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Review

Molecular imprinting used for chiral separations

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Abstract

Molecular imprinting is a promising technique for the preparation of synthetic polymers of predetermined specificity. Functional monomers are copolymerized with crosslinkers in the presence of the desired molecule, the imprint molecule. The use of these polymers as chiral stationary phases is discussed. Other applications, such as antibody-mimics, enzyme-like catalysts and sensors, are also focused upon.

Contents

1. Introduction

The idea to create a host, specific for a desired molecule, by coordinating the assembly of functional monomers around the molecule of interest (the imprint molecule) has been considered and discussed for quite some time. It is only recently, however, that the techniques required have been sufficiently developed to allow a realization of this dream [1]. Two essentially different approaches have been developed: covalent and non-covalent molecular imprinting. In both cases, the functional monomers, chosen so as to allow interactions with the functional groups of

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the imprint molecule, are polymerized in the presence of the imprint molecule.

In the covalent approach, the imprint molecule is covalently coupled to a polymerizable molecule. After copolymerization with a crosslinker, the imprint molecule is chemically cleaved from the highly crosslinked polymer. The binding of this type of polymer relies on reversible covalent bonds. Covalent imprinting has been used in the preparation of polymers selective for derivatized and free sugars [2–4], glyceric acid and its derivatives [5–7], amino acids and amino acid derivatives [8–10], mandelic acid [11], aromatic ketones [12,13], dialdehydes [14], transferrin [15] and bis-NAD [16].

In the non-covalent approach, the imprint molecules are mixed with functional monomers capable of interacting non-covalently with the imprint molecules. The functional monomers are copolymerized with crosslinkers to yield a highly crosslinked and rigid polymer. The imprint molecules are subsequently removed from the polymer, leaving recognition sites complementary to the imprint species in shape and in the positioning of the functional groups. The recognition of the polymer constitutes an induced molecular memory, which makes the recognition sites capable of selectively recognizing the imprint species. The imprint molecules interact, during both the imprinting procedure and the rebinding, with the polymer via non-covalent interactions, e.g. ionic, hydrophobic and hydrogen bonding (Fig. 1). Non-covalent molecular imprinting has been applied to the preparation of polymers selective for dyes [15,17,18], diamines [17,19], vitamins [20], amino acid derivatives [21–44], peptides [33,44,45], β-adrenergic blockers [46], theophylline [47], diazepam [47], nucleotide bases [48] and naproxen [49]. A combination of covalent and non-covalent molecular imprinting has also been reported [50], where the monomers and the imprint molecules were covalently coupled during the polymerization, whereas the subsequent rebinding took place by non-covalent interactions.

Over a relatively short period, molecularly imprinted polymers (MIPs) have been developed for a broad range of potential applications. Three main areas can be foreseen for the MIPs: (i) as tailor-made separation materials, (ii) as

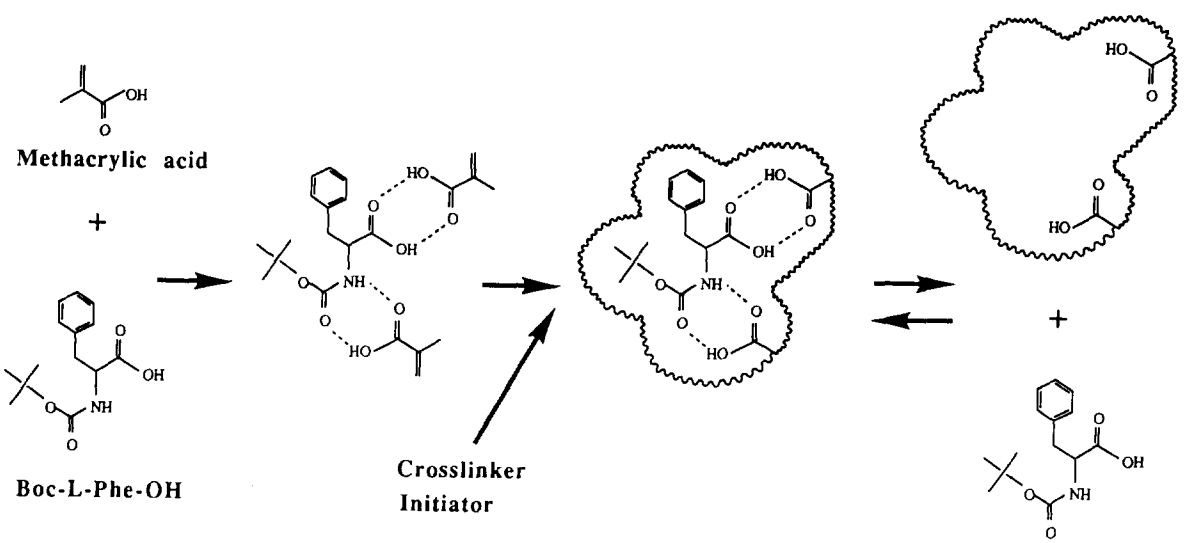

Fig. 1. Schematic representation of the concept of non-covalent molecular imprinting with an amino acid derivative (Boc-L-Phe-OH) as the imprint molecule and methacrylic acid as the functional monomer. Methacrylic acid interacts, via hydrogen bonds, with the carboxyl and carbamate functionalities of the imprint molecule. Crosslinker is added and the polymerization is initiated. The interactions are maintained in the rigid bulk polymer formed. The imprint molecule is extracted from the polymer, leaving a recognition site selective for the imprint molecule. The polymer is able to recognize and rebind the imprint molecule.

enzyme mimics or catalytically active polymers in enzyme technology and organic synthesis, and (iii) as sensors in biosensor-like configurations, whereby the polymers are used as substitutes for the biological materials normally employed. Most of the following examples are taken from work utilizing the non-covalent approach, as this seems to be a more direct approach for the applications to be discussed, especially those involving chiral separation. For more details on covalent imprinting we refer to reviews on this topic [51,52].

2. Molecularly imprinted separation materials

Molecular imprinting has become a simple and straightforward technique for preparing synthetic polymers of predetermined selectivity. Normally, the polymers are produced by bulk polymerization of a mixture of functional monomers and crosslinking monomers arranged around the imprint molecule, followed by grinding to particles and extraction of the imprint species. The particle size is usually in the 25 μm range, suitable for use as stationary phases in high-performance liquid chromatography (HPLC) [24–38,40,42–46,48,49] and thin-layer chromatography (TLC) [53]. Alternative approaches include the preparation of continuous polymer rods [41], the preparation of polymer beads by suspension polymerization and the preparation of composite materials, e.g. polymer-coated silica particles [15,18] and polymer beads grafted with imprinted polymers [54]. Figs. 2 and 3 show the functional monomers and the crosslinkers that have been utilized in non-covalent molecular imprinting.

A number of non-covalent molecularly imprinted copolymers have been reported (Table 1). The functional monomers were in general chosen so as to facilitate specific interactions

Table 1
Non-covalently molecularly imprinted polymers prepared by copolymerization of functional monomers and crosslinkers

Functional monomer	Crosslinker	Ref.
Methyl methacrylate (**3**)	N,N′-Methylenediacrylamide (**11**), N,N′-phenylenediacrylamide (**12**), 3,5-bis(acryloylamido)benzoic acid (**13**)	[17]
Methyl methacrylate (**3**)	N,N′-Methylenediacrylamide (**11**), N,N′-phenylenediacrylamide (**12**)	[18]
p-Vinylbenzoic acid (**4**), ethylstyrene (**5**)	Divinylbenzene (**15**)	[21]
Acrylic acid (**1**)	Ethylene glycol dimethacrylate (**14**)	[21,41]
Acrylic acid (**1**)	N,O-Bisacryloyl-L-phenylalaninol (**16**)	[22]
Methacrylic acid (**2**)	Ethylene glycol dimethacrylate (**14**)	[24–39,42–47,53]
Itaconic acid (**6**)	Ethylene glycol dimethacrylate (**14**)	[46]
2-Acrylamido-2-methyl-1-propane-sulphonic acid (**7**)	Ethylene glycol dimethacrylate (**14**)	[19]
Methacrylic acid (**2**)	Ethylene glycol dimethacrylate (**14**), N,N′-1,3-phenylene-diacrylamide	[48]
1-Vinylimidazole (**8**)	Ethylene glycol dimethacrylate (**14**)	[40]
4-Vinylpyridine (**9**)	Ethylene glycol dimethacrylate (**14**)	[40,49]
2-Vinylpyridine (**10**)	Ethylene glycol dimethacrylate (**14**)	[42]
Methacrylic acid (**2**), 2-vinylpyridine (**10**)	Ethylene glycol dimethacrylate (**14**)	[42]
Methacrylic acid (**2**)	Trimethylolpropane trimethacrylate (**18**)	[44]
Methacrylic acid (**2**)	pentaerythritol triacrylate (**17**)	[44]
Methacrylic acid (**2**)	pentaerythritol tetraacrylate (**19**)	[44]

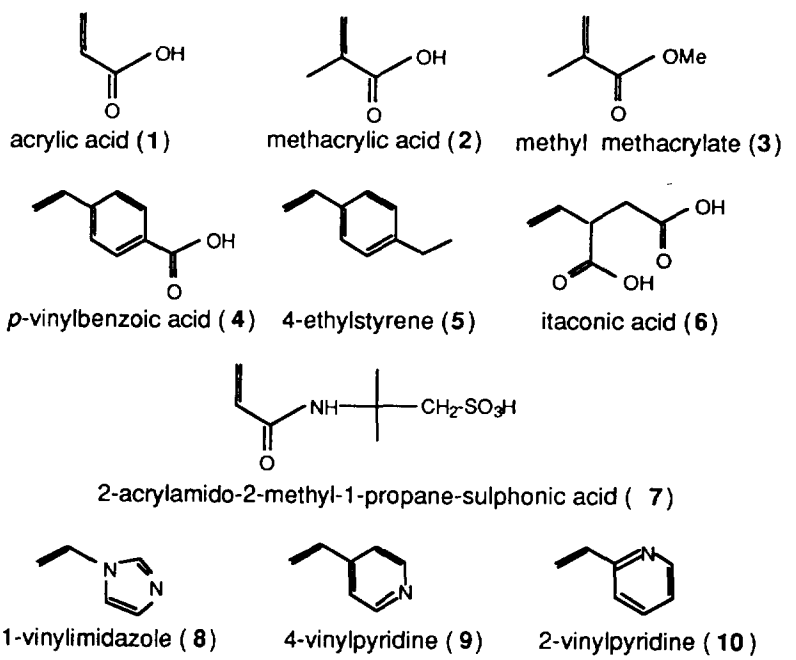

Fig. 2. Functional monomers used in non-covalent molecular imprinting.

with the functional groups of the imprint molecules. The most widely used functional monomer is methacrylic acid (MAA) (**2**, Fig. 2). It is assumed to interact via ionic interactions with amines and via hydrogen bonds with amides, carbamates and carboxyls.

The ionic interaction is stronger than the hydrogen bonding interaction, a fact which is reflected in better selectivities of polymers interacting with the imprint molecules via ionic bonds than of polymers interacting only via hydrogen bonds. The introduction of 4-vinylpyridine (**9**, Fig. 2) as a functional monomer in non-covalent MIPs made the ionic interactions possible between the recognition sites of the polymers and the imprint molecules containing the carboxyl functionality [40,49]. This resulted in better selectivities for such imprint species compared to the selectivities that have been achieved with polymers prepared with methacrylic acid [35]. The related monomer 2-vinylpyridine (**10**, Fig. 2) has also been shown to be useful for this purpose, either as sole functional monomer or in conjunction with methacrylic acid [42].

Ethylene glycol dimethacrylate (EDMA) (**14**, Fig. 3) has been extensively used as crosslinker in non-covalent MIPs. Recently, crosslinkers containing three or four vinyl groups have also been investigated [44]. Pentaerythritol triacrylate (PETRA) (**17**, Fig. 3) and trimethylolpropane trimethacrylate (TRIM) (**18**, Fig. 3), both being pentaerythritol derivatives with three vinyl groups, were shown to be superior to EDMA (**14**, Fig. 3), in that the resulting polymers exhibited better load capacities, selectivities and resolving capabilities when used as stationary phases in liquid chromatography.

2.1. Chiral separations

More than half of all drugs on the market are asymmetric molecules [55]. Some 90% of these are administered as racemates. Since all biological systems in nature are based on optically active molecules (proteins, enzymes, receptors, sugars, etc.), it is no surprise that the two enantiomers of a racemic drug might interact with the biological system differently. One of the enantiomers may have pharmacologically different or unwanted side effects [56–58]. The same

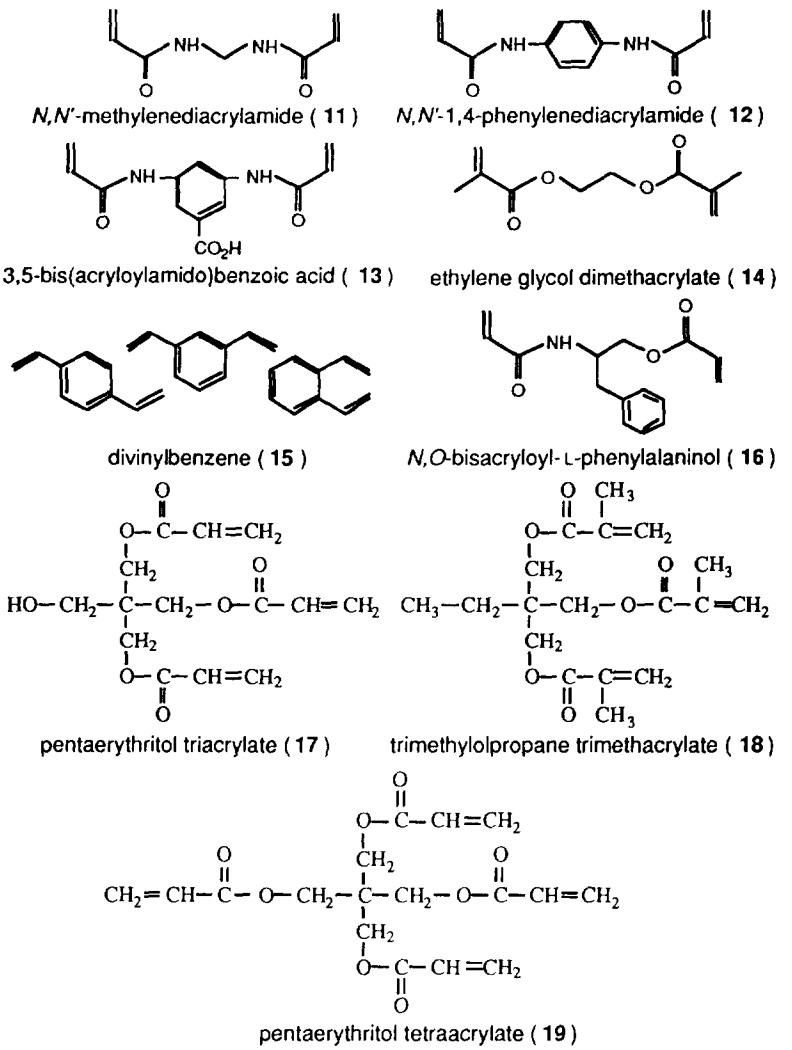

Fig. 3. Crosslinking monomers used in non-covalent molecular imprinting.

is true for racemic pesticides and herbicides; often only one of the enantiomers possesses the desired activity.

These facts moved the Food and Drug Administration in 1992 to issue a *Policy Statement for the Development of New Stereoisomeric Drugs*, stating that for every new racemic drug, the two enantiomers must be treated as separate substances in pharmacokinetic and toxicological profiling. The European Community has also recently issued statements about chiral active substances. This increasing demand for optically pure compounds has resulted in an interest in asymmetric synthesis and development of tools for efficient chiral separations. There is a need for both preparative methods to purify optically active compounds, as well as analytical methods to be able to perform pharmacokinetic studies and to control the enantiomeric excess and the optical purity of chiral precursors and final products of asymmetric syntheses.

Chiral separation by liquid chromatography is a widely studied area. Four main approaches have been developed: (i) derivatization with a

chiral reagent and separation of the resulting diastereomers on a non-chiral stationary phase, (ii) direct separation on a non-chiral stationary phase with the use of a chiral mobile phase additive, (iii) derivatization with a non-chiral reagent and separation on a chiral stationary phase (CSP) and (iv) direct separation on a CSP. The CSP consists of a chiral selector, sometimes immobilized to a stationary phase to improve its stability. A broad range of CSPs have been developed, e.g. the 'Blaschke-type CSPs' [59–61], the 'Pirkle-type CSPs' [62–64], polysaccharides, such as cellulose [65,66], amylose [67] and cyclodextrins [68,69], and immobilized proteins and enzymes [70–74].

With all due respect to the current methods for asymmetric synthesis, enzymatic resolution and techniques for the separation of enantiomers including the more traditional CSPs, the field of chirotechnology demands more. When an optically active compound is successfully imprinted, the polymer formed is able to discriminate between the imprint molecule and its antipode. Non-covalent MIPs have therefore proven useful as CSPs. We feel that the technique of molecular imprinting has a great potential in this context as the concept permits, at least in principle, the manufacturing of specific tailor-made polymers for a given separation process.

2.1.1. Chromatographic conditions

In general, the MIPs are packed into analytical HPLC columns. The applied flow-rates are in the range of 0.1–1.0 ml/min. The eluents, consisting of neat solvents or mixtures of solvents such as acetonitrile, chloroform and heptane, with or without added acetic acid, are chosen so as to give appropriate retentions. It is considered to be advantageous if the eluent is identical with the polymerization solvent, in order to resemble the conditions during the formation of the recognition sites as much as possible, but it is not a prerequisite. There are usually no chromatographic problems due to swelling or shrinking of the polymers when the eluents are changed, since the polymers are highly crosslinked. The elutions are generally performed at ambient temperature. In some cases, however, elevated temperatures have been applied [23,28].

2.1.2. Load capacity

Typically, the resolution of up to 500 μg of racemic amino acid derivatives or short peptides requires approximately 1 g of dry polymer. This is fine for analytical separations or if one wishes to carry out enantiopolishing on a commercial scale, i.e. the removal of small amounts of a contaminating optical isomer. Recently, however, we have developed a new polymer system in our laboratory which is superior to previously reported MIPs [44]. Considerably higher load capacities and selectivities were observed (1 mg of racemate per g polymer leading to baseline separation), making large-scale processes soon realistic.

2.1.3. Column efficiency

The last eluting peak, i.e. the enantiomer used as imprint molecule, is usually broadened due to tailing. This is likely due to the slow kinetics of the binding and the dissociation and to the fact that the recognition sites are heterogeneous. Some of the sites possess very good recognition for the imprint molecule, whereas others are less specific. The elution rate of the last eluting compound can, however, be enhanced with gradient elution, which results in sharper peaks [44].

It is important to have in mind that the polymer particles are prepared by grinding the bulk polymers. The particles are therefore highly irregular and not of uniform size, which also affects the column efficiency. New methods for the preparation of spherical beads of uniform size are, however, under development in our laboratory. This will most likely improve the column efficiency.

2.1.4. Selectivity

A characteristic feature of CSPs prepared by molecular imprinting is that the elution order of the enantiomers is easily predicted, because this is solely dependent on which enantiomer is used as imprint molecule. One is thus not confined to the trial and error approach inherent with the

old CSPs. This was demonstrated, for example, with copolymers of 4-vinylpyridine and ethylene glycol dimethacrylate imprinted with Z-aspartic acid [40]. When the imprint molecule was of L-configuration, the L-enantiomer was more retarded than the D-enantiomer, and vice versa when the imprint molecule was of D-configuration. In this context it should be mentioned that when the racemate was present during the polymerization, no chiral resolution could be achieved on the resulting polymer. Hence, the chiral information is introduced during the imprinting procedure and is not inherent to the polymer as such.

Chiral resolution of various racemic amino acid derivatives, peptides, other organic acids and some drugs has been studied on non-covalent molecularly imprinted stationary phases. Some examples are given in Table 2. The first reported drugs separated by this technique were β-blockers. For example, (*R*,*S*)-timolol was separated with baseline separation [46]. Another drug that was recently separated into its optical antipodes is naproxen, a non-steroidal anti-inflammatory drug (NSAID) [49].

In some cases, an extremely high selectivity for the imprint molecule was achieved. For example, racemic Z-aspartic acid, but not racemic Z-glutamic acid, was resolved on a Z-L-aspartic acid-imprinted 4-vinylpyridine–EDMA copolymer, and vice versa when a polymer imprinted with Z-L-glutamic acid was investigated [40]. Despite the small difference between Z-aspartic acid and Z-glutamic acid, the polymers were able to discriminate between these two species. The same high selectivity has also been shown on Z-L-aspartic acid- and Z-L-glutamic acid-imprinted methacrylic acid–EDMA copolymers [35].

High selectivity towards the imprint species was also observed on methacrylic acid–EDMA copolymers imprinted with some other N^{α}-protected amino acids [43]. The resolving capabilities of various racemic N^{α}-protected amino acids were tested and it was concluded that the separation factors were in all cases highest for the racemate of the molecule present during the polymerization. For example, separation of racemic Boc-phenylalanine, Fmoc-phenylalanine and Z-alanine on a Z-L-phenylalanine-imprinted CSP resulted in lower separation factors than when racemic Z-phenylalanine was separated.

Noteworthy are the recently reported extremely high separation factor (α) of 17.8 obtained for

Table 2
Chromatographic resolution on non-covalently molecularly imprinted CSPs (N.S. means not specified)

Imprint molecule	α	R_s[a]	f/g[b]	Ref.
Z-L-Ala-OH	1.93	N.S.	N.S.	[43]
H-L-Arg-OEt	1.5	N.S.	N.S.	[32]
Z-L-Asp-OH	2.81	1.22	0.81	[40]
Z-L-Glu-OH	2.45	3.10	1.00	[44]
H-L-Leu-βNA	3.8	N.S.	N.S.	[33]
H-L-Phe-OEt	1.3	0.3	N.S.	[29]
H-L-*p*-NH_2Phe-OEt	1.8	0.8	N.S.	[29]
H-L-Phe-NHEt	2.0	0.5	N.S.	[29]
H-L-Phe-NHPh	13	N.S.	N.S.	[38]
H-L-*p*-NH_2Phe-NHPh	8.38	N.S.	N.S.	[31]
H-D-*p*-NH_2Phe-NHPh	15	N.S.	N.S.	[32]
H-L-Phe-NMePh	2.03	N.S.	N.S.	[30]
H-L-Me_2Phe-NHPh	3.7	N.S.	N.S.	[33]
Boc-L-Phe-OH	2.14	N.S.	N.S.	[36]
Dansyl-L-Phe-OH	3.15	1.6	0.96	[42]
Fmoc-L-Phe-OH	1.36	N.S.	N.S.	[43]
Z-L-Phe-OH	2.29	3.14	1.00	[44]
Boc-L-Phe-NHPh	2.95	N.S.	N.S.	[43]
Pyridoxyl-L-Phe-NHPh	2.50	N.S.	N.S.	[87]
Pyridylmethyl-L-Phe-NHPh	8.4	N.S.	N.S.	[33]
H-L-Pro-NHPh	4.5	N.S.	N.S.	[33]
Boc-L-Pro-OSu	1.25	0.8	N.S.	[35]
H-L-Trp-OEt	1.8	0.5	N.S.	[29]
Ac-D-Trp-OMe	3.92	2.2	1.0	[42]
Boc-L-Trp-OH	4.35	1.9	1.0	[42]
Z-L-Trp-OH	1.67	0.1	N.S.	[35]
Z-L-Trp-OMe	1.28	0.2	N.S.	[35]
Z-L-Tyr-OH	2.86	5.47	1.00	[44]
H-L-Phe-Gly-NHPh	5.1	N.S.	N.S.	[33]
Boc-L-Phe-Gly-OEt	3.04	3.44	1.00	[44]
Z-L-Ala-L-Ala-OMe	3.19	4.50	1.00	[44]
Ac-L-Phe-L-Trp-OMe	17.8	N.S.	1.00	[45]
Z-L-Ala-Gly-L-Phe-OMe	3.60	4.15	1.00	[44]
(*S*)-(−)-timolol	2.9	2.0	N.S.	[46]
(*S*)-naproxen	1.65	N.S.	0.83	[49]
(*R*)-phenylsuccinic acid	3.61	2.0	1.0	[42]
L-mandelic acid	1.41	N.S.	0.70	[40]

[a] The resolution factors (R_s) were calculated according to Ref. [85].
[b] The resolution factors (f/g) were calculated according to Ref. [86].

the racemic system of Ac–Phe–Trp–OMe [45] and the high resolution factors obtained when various racemic amino acid derivatives and peptides were resolved [44].

2.2. Separation of macromolecules

Most imprinting studies, involving both the imprinting and the separation steps, have been performed in organic solvents. Efforts are now being made to accomplish this also in aqueous systems. Likewise, the majority of imprinting studies have been carried out with small molecules. An important extension would be the use of macromolecules such as proteins, an area of increasing interest in the scientific community. For these studies we use a variant of molecular imprinting which we have named surface imprinting [75,76].

3. Other areas of application

3.1. Antibody mimics

Another area for which imprints have been tested is their suitability as antibody mimics. Imprints against the bronchodilating drug theophylline and against the tranquillizer diazepam have shown astonishingly specific recognition. In fact, when these MIPs were tested in competitive radioimmuno-style assays, their recognition of related structures was either non-existent or far below that of the original imprint molecule [47,77]. Amazingly, the cross-reactivity profiles of these MIPs were practically identical to those reported for monoclonal antibodies against these drugs. The anti-theophylline MIPs were used for the determination of theophylline concentrations in patient serum samples, pointing towards their use as stable alternatives to antibodies in conventional immunoassays. The measured dissociation constants were in the micromolar range. Some of our more recent studies have involved the successful preparation of imprints against morphine and Leu-enkephalin, leading to what could be considered as an artificial opioid receptor. It will be interesting to study and compare molecular recognition in these receptor binding site mimics.

3.2. Enzyme mimics

Scientists have long attempted to create synthetic polymers with enzyme-like properties, i.e. polymers expressing substrate-selective catalytic behaviour, although progress until now has been extremely modest. In light of the impressive results obtained in mimicking enzyme and antibody binding sites by molecular imprinting, an obvious extension would be to utilize the same technique to develop polymer systems possessing various catalytic functionalities in the binding sites [78,79]. In one of the earliest studies on this topic (in parallel with the ongoing work on catalytically active antibodies [80]), imprints were prepared against a transition-state analogue. A polymer was prepared with sites selective for *p*-nitrophenol methylphosphonate, a transition-state analogue for the hydrolysis of *p*-nitrophenyl acetate [78]. The MIP demonstrated preferential binding of the transition-state analogue and induced a small increase in the rate of hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol and acetate. This rate enhancement was specifically inhibited by the transition-state analogue, providing evidence that the catalysis achieved was taking place in the sites selective for the transition state analogue.

Attempts to direct organic reactions using MIPs are obviously related in nature to the more enzyme-like systems described above. The most impressive study to date, in terms of mediating reaction selectivity, demonstrated the selective reduction of a 3,17-diketo steroid. In this case, the reaction could be directed, almost exclusively, to reduction at a predetermined keto group on the steroid. A reactive $LiAlH_2$ group was selectively positioned in the surrounding imprinted polymer, thus leaving the other keto group unreduced [81].

More recently, efforts in two laboratories [82,83] have led to the development of polymer systems showing modest catalytic activity for the β-elimination of HF from 4-fluoro-4(*p*-nitrophenyl)-2-butanone. In one system [82], a car-

boxylic group was positioned opposite to the fluorine. This was achieved by imprinting with a corresponding molecule containing a base, in order to direct the positioning of the carboxylic group in the active site. This is similar to the studies on catalytic antibodies in which aspartate and glutamate residues in the antibody combining sites were aligned to catalyze the elimination of HF from a β-fluoroketone by abstraction of an α-carbon proton [88]. We believe that the complementarity obtained allows the negatively charged carboxylate monomer residue to function as a general base.

In summary, the results obtained so far from catalytically active polymers are modest. However, the stability of such preparations, along with the possibility of introducing totally new catalytic properties, suggests this as an area worthy of intense effort [84]. The chance of success utilizing the imprint as a model for directing catalytic stereo- and regioselective reactions is not too remote. It is quite conceivable that with such catalytic systems chiral discriminations might be achieved.

3.3. Substrate-selective sensors

With the refinement of molecular imprinting as demonstrated in the successful chiral separation of compounds or in the preparation of antibody or receptor binding-site mimics, an obvious additional application would be the use of such substrate-selective polymers as sensor components. Despite the current wide range of biosensor applications, there is much room for improvement. On substituting the bio-part in an enzyme or antibody-based sensor with catalytically active or ligand-specific polymers prepared from specific molecular imprints, a more robust sensing element system would be obtained. Furthermore, in many cases no suitable biomolecule is available and it is here that molecular imprinting has its great potential, namely creating the custom-tailored binding site for a given molecule. Work in this area has recently begun to show success, and only few published data are available. In one example, a separation process differentiated optical isomers of amino acid derivatives on a column containing molecular imprints made against one enantiomer using streaming potential measurements [34]. Finally, in studies using imprinted polymer membranes as a sensing layer in a field effect device, a lowering of the capacitance was observed on specific binding of the original imprint molecule [39].

All these studies are preliminary but there is no doubt that we will see, in the near future, many such sensoring systems utilizing synthetic binding sites. The systems discussed above can also be considered as high-specificity chemical sensors. The potential for such devices as a kind of artificial nose in single- and multiple-compound determinations appears promising and should constitute a valuable extension to existing chemical and bio-sensor systems.

4. Concluding remarks

We believe that the possibility of making stereospecific polymers by the technique of molecular imprinting will be extended further and find increasing application. The remarkable stability of the acrylic polymers, expressing unimpaired molecular memory after repeated use (100-fold) and over extended periods of time (several years), strongly support this notion.

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