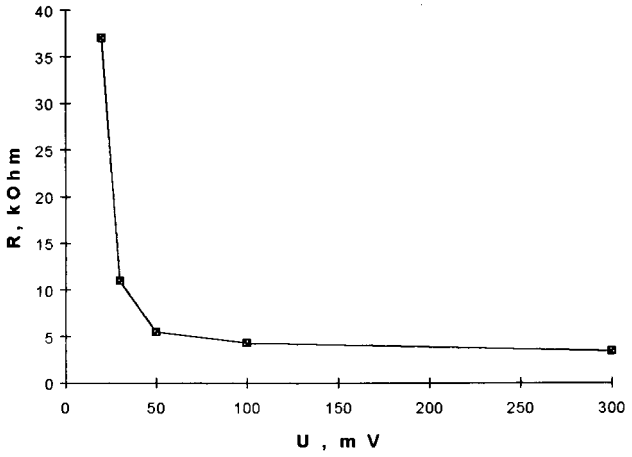

Figure 5. Dependence of the polymer P5 electroresistance on applied voltage.

with the templates, could be optimally detected at an applied voltage of 10−30 mV. At lower voltages, the reproducibility of the measurements was insufficient, whereas at higher voltages, changes in membrane electroresistance, induced by template binding, were negligible. Thus, the 20 mV used in Figure 2 represents the optimal voltage.

Membranes prepared from P1, P2, and P4 showed clear saturation phenomena at 20 μM template concentration (Figure 2). Such saturation was absent in the case of P3 and P5. A possible explanation for this phenomenon may be drawn from the fact that the individual cavities in the polymer have varying degrees of affinity and selectivity similar to those of polyclonal antibodies.[9,10] The binding of the template molecules to high-affinity sites, concentrated inside the polymeric domains led to their shape changes. Low-affinity sites were probably concentrated on the surface of the polymer pores and bound the templates without causing reorganization of the polymer structure. Differences in the proportion of high- to low-affinity sites[17] of the polymers might explain the distinct saturation phenomena. A detailed investigation of the processes involved in polymeric membrane−template interactions is in progress.

In the sensor technology, several factors are of main interest: sensitivity, selectivity, response reproducibility, and stability. As for the sensitivity of the imprinted polymers, it was shown that analytes in the micromolar concentration can be detected (Figure 2).

The selectivity of the imprinted polymers was studied in an earlier work with an atrazine-imprinted membrane,[6] and it was shown that introduction of substrates with a molecular structure similar to atrazine in the electrochemical cell (triazine and simazine) resulted in changes of less than 50 Ω, confirming high selectivity of the molecular imprinted polymers. In our experiments, the highest responses were always observed for the template.

The reproducibility of the sensor responses was determined to a large extent by the conditions of the membrane preparation and the washing step. The response deviation was observed at about 20−30% for different membranes with the same composition. The reproducibility of the conductometric measurements, using the same membrane was about 10%. Further improvements in the reproducible preparation of thin imprinted membranes must be achieved in order to enable practical application of these materials in sensor technology.

The stability of the membranes was investigated by repeating measurements of P2 for atrazine during a 4 month period. It was found that imprinted membranes, prepared as described above, could have been stored at room temperature in a buffer solution for 4 months without showing any loss of their sensitivity.

In conclusion, a number of new polymeric membranes were prepared by molecular imprinting and tested as novel materials for sensor development. These membranes enabled the detection of the templates in solution in the range of 1−50 μM. The high specificity and stability exhibited by these membranes make them a promising alternative to enzymes, antibodies, and natural receptors in sensor technology.

Although our current research is still in a preliminary stage, it seems that the proposed technique could provide a useful and easy procedure for the preparation of imprinted membrane based sensor systems with high selectivities and sensitivities for many classes of organic substances.

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