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Molecularly Imprinted Polymers for Biomolecular Recognition

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Summary

Molecular imprinting of polymers is a concept for the synthetic formation of structurally organized materials providing binding sites with molecular selectivity. Compared to biological receptors, these polymeric recognition systems have the advantage of superior chemical and mechanical stability with potential applications in areas such as biomimetic catalysis and engineering, biomedical analysis, sensor technology, or the food industry. In particular, molecularly imprinted polymers (MIPs) providing selectivity for biorelated molecules are gaining substantial importance. In this context, a self-assembly approach for the synthesis of imprinted polymers against the flavonol quercetin is presented, which is exemplary for the biologically relevant group of flavonoid compounds. The creation of synthetic selective recognition sites for this biomolecule is demonstrated by comparing the separation capabilities of imprinted and nonimprinted polymer particles for several structurally related molecules via high-performance liquid chromatography experiments. The developed quercetin-MIP enables selective extraction of quercetin even from complex mixtures, demonstrating the potential for designing biomimetic recognition materials with improved selectivity for biomolecules with tunable functionality at a nanoscale.

Key Words: Molecularly imprinted polymers; synthetic receptors; biomimetic recognition; flavonoids; quercetin; high-performance liquid chromatography.

1. Introduction

For the last two decades, the concept of designing synthetic molecular recognition materials that can mimic biological functions has generated substantial interest ***(1–7)*** and stimulated research on structurally organized materials. Particularly at a nanoscale level, the technique of molecular imprinting—creating synthetic recognition sites within macromolecular matrices by template inclusion during polymerization—has demonstrated potential for a variety of

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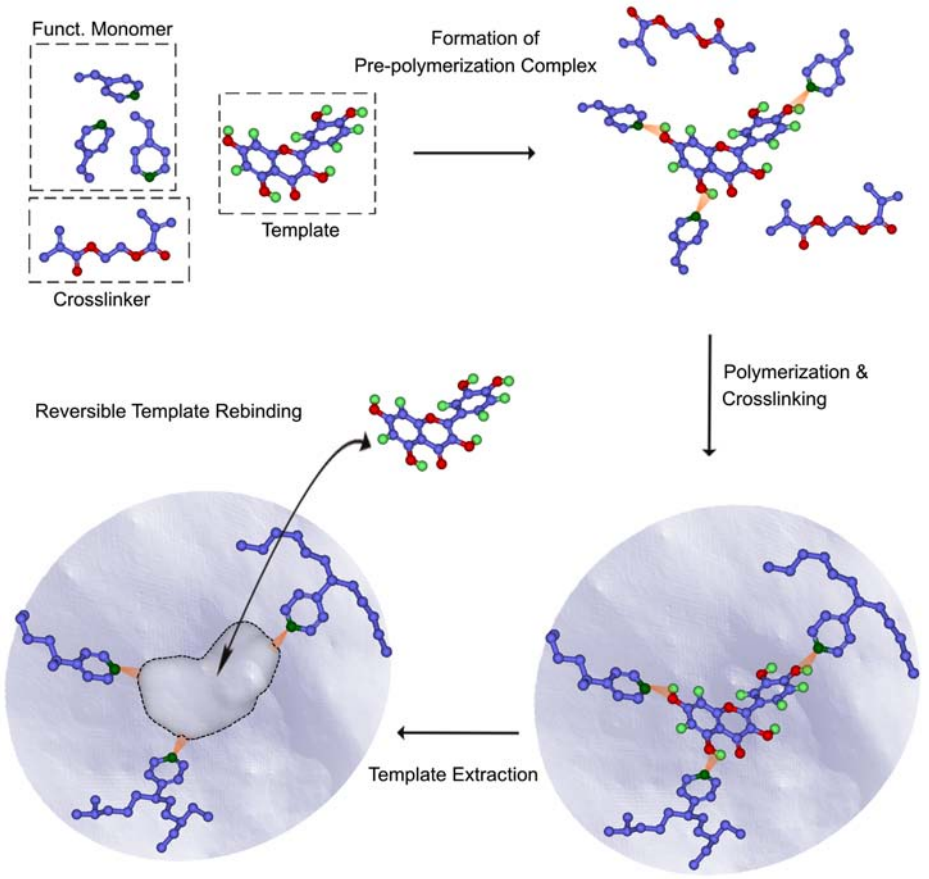

Fig. 1. Schematic concept of molecular imprinting.

applications ***(8,9)***. Generally, this methodology is based on utilizing the functionalities of a target molecule (template) to assemble its own recognition cavity by forming interactions with "complementary" functional groups of appropriate functional monomers. These interactions are provided by either cleavable covalent bonds or noncovalent interactions, which are then "frozen" in position by polymerization carried out in the presence of a high concentration of crosslinker ***(10–13)***. Subsequent removal of the template creates binding pockets within the polymer matrix that memorize the spatial arrangement of functional groups and the size and shape of the target molecule. Ideally, highly selective recognition of the imprinted analyte is thereby ensured, which favors a reversible rebinding process and selective retention of the templated analyte within the biomimetic recognition matrix (*see* **Fig. 1**).

So far, molecular imprinting has mainly been demonstrated for templates with a relatively low molecular weight ***(14–16)***. In the past few years, successful preparation of molecularly imprinted polymers (MIPs) specifically targeting practically relevant biomolecules such as flavonoids ***(17,18)***, mycotoxins ***(19)***, proteins, or carbohydrates ***(20–26)*** has been discussed in the literature. Nevertheless, the formation of synthetic receptors for macromolecules remains a challenging task, and appropriate procedures for the preparation of MIPs selective for specific biomolecules are of substantial biological and biomedical interest.

In the present study, imprinted polymers against the flavonol quercetin were successfully synthesized and characterized via high-performance liquid chromatography (HPLC) experiments. Because quercetin represents a biomolecule with several functionalities enabling noncovalent interactions with functional monomers, this approach is a suitable example for the formation of synthetic recognition matrices selective for biomolecules.

2. Materials

1. Laboratory mortar grinder.
2. Standard testing sieve (25 µm).
3. HPLC column slurry packer (pressure: 10,000 psi maximum).
4. Blank stainless steel HPLC columns (150 mm or 250 × 4.6 mm id).
5. HPLC system with a UV/Vis diode array detector.
6. Template analyte (quercetin dihydrate).
7. Acetone, purissim (>99%) over molecular sieve (used as porogenic solvent).
8. 4-Vinylpyridine ([4-VP] functional monomer). Reactive monomer; store under refrigeration below –10°C (+14°F). This monomer polymerizes gradually at room temperature. At elevated temperatures, polymerization could occur, generating heat and pressure, which could rupture a closed container.
9. Ethyleneglycol dimethacrylate ([EGDMA] crosslinker).
10. 2,2'-Azobisisobutyronitrile (polymerization initiator).
11. Morin.
12. (+) Catechin.
13. Rutin.
14. 2-Carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone (C-fla).

3. Methods

The methods described next outline synthesis of the molecularly imprinted block-polymer; the preparation steps including grinding, sieving, and sedimentation required before application of the polymer; separation experiments applying the prepared polymer particles as HPLC separation matrix; and evaluation of the obtained results.

3.1. Synthesis of Polymer

3.1.1. Imprinted Polymer

The pre-polymerization mixture was prepared as follows: 1 mmol of quercetin as the template molecule was dissolved in 15 mL of acetone in a glass vial (30-mL volume). Then 8 mmol of the functional monomer 4-vinylpyridine (*see* **Note 1**), 40 mmol of the crosslinker ethyleneglycol dimethacrylate (*see* **Note 2**), and 2% (w/w) 2,2'-azobisisobutyronitrile were added in the denoted order. The solution was next cooled in an ice bath and purged with nitrogen for 5 min. Finally, the polymerization was started (*see* **Note 3**) by thermal initiation at 60°C in a water bath (*see* **Note 4**).

3.1.2. Control Polymer

To assess properly the imprinting effect obtained for the target analyte, a nonimprinted control polymer is prepared for control experiments. The same procedure as described in **Subheading 3.1.1.** is applied to synthesize the nonimprinted polymer, but without adding the template.

3.2. Processing of Synthesized Polymers

The following steps describe the processing of the block-polymer for application as a separation matrix in liquid chromatography (the same procedure as applied for imprinted and control polymer).

1. Crush a polymer block into small particles using a porcelain mortar and pestle.
2. Grind the polymer particles for 3 min in a mechanical mortar.
3. Wet-sieve the polymer particles with acetone using a 25-µm sieve.
4. After drying, subject the polymer particles, which are detained by the sieve, to additional grinding and wet-sieving by repeating **steps 2** to **4** until most of the polymer particles are sieved.
5. Sediment the collected acetone-polymer particle mixture in approx 250 mL of acetone to eliminate fine particles of grain sizes <5 µm. After 1 h, discard the supernatant and add fresh acetone to the precipitated polymer particles. Several sedimentation steps are necessary to eliminate the majority of fine particles (*see* **Notes 5** and **6**).
6. Wash the polymer particles with methanol and dry at 45°C for 24 h in an oven.

3.3. HPLC Separation Experiments

Selective separation matrices based on molecularly imprinted polymers should exhibit selective retention behavior for the template analyte when evaluating the separation capabilities of imprinted and control polymers for the template molecule and structurally related compounds (*see* **Table 1**). Therefore, the imprinted polymer particles and the control polymer particles were applied as stationary phase during HPLC experiments ***(27)*** (*see* **Note 7**).

1. Sonicate 3 g of polymer particles (either imprinted or control polymer) in approx 50 mL of acetone and pack into stainless-steel HPLC columns (250 × 4.6 mm id) with acetone at 200 bar using an air-driven fluid pump (slurry packer) (*see* **Note 8**).
2. Mount the packed HPLC columns (containing either imprinted polymer particles or control polymer particles) into the HPLC system, and perform a template extraction step in order to create the molecule-specific recognition cavities within the polymer matrix.
3. Extract the template molecules from the polymer particles by flushing the columns with methanol:acetic acid (HAc) (7:1 [v/v]) at a flow rate of 1 mL/min until a stable baseline is recorded. To establish comparable conditions, subject the control column to the same procedure. Then equilibrate each column (until a stable baseline is recorded) with acetonitrile (MeCN):H_2O:HAc (80:10:10 [v/v/v]) as the mobile phase (*see* **Note 9**), and evaluate with respect to their separation and recognition properties for the template quercetin and several structural analogs (*see* **Fig. 2**).
4. Using the HPLC column packed with control (nonimprinted) polymer particles as reference, determine the selectivity for the template analyte and crossreactivity of the imprinted polymer.
5. Perform all elutions at ambient temperature at a mobile phase flow rate of 1 mL/min, and monitor spectrophotometrically at 200 to 450 nm using a diode array UV/Vis detector.
6. For each chromatographic run, inject 2 µg of the investigated compounds dissolved in 20 µL of the mobile phase using 0.2 µL of acetone as void marker.

3.4. Evaluation

Several characteristic parameters can be interpreted as the main evidence that an imprinting effect has been achieved (*see* **Fig. 2** and **Table 2**). First, the retention time of quercetin in the column packed with the quercetin imprinted polymer is approx 10 times higher compared to the column packed with the control polymer. The imprinted column shows some cross-selectivity for structurally related compounds, as shown in **Table 2**. Nevertheless, the strongest difference in retention time between the imprinted and the control column occurs for the imprint molecule quercetin (retention index [RI] value of 1). According to **Table 2**, three analytes (Rutin, [+] Catechin, and C-fla) have low crossreactivity with the generated antiquercetin recognition cavities, whereas morin, with an RI value of 0.7 (*see* **Note 10**), shows considerable crossreactivity owing to the closest structural similarities of the molecular structure to the imprint molecule quercetin. Second, the large difference in capacity factors for quercetin when comparing imprinted and control polymer ($k'_{MIP} = 10$, $k'_{CTL} = 1.4$ in MeCN:H_2O:HAc [80:10:10]) indicated a strong imprinting effect. Third, the considerably pronounced tailing of the flavonoid peaks in the imprinted column is an indication of a heterogeneous binding site distribution with—more or less—specific and nonspecific sites distributed within the poly-

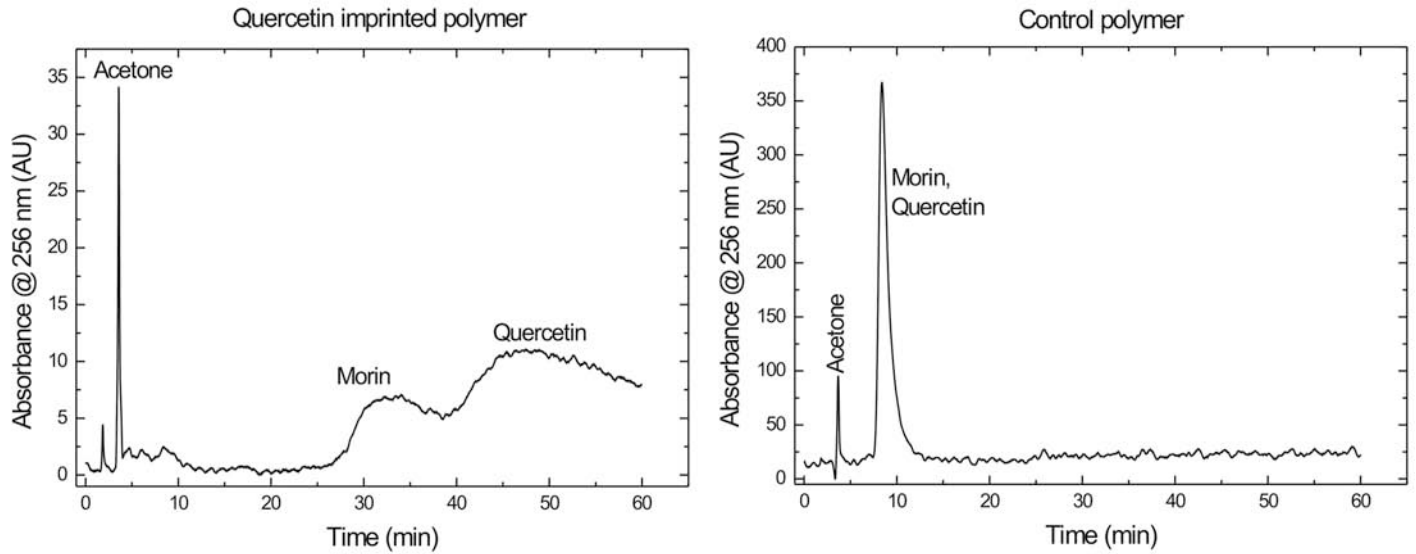

Fig. 2. Chromatograms for **(A)** imprinted polymer and **(B)** control polymer with acetone, morin, and quercetin (4-VP/EDMA copolymer) in MeCN:H_2O:HAc (80:10:10 [v/v/v]) as mobile phase (flow rate: 1 mL/min).

Table 1
Selected Flavonoid Compounds Used in HPLC Experiments

Name	**Quercetin**	**Morin**	**C-Fla**
Class	flavonol	flavonol	isoflavone
Formula	$C_{15}H_{10}O_7$	$C_{15}H_{10}O_7$	$C_{19}H_{16}O_7$
CAS#	117-39-5	480-16-0	15485-76-4
Structure			
Name	**(+)-Catechin**	**Rutin**	
Class	flavanol	flavonol glycoside	
Formula	$C_{15}H_{14}O_6$	$C_{27}H_{30}O_{16}$	
CAS#	154-23-4	153-18-4	
Structure			

Table 2
Results for Capacity Factors, Separation Factors, and RIs for Imprinted and Nonimprinted 4-VP/EDMA Copolymer in MeCN:H_2O:HAc (80:10:10 [v/v/v]) as Mobile Phase[a]

	t_{CTL}	k'_{CTL}	t_{MIP}	k'_{MIP}	α_{CTL}	α_{MIP}	RI value
Acetone	3.5	—	3.6	—	—	—	—
Quercetin	**8.4**	**1.4**	**39.5**	**10.0**	**1.0**	**1.0**	**1.0**
Morin	8.4	1.4	27.8	6.8	1.0	1.5	0.7
(+) Catechin	4.9	0.4	5.9	0.7	3.5	15.2	0.2
Rutin	4.3	0.2	4.9	0.4	6.3	27.9	0.2
C-fla	4.5	0.3	4.7	0.3	5.0	33.5	0.2

[a]Flow rate: 1 mL/min. Bold numbers indicate values for the tempated analyte.

mer ***(28)***. The print molecule interacts differently with these binding sites; it is retained for a longer time in the column owing to these interactions and, consequently, produces a broader peak with pronounced tailing. These results emphasize that HPLC columns packed with imprinted polymer particles are suitable for the separation of structurally closely related analytes based on biomimetic recognition mechanisms within a synthetic receptor matrix.

3.5. Application: Molecularly Imprinted Solid-Phase Extraction for Determination of Quercetin From Red Wine

Because HPLC characterization of molecularly imprinted polymers for quercetin has proven the existence of a substantial imprinting effect, the polymer was used as sorbent material for the solid-phase extraction of quercetin from red wine ***(18)***. The molecularly imprinted polymer enabled facile sample

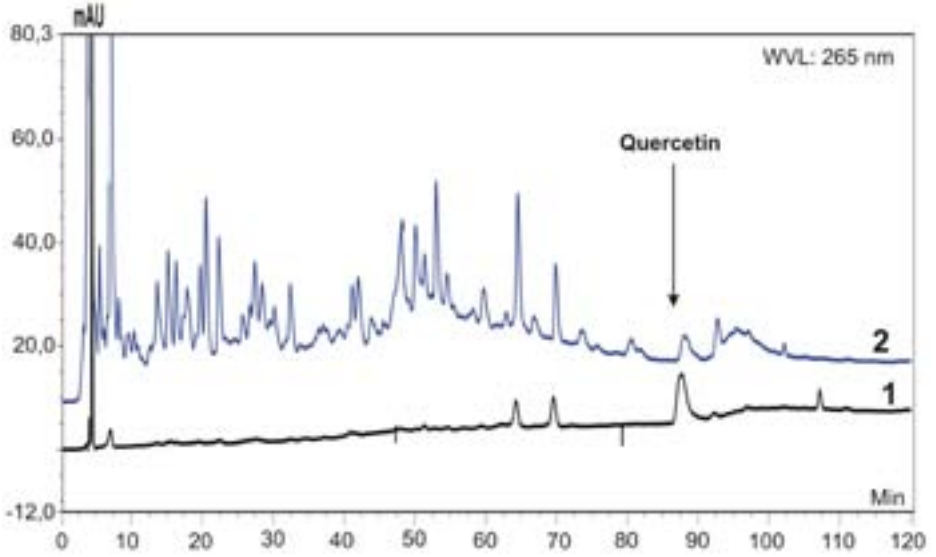

Fig. 3. Chromatogram of a red wine (Merlot) registered at 265 nm (2) before and (1) after MIP solid-phase extraction; (1) after 1.75 mL of elution. (From **ref.** ***18*** with permission.) WVL, wavelength.

cleanup prior to HPLC measurements and selective enrichment of quercetin from the red wine without any further sample cleanup procedures (*see* **Fig. 3**).

4. Notes

1. Prior to polymerization, 4-vinylpyridine has to be freshly distilled using a microdistillation apparatus in order to remove the inhibitor. The distilled 4-vinylpyridine solution can then be stored in a freezer at temperatures below –10°C for several months.
2. Depending on the type of template analyte, different functional monomers (including mixtures of functional monomers), crosslinkers, and solvents can be applied ***(12,29,30)***. One main prerequisite is adequate solubility of sufficient amounts of the selected compounds in the solvent of choice. A typical functional monomer-to-solvent ratio is 3:4 (v/v). The molar ratio selected for the imprinting approach of quercetin is 1:8:40 (template:functional monomer:crosslinker). Depending on the required amount of functional monomer and selected crosslinker, ratios typically vary between 1:4:20 and 1:10:60 ***(30–32)***. Resulting from losses during the sedimentation procedure (*see* **Note 5**), a minimum of 0.18 mmol of template in the prepolymerization mixture should be used. A minimum of 2 g of polymer material after the final sedimentation step is necessary for a successful HPLC column packing process. The functional monomer should be selected according to the potential maximum number of interaction sites, because the for-

mation of stable complexes between template molecules and functional monomers in the pre-polymerization mixture and during polymerization will determine the quality of the imprint. The ratio of template to functional monomer and to crosslinker for a defined system is frequently determined by trial and error after characterization of the binding properties of the resulting imprinted polymer. Methods such as nuclear magnetic resonance (NMR) studies of the pre-polymerization mixture facilitate the selection process of suitable ratios between template and functional monomer involved in the formation of complex ***(33,34)***.

3. Polymerization must be performed in an airtight glass vial, because oxygen acts as an inhibitor during a radical polymerization.
4. Depending on the properties of the template, thermal polymerization at temperatures <60°C (radical starter 2,2'-azobis-[2,4-dimethylvaleronitrile]) and ultraviolet polymerization (at room temperature or lower) offer alternatives for starting the radical copolymerization.
5. An ultrasonication processing step is applied prior to each sedimentation to ensure complete mixing between polymer particles and solvent. The accurate removal of fine particles is a crucial step, because the application of fine particles (<10-µm diameter) during packing of HPLC stationary-phase materials can result in high back-pressures in the HPLC column. The reduction of the particle size distribution is also necessary for better HPLC column performance, because using particles with a wide size distribution will lead to interfering peak-broadening effects.
6. A clear supernatant solution obtained after 20 to 30 min indicates sufficient removal of most of the fine particles.
7. Radioligand ***(35,36)*** and competitive fluorescence ***(37)*** binding assays are additional qualified techniques to evaluate the selective recognition performance of imprinted polymers. Analytical techniques such as NMR ***(33,34)***, infrared spectroscopy, or mass-sensitive devices ***(38)*** can provide supplementary information on the recognition (binding) events. However, in our personal opinion and experience, separation techniques related to affinity chromatographic concepts are particularly suitable for analyzing recognition properties of MIPs and are among the most promising applications of MIP-based recognition matrices for biomolecules.
8. The accurate and careful packing of HPLC columns is of great importance, because enclosure of air bubbles or nonuniform packed columns will result in diminished HPLC performance.
9. The proper choice of mobile phase is crucial and strongly depends on the nature of interactions between the analyte and the stationary phase. It has been shown in the literature that the application of the same solvent previously used for polymerization ***(39)*** is beneficial for maximizing the recognition properties in chromatographic applications. Although organic solvents may enhance, e.g., hydrogen bonds and ionic interactions, hydrophobic interactions are mainly amplified when applying aqueous media.
10. Capacity factors are calculated as $k' = (t - t_0)/t_0$, in which t is the retention time for the compound and t_0 corresponds to the retention time for the void marker

(acetone in this experiment). Separation factors are calculated as $\alpha = k'_{TM}/k'_{TS}$, with TM indicating the template molecule and TS the respective test substance. RI is calculated as $RI = \alpha_{CTL}/\alpha_{MIP}$, in which MIP and CTL indicate the MIP and control polymer, respectively. The RI value for the template analyte is 1 per definition and is taken as a reference. Smaller RI values for structurally related molecules indicate less interaction among the analyte, mobile phase, and stationary phase, yielding information on the selectivity and crossreactivity of the MIP.

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