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Separation of amino acids, peptides and proteins on molecularly imprinted stationary phases

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Abstract

Stationary phases, to be used in high-performance liquid chromatography, were tailor-made for the separation of amino acids, peptides and proteins. The stationary phases were prepared by molecular imprinting, applying two different approaches. Low-molecular-mass compounds were imprinted in bulk polymers by copolymerization of functional monomers and cross-linkers in the presence of the compound of interest, the print molecule. These polymers were, after extraction of the print molecule, successfully applied as chiral stationary phases, showing high resolution and load capacity. The development of a surface-imprinting approach for the preparation of stationary phases selective for proteins is also discussed.

1. Introduction

Molecular recognition plays a central role in interactions between biochemically important species. These interactions rely on selective binding between the recognition sites and the ligands. The aim in the design of synthetic receptors or binding sites has been to construct host systems possessing steric and electronic features complementary to those of the guest to be bound by the host. This has resulted in hosts such as crown ethers, cyclodextrins, cyclophanes and various molecular clefts and cavities [1–3].

Another approach to create receptor-like binding sites is molecular imprinting, sometimes referred to as template polymerization [4–6]. The binding sites are tailor-made by the copolymerization of functional monomers and cross-linkers in the presence of a print molecule. The print molecule is subsequently removed from the polymer, leaving recognition sites complementary to the print molecule in shape and in the positioning of functional groups. The polymer is able to selectively recognize and rebind the print molecule (Fig. 1). Molecularly imprinted polymers prepared according to this approach have proven to be useful as chiral stationary phases (CSPs) [7–15].

The technique of molecular imprinting can be applied to macromolecules too [16,17]. Glad et al. [18] published in 1985 a surface-imprinting approach for the preparation of adsorbents selective for the glycoprotein transferrin. In a more recent approach we have developed a method for the imprinting of proteins containing surface-exposed histidines [19].

Some aspects of the separation of amino acid

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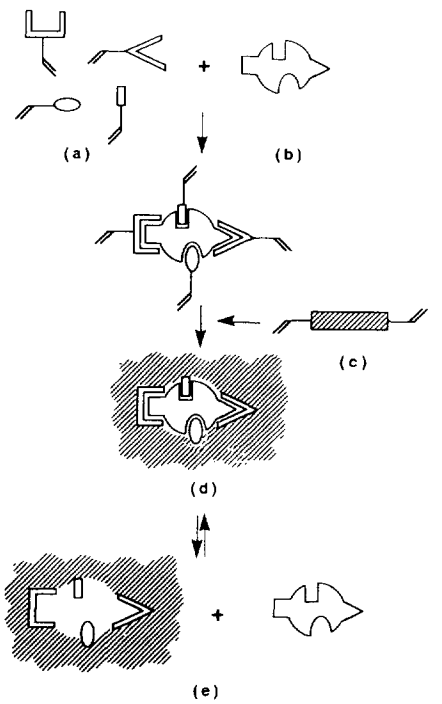

Fig. 1. Schematic representation of the concept of molecular imprinting. Functional monomers (a) interact non-covalently with a print molecule (b). Cross-linker (c) is added and the polymerization is initiated. The interactions between the functional groups of the polymer and the functional groups of the print molecule are maintained in the rigid bulk polymer (d). The print molecule is extracted from the polymer, leaving specific recognition sites, complememtary to the print molecule in the positioning of the functional groups and in shape (e). The polymer is able to selectively rebind the print molecule.

derivatives, peptides and proteins on molecularly imprinted stationary phases are detailed in this paper.

2. Experimental

2.1. Chemicals

Amino acid derivatives and peptides were purchased from Sigma (St. Louis, MO, USA), Bachem (Bubendorf, Switzerland), Nova Biochem (Läufelfingen, Switzerland) or synthesized according to König and Geiger [20] using N,N'-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. Methacrylic acid (MAA) and 2,2'-azoisobutyronitrile (AIBN) were obtained from Janssen Chimica (Geel, Belgium). Ethylene glycol dimethacrylate (EDMA) and 4-vinylpyridine (4VPy) were from Merck–Schuchardt (Germany). Trimethylolpropane trimethacrylate (TRIM) was from Aldrich (Steinheim, Germany). Bovine pancreas ribonuclease A (RNase A) and bovine serum albumin (BSA) were obtained from Sigma.

2.2. Preparation of bulk polymers

MAA and 4VPy were used as functional monomers. EDMA or TRIM were used as cross-linkers. Print molecule, functional monomer, cross-linker and solvent (according to the amounts given in Table 1) were mixed with the initiator AIBN. The mixtures were deoxygenated with a stream of nitrogen for 10 min and then irradiated at 366 nm for 24 h in 4°C (polymers 1, 2, 5 and 6) or −20°C (polymers 3 and 4). The bulk polymers were ground in an end runner mill Model RM O (Retsch, Haan, Germany) and wet-sieved by hand with water

Table 1
Polymer compositions

Polymer	Print molecule, mmol	Functional monomer, mmol	Cross-linker, mmol	Polymerization solvent, ml
1	Boc-L-Phe-OH, 2.5	MAA, 10	EDMA, 50	Chloroform, 15
2	Z-L-Phe-OH, 2.5	MAA, 10	EDMA, 50	Chloroform, 15
3	Z-L-Asp-OH, 1.0	4VPy, 12	EDMA, 60	Tetrahydrofuran, 18
4	Z-L-Glu-OH, 1.0	4VPy, 12	EDMA, 60	Tetrahydrofuran, 18
5	Z-L-Ala-L-Ala-OMe, 5.0	MAA, 30	TRIM, 30	Chloroform, 20
6	Z-L-Ala-Gly-L-Phe-OMe, 3.75	MAA, 30	TRIM, 30	Chloroform, 20

and ethanol through a 25-μm sieve (Retsch). The particles which passed the sieve were collected and dried on a sintered glass funnel. The particles were sedimented in acetone and the fines were removed by decantation.

2.3. High-performance liquid chromatography

The sieved and sedimented particles were slurried in chloroform–acetone (17:3, v/v) and packed with acetone as solvent into stainless-steel HPLC columns at 300 bar using an air-driven fluid pump (Haskel, Burbank, USA). The print molecules were extracted from the polymers by eluting with methanol–acetic acid (9:1, v/v) (polymers 1 and 2), tetrahydrofuran–acetic acid (7:3, v/v) (polymers 3 and 4) or methanol–acetic acid (7:3, v/v) (polymers 5 and 6). The HPLC analyses were performed using a Kontron HPLC comprising a Model 420 HPLC pump, a Model 425 gradient former and a Model 432 variable-wavelength absorbance detector. The pump was controlled by a Toshiba T 1000 personal computer. The elutions were performed at ambient temperature and monitored spectrophotometrically at 260–265 nm.

2.4. Preparation of protein-imprinted silica particles

Methacrylate silica and N-(4-vinyl)-benzyl iminodiacetic acid were prepared as previously described [19]. The RNase A-imprinted silica (prepared in the presence of RNase A) and the reference silica (prepared in the presence of BSA) were also prepared as previously described [19].

2.5. Chromatographic evaluation of protein-imprinted stationary phases

The silica particles were slurried in water–N,N-dimethylformamide (7:3, v/v) and packed at 200 bar into PTFE-coated stainless-steel columns (50 × 4.6 mm) with water–N,N-dimethylformamide (7:3, v/v) as packing solvent. The chromatographic evaluation was performed in the HPLC mode using the same HPLC system as described above. The flow-rate was 0.5 ml/min and the elution was monitored spectrophotometrically at 280 nm. The columns were washed by injecting 5 ml 0.1 *M* EDTA and 5 ml 6 *M* urea, repeated five times each. The eluents for the separation studies were [20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) pH 7.0, 0.5 *M* NaCl]–N,N-dimethylformamide (7:3, v/v) with and without 0.5 m*M* $ZnCl_2$.

3. Results and discussion

3.1. Molecularly imprinted bulk polymers

Molecularly imprinted bulk polymers, selective for various amino acid derivatives and peptides were prepared by copolymerization of functional monomers and cross-linkers in the presence of the print molecules (Table 1). The functional monomers were chosen so as to interact non-covalently with the functional groups of the print molecules. The polymers were prepared with varying molar ratios of monomers to print molecules. The ground, sieved and extracted polymers were used as stationary phases in HPLC. The polymers described in Table 1 resulted in the highest separation factors of the polymers prepared.

A MAA–EDMA copolymer imprinted with the N^{α}-protected amino acid Boc–L-Phe–OH (Boc = *tert.*-butyloxycarbonyl) (polymer 1, Table 1) was investigated by frontal chromatography [9]. The dissociation constant for Boc–L-Phe–OH was determined to be lower than for Boc–D-Phe–OH (6.3 and 8.1 m*M*, respectively). This shows that the affinity for the L-enantiomer was higher than for the D-enantiomer, as was expected since the L-enantiomer was used as print molecule. The number of binding sites giving rise to these dissociation constants were equal for both enantiomers (28 μmol/g dry polymer).

To obtain a stereospecific recognition, a "three-point" interaction is required [21]. In the case of a polymer imprinted with an N^{α}-protected amino acid and prepared using MAA as the functional monomer, it is believed that the

carboxy and carbamate functions of the print molecule interact via hydrogen bonds with the positioned carboxy groups of the polymer (Fig. 2). This gives, however, only two interaction points. Therefore, at least one additional interaction must exist to explain the observed stereospecific recognition. A polymer imprinted with Z–L-Phe–OH (Z = benzyloxycarbonyl) (polymer 2, Table 1) was able to resolve racemic Z–Phe–OH with a separation factor (α) of 1.84 (Table 2). Changing the N^{α}-protecting group of the racemic phenylalanine derivative to be separated on the CSP to Boc, 9-fluorenylmethyloxycarbonyl (Fmoc) or acetyl (Ac), instead of Z, resulted in lower separation factors (Table 2). The same trend, i.e. low separation factors with non-imprinted molecules, was seen when these racemates were separated on Boc–L-Phe–OH- and Fmoc–L-Phe–OH-imprinted polymers. Changing the amino acid side chain of the racemate to be separated on the Z–L-Phe–OH-imprinted stationary phase from that of phenylalanine (Z–Phe–OH) to that of alanine (Z–Ala–OH) also resulted in a lower separation factor (Table 2). This shows that both the N^{α}-protecting group and the amino acid side chain interacts with, and are recognized by the binding sites in the polymer [12].

A Boc–L-phenylalanine anilide-imprinted MAA–EDMA copolymer was able to resolve the racemate of the print molecule efficiently ($\alpha = 2.95$). Racemic Boc–phenylalanine *p*-nitroanilide and racemic Boc–phenylalanine glycine ethyl ester were less well resolved on this CSP ($\alpha = 1.68$ and 1.52, respectively), showing that also the C^{α}-protecting group of the amino acid is of importance for the recognition [12].

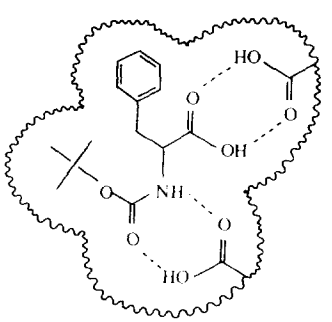

Fig. 2. Schematic representation of Boc–Phe interacting with the carboxyl groups of the polymer via hydrogen bonds.

Table 2
The influence of the N^{α}-protecting group and the amino acid side chain in the recognition

Separated species	Separation factor (α)[a]
Z–D,L-Phe–OH	1.84
Boc–D,L-Phe–OH	1.31
Fmoc–D,L-Phe–OH	1.21
Ac–D,L-Phe–OH	1.21
Z–D,L-Ala–OH	1.28

Separation of 80 nmol of the indicated racemates on a Z–L-Phe–OH-imprinted CSP (polymer I) using isocratic elution with chloroform as the eluent at 0.5 ml/min (column size: 100 × 4.5mm).

[a] The separation factor (α) was calculated as $\alpha = (t_L - t_0)/(t_D - t_0)$, where t_L and t_D are the retention times of the L- and the D-enantiomer, respectively, and t_0 is the void volume.

The most widely applied functional monomer in non-covalent molecular imprinting is MAA. It has been shown to interact via ionic interactions with amines on the print molecules and via hydrogen bonds with amides, carbamates and carboxyls on the print molecules. The introduction of 4VPy as a functional monomer in non-covalent molecular imprinting made ionic interactions between the recognition sites of the polymers and print molecules containing the carboxy group possible [10]. 4VPy–EDMA copolymers prepared in the presence of Z–L-Asp–OH (polymer 3, Table 1) or Z–L-Glu–OH (polymer 4, Table 1) were able to resolve racemic mixtures of their respective print molecules. Resolution of the racemate of Z–Glu–OH on the Z–L-Asp–OH-imprinted polymeric CSP was not possible. The reverse was also not possible, namely the separation of Z–D,L-Asp–OH on the Z–L-Glu–OH-imprinted stationary phase. Z–Asp–OH and Z–Glu–OH differ by only one methylene group. Despite this small

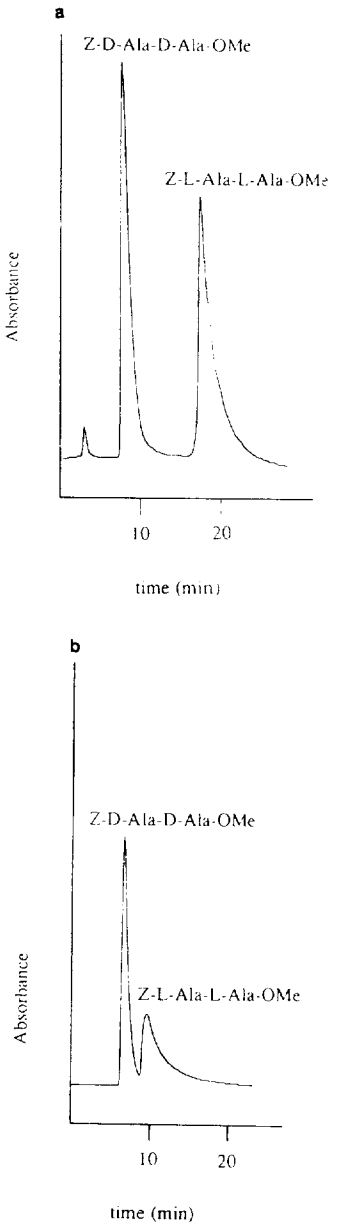

Fig. 3. Separation of mixtures of Z-L-Ala-L-Ala-OMe and Z-D-Ala-D-Ala-OMe on a Z-L-Ala-L-Ala-OMe-imprinted CSP (polymer 5) packed into an HPLC-column (250 × 4.6 mm). (a) A 100-μg amount was separated using gradient elution at 1 ml/min with chloroform–acetic acid (99.75:0.25) and chloroform–acetic acid (8:2) (A) as the eluents (gradient: 0–10 min, 0% A; 10–18 min, 0–5% A; 18–22 min, 5% A; 22–24 min, 5–0% A). (b) A 1-mg amount was separated using isocratic elution at 1 ml/min with chloroform–acetic acid (99.75:0.25) as the eluent.

difference, the imprinted polymers are able to discriminate between the species.

Molecularly imprinted polymers with high load capacity and excellent resolving capability were prepared using the branched cross-linker TRIM [13,14]. Fig. 3a shows the resolution of 100 μg of a racemic mixture of the dipeptide Z–Ala–Ala–OMe on a Z–L-Ala–L-Ala–OMe-imprinted MAA–TRIM copolymer (polymer 5, Table 1) packed into an analytical column (250 × 4.6 mm). Gradient elution was used to enhance the elution rate of the enantiomer used as print molecule, since it was much more retarded than the non-imprinted optical antipode. The separation factor was 3.2 and the resolution factor was 4.5. The load capacity of MAA–TRIM copolymers are higher than what has been reported for MAA–EDMA copolymers. Fig. 3b shows the elution profile when 1 mg was sepa-

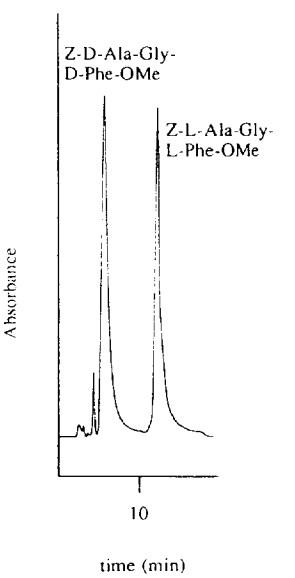

Fig. 4. Separation of 100 μg of a mixture of Z–L-Ala–Gly–L-Phe–OMe and Z–D-Ala–Gly–D-Phe–OMe on a Z–L-Ala–Gly–L-Phe–OMe-imprinted CSP (polymer 6) packed into an HPLC column (250 × 4.6 mm) using gradient elution at 1 ml/min with chloroform–acetic acid (99:1) and chloroform–acetic acid (9:1) (A) as the eluents (gradient: 0–7 min, 0% A; 7–9 min, 0–100% A; 9–17 min, 100% A; 17–22 min, 100–0% A).

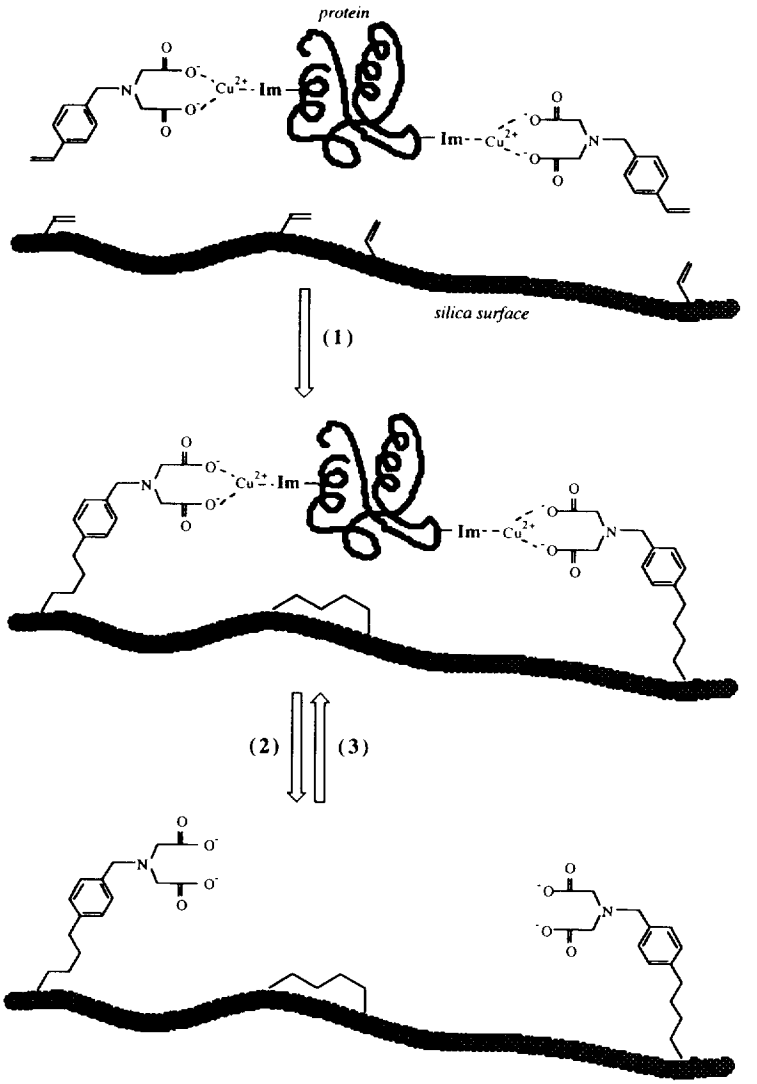

Fig. 5. Schematic representation of protein imprinting on silica-surfaces derivatized with methacrylate groups. Imidazole groups (Im) of surface-exposed histidines on the protein and the monomer N-(4-vinyl)-benzyl iminodiacetic acid coordinate metal ions. The derivatized silica particles are added and the polymerization is initiated (1). The protein and the metal ions are subsequently removed, e.g. by treatment with EDTA (2). The metal-binding ligands are positioned so as to, in the presence of metal ions, selectively recognize and rebind the protein (3).

rated using isocratic elution. The enantiomers were resolved with almost baseline resolution.

Another example of a chiral separation on an imprinted MAA–TRIM copolymer is shown in Fig. 4. A mixture of Z–L-Ala–Gly–L-Phe–OMe and Z–D-Ala–Gly–D-Phe–OMe was resolved on a CSP imprinted against Z–L-Ala–Gly–L-Phe–OMe (polymer 6, Table 1) with a separation factor of 3.6 and a resolution factor of 4.1.

3.2. Surface imprinting of proteins

A metal-binding monomer, N-(4-vinyl)-benzyl iminodiacetic acid, and the imidazole groups of the surface-exposed histidines of RNase A were allowed to coordinate metal ions, as shown schematically in Fig. 5 [19]. Silica particles, derivatized with methacrylate groups, were added and the polymerization was initiated. The

protein was subsequently removed by treatment with EDTA and urea. The resulting silica particles were used as a stationary phase in HPLC. This RNase A-imprinted stationary phase was compared with a stationary phase consisting of silica particles prepared in the presence of BSA instead of RNase A. It was concluded that the RNase A-imprinted stationary phase showed, in the presence of metal ions, higher affinity for RNase A than what the reference stationary phase did.

4. Conclusions

Molecular imprinting has proven to be a useful technique for the preparation of stationary phases selective for amino acid derivatives, peptides and proteins. The technique can also be applied to other types of compounds, exemplified by β-adrenergic blockers [15] and non-stereoidal anti-inflammatory drugs [11]. The polymers are easily prepared, inexpensive and possess excellent chemical, physical and mechanical stability.

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