Imprinted Polymers with Memory for Small Molecules, Proteins, or Crystals**

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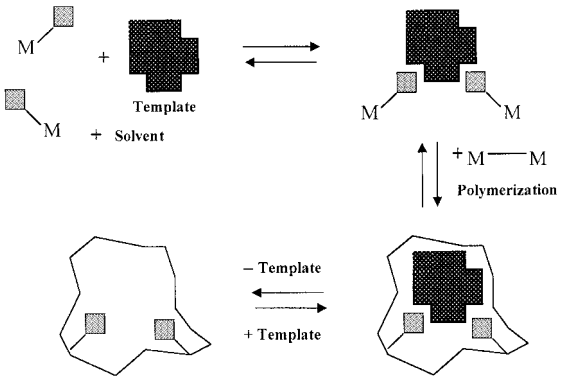
Introduction

Molecular recognition is associated with biological processes such as the immuno response, ligand–receptor interactions, and enzyme catalysis. The ability of biological hosts to strongly and specifically bind to a particular molecular structure is a key factor in the biological machinery. Well-known examples are the sensitivity of the immuno response,[1] where antibodies are generated in response to minute amounts of a foreign antigen, or the energy saved by enzymes due to their ability to stabilize the transition state of the reaction to be catalyzed.[2] With biological examples as models, chemists hope to be able to mimic these properties for various applications.[3–5] For instance stable recognition elements capable of strongly and selectively binding molecules could be used in robust, sensitive analytical methods for the analysis of trace levels of compounds in complex matrices. Alternatively such recognition elements could be used to separate undesirable compounds from foods or biological fluids, for targeted delivery of drugs, or for demanding separations at a preparative level in the fine-chemical industry.

Robust molecular recognition elements with antibody-like ability to bind and discriminate between molecules or other structures can today be synthesized using molecular imprinting techniques.[6, 7] This includes the synthesis of cross-linked polymers in the presence of templates which may be small molecules, biological macromolecules, microorganisms, or whole crystals (Scheme 1). Functional monomers that can be linked covalently or noncovalently to the template bind the template to the monomer or growing polymer during synthesis and, after template removal, provide the subsequent binding interactions (Table 1). Removal of the template from the formed polymer thus generates a structure complementary to the template structure or to an analogous structure. This simple and appealing concept has for more than 50 years[8] attracted chemists from several disciplines in their search for solutions to their particular problems. Nevertheless advances in the field have been slow and real applications that can be commercially exploited are still lacking. However, in view of some recent promising developments this may be about to change. Combinatorial techniques for rapid, parallel synthesis and testing of large groups of materials promise, for instance, to accelerate the development of new recognition elements significantly.[9, 10] Furthermore clever design of linkers to position the binding-site functional groups at distances allowing subsequent rebinding by electrostatic interactions has resulted in materials that exhibit excellent recognition properties for highly interesting targets such as peptides[11] and steroids.[12] Also larger molecules such as proteins can now be recognized by surface imprinting techniques.[13] Thus, template imprinting is not restricted to low molecular weight targets. Recent contributions have shown that even imprinting of whole cells[14] and inorganic crystals[15] generates structures capable of efficient recognition of the corresponding template structure. Finally, imprinting of metal ions for sequestration or sensing[7] and molecularly imprinted enzyme mimics[4] are burgeoning areas, but outside the scope of this contribution. This highlight will summarize some of the most promising recent developments for the generation of templated binding sites and then provide connections to potential applications.



Scheme 1. Imprinting of network polymers with various templates. The template can be a small molecule, protein, cell, or crystal.

Noncovalent Imprinting for the Recognition of Small Molecules

The most successful noncovalent imprinting systems are based on commodity acrylic or methacrylic monomers, cross-linked with ethyleneglycol dimethacrylate (EDMA). Methacrylic acid (MAA) is, to date, the most widely used functional monomer, as can be seen from the numerous examples in the literature.[7, 16] The most common procedure applied to imprinting with L-phenylalanine anilide (L-PA) is outlined in Scheme 2.[17, 18]

The broad applicability of MAA as a functional monomer is related to the fact that the carboxylic acid group serves well as a hydrogen-bond and proton donor and as a hydrogen-bond acceptor.[19] In aprotic solvents such as acetonitrile, carboxylic acids and amine bases form contact hydrogen-bonded assemblies, where the association strength for a given acid increases with the basicity of the base.[20] Thus templates containing Brønsted basic or hydrogen-bonding functional groups are potentially suitable templates for the MAA/EDMA system.[10, 17, 18] Furthermore, hydrogen bonds can form with templates containing acid,[21] amide,[22] or functionalized

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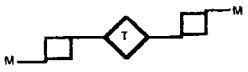
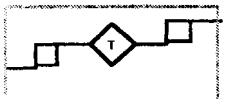
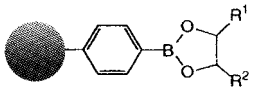
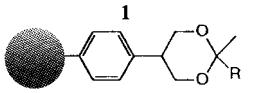
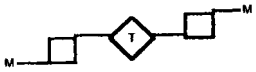
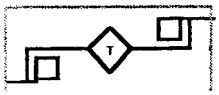
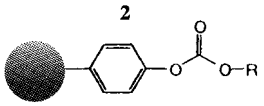
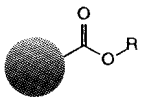
Table 1. Approaches for imprinting small molecules.[a]

Synthesis	Rebinding	Attachments	Ref.
covalent 	covalent 		[40]
		1 	[41]
covalent 	noncovalent 	2 	[12]
		3 	[31]
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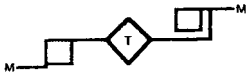
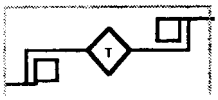
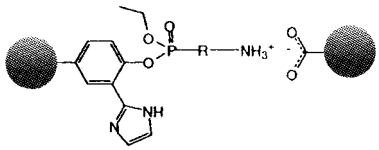
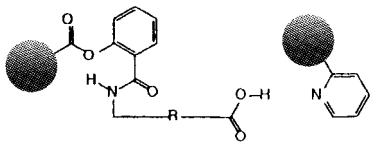
covalent – noncovalent 	noncovalent 		[42]
		5 	[11]
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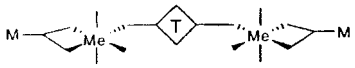
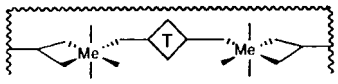
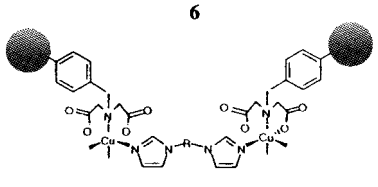
metal ion mediated 	metal ion mediated 		[43]
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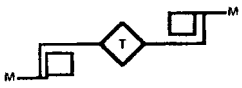
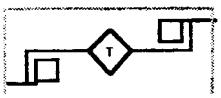
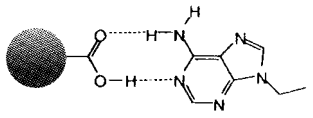
noncovalent 	noncovalent 		[23]
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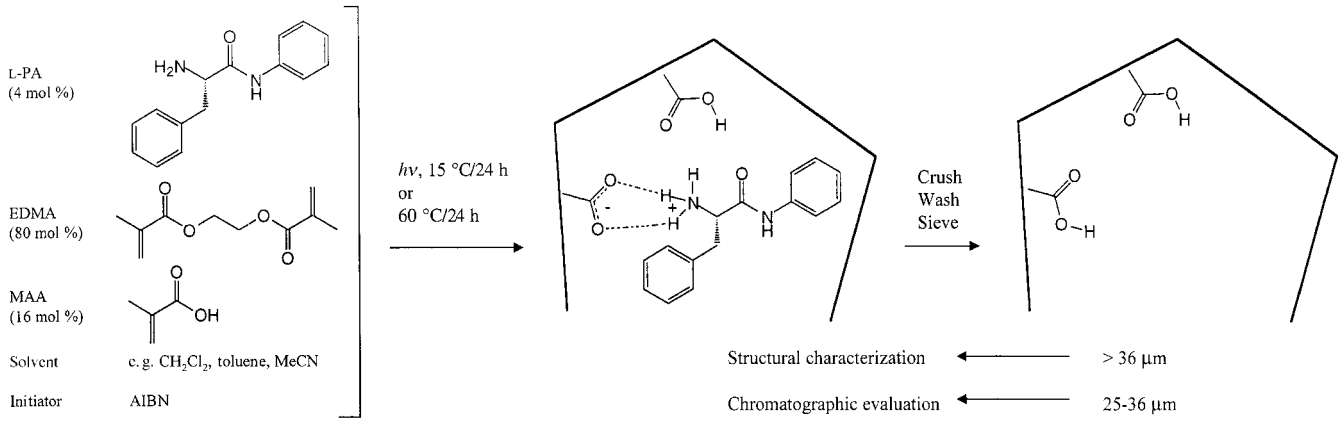
[a] T = template, M = polarizable group.



Scheme 2. Imprinting protocol for the recognition of L-phenylalanine anilide (L-PA). AIBN = azobisisobutyronitrile, EDMA = ethyleneglycol dimethacrylate, MAA = methacrylic acid.

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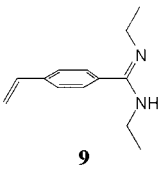
nitrogen heterocycles,[23, 24] the latter two being particularly stable. However, other criteria are that the template is soluble in the most common solvents or monomers used in the imprinting step, that it is available in preparative amounts, and that it is stable under the conditions of the polymerization and does not undergo reactions with free radicals. Provided that these criteria are fulfilled the resulting molecularly imprinted polymers (MIPs) usually exhibit a significant and selective rebinding of the template when compared to non-imprinted control polymers.

The potential for a given monomer template pair to produce templated sites can be predicted by measuring the stability constants, for example by spectroscopic techniques, in a homogeneous solution mimicking the monomer mixture prior to polymerization.[17, 25] This can ultimately be used as a preliminary screening procedure to search for suitable functional monomers. With 9-ethyladenine (**8**, see Table 1) as template a comparison can be made between the solution stability of the complex and the binding constant and site density obtained in experiments for batch rebinding to the imprinted material. 9-Ethyladenine associates with butyric acid in chloroform at three sites with estimated association constants of 114, 41, and $5\,\text{M}^{-1}$ respectively.[26] Assuming similar association constants between MAA and this template prior to polymerization the major part of the template would be present in the complexed form. The corresponding imprinted polymer prepared in chloroform exhibited an adsorption isotherm that could be fitted to a bi-Langmuir adsorption model, resulting in a class of high-energy binding sites with a binding constant (K_a) in chloroform of $76\,000\,\text{M}^{-1}$, similar to the binding energies observed for designed synthetic receptors of the same molecule.[23] Also of interest is the fact that more than 30% of the added template resulted in high-affinity binding sites (site density $n = 20\,\mu\text{mol g}^{-1}$). Usually, however, the yield is considerably lower, often in the range of only a few percent. Subtracting the binding to a reference polymer imprinted with benzylamine from the binding to a polymer imprinted with 9-ethyladenine gave a mono-Langmuir isotherm (K_a $79\,000\,\text{M}^{-1}$), thus indicating that the sites probed in this experiment are isolated and relatively uniform. The remaining sites interact nonselectively with solutes binding to carboxylic acids and limit the degree of separation that can be achieved.

In spite of the pronounced recognition observed for a number of important targets with the use of MAA as functional monomer, recognition of any given target molecule requires access to functional monomers targeted towards structural features specific for particular compounds or classes of compounds. Based on the structural features of the templates that generate good sites in the MAA system, an interesting possibility would be to incorporate these structures in new functional monomers for the recognition of carboxylic acids. This concept is somewhat similar to the reciprocity concept in the design of chiral stationary phases. Thus, Wulff et al. synthesized *N*,*N*′-diethyl-*p*-vinylbenzamidine (**9**) and showed that this monomer could be used to generate high-fidelity sites for the molecular recognition of carboxylic acids as well as sites exhibiting catalytic esterolytic activity.[27] The binding is strong enough here to provide efficient recognition also in aqueous media. Furthermore, since the functional monomer is quantitatively associated with the template prior to polymerization, the nonspecific binding is minimized. Functional-group complementarity is thus one basis for the choice of functional monomer. In the case of templates with low polarity and with few functional groups that can interact with functional monomers, it may be beneficial to use amphiphilic monomers stabilizing the monomer template assemblies by hydrophobic and van der Waals forces. Thus cyclodextrins have been used to template binding sites for cholesterol (**10**, β-CD = β-cyclodextrin)[28] or to enhance the selectivity in the imprinting of amino acids.[29] Monomers based on bile acids or cholesterol (**11a**) have also been used to generate binding sites for cholesterol (**11b**).[30] In a comparison of a number of common adsorbents, the adsorbents imprinted with cholesterol exhibited the largest uptake of cholesterol from liquids mimicking intestinal fluids at physiologically relevant cholesterol concentrations.



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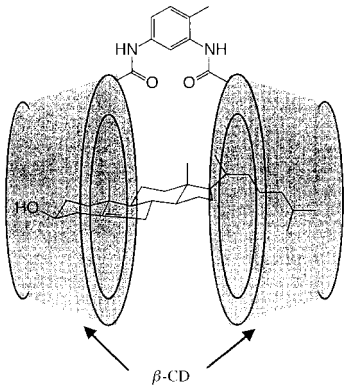

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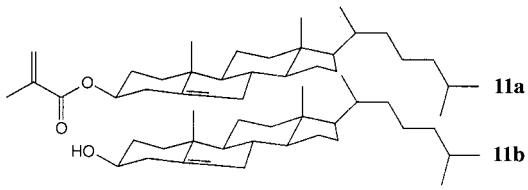


11a

11b

In summary the simple mix-and-bake procedure described here can be used to generate molecular recognition elements for a large variety of small molecules. However, important challenges remain in the molecular imprinting of larger complex biomolecules.[36]

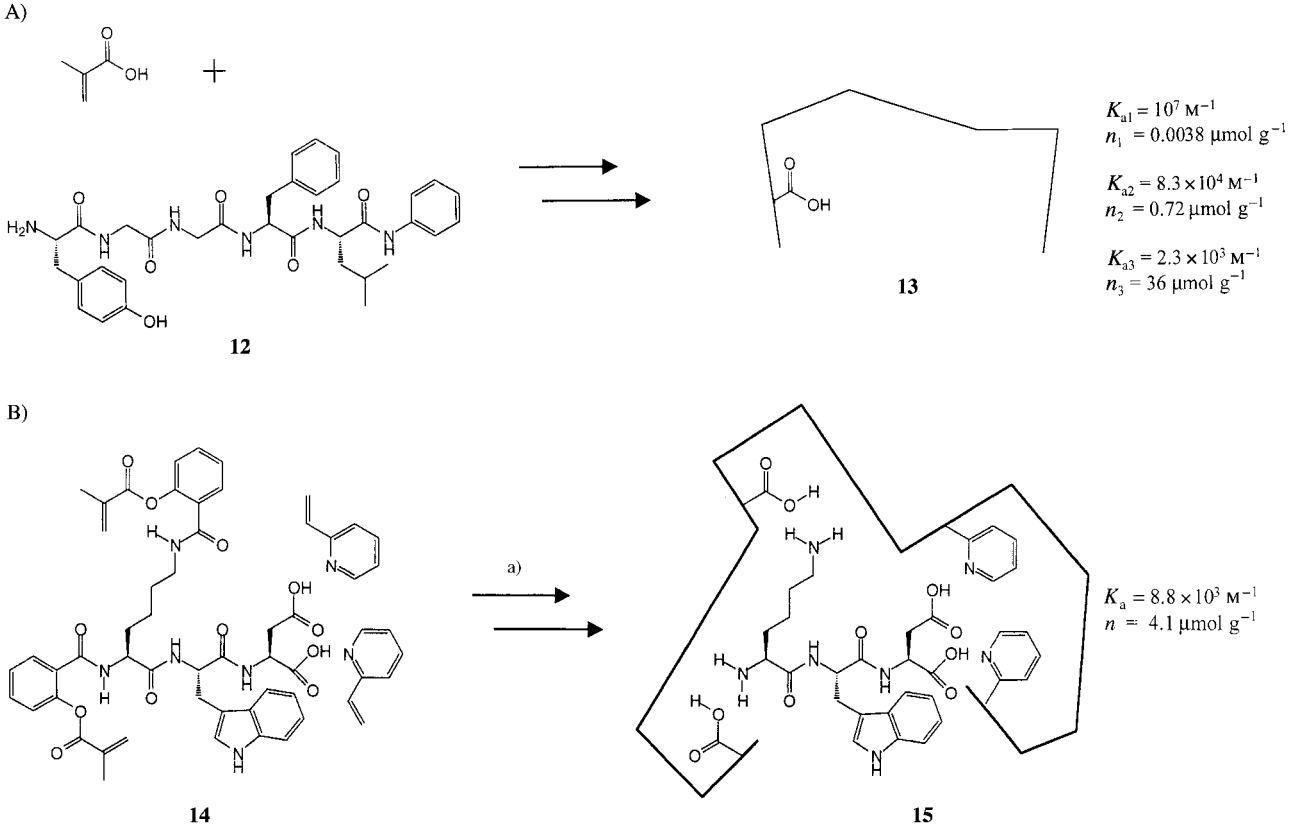
Molecular Recognition of Oligopeptides

Stable molecular recognition elements designed towards amino acid sequences and isomers of peptides could potentially be used for demanding separations of peptide mixtures, in peptide sensors, in therapeutic applications, or as biological

model systems. Amino acid amides and smaller peptides have been used as templates in the MAA/EDMA protocol (Scheme 2), resulting in materials showing strong and selective binding of the amino acid or peptide target. Thus Leu-enkephalin anilide (**12**) was imprinted to generate a binding site for Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) (**13**).[22] The resulting material exhibited high binding constants for **13** in organic as well as in organic/aqueous solvents (Scheme 3 A). The high affinities for the peptide target allow these materials to be used as antibody substitutes in competitive assays and for other analytical applications. However, as noted above the nonspecific binding to these materials is considerable and the density of high-energy binding sites is low. This essentially limits their use to analytical applications. Solubility is another problem. Many peptides are only sparingly soluble in commonly used monomer–solvent systems. Therefore, addition of polar solvents prior to polymerization or the use of more soluble analogues may be required. The former results in destabilization of the functional monomer–template assemblies, which are held together by electrostatic interactions, and a lower yield of templated sites. The latter, on the other hand, may result in a site lacking complementarity to the target peptide.

One possible way to circumvent these problems is to attach the template covalently to the functional monomer and, after polymerization and template removal, allow the rebinding to take place by noncovalent association (see above). This is feasible in the case of a hydrolyzable ester (such as **4**, Table 1),[31] amide, or imine bond,[32] where subsequent rebinding can take place by electrostatic interactions between the respective amine and carboxylic acid. After disappointing results in terms of template removal, binding capacity, and selectivity, this approach has not been pursued further. Reasons for the poor results were the intrinsic stability of the monomer–template bonds and an incorrect positioning of the functional group for optimal noncovalent recognition. One solution to these problems was introduced by the group of Whitcombe and Vulfson.[12] They used easily cleavable sacrificial spacers between the monomer and the template (**3** and **6**, Table 1) to correctly position functional groups for rebinding.

So far the approach has been used to generate recognition elements for cholesterol, polychlorinated biphenyls (PCBs), and peptides. As an example of the latter, the tripeptide Lys-Trp-Asp (**15**, Scheme 3 B) was used as template.[11] It was covalently linked to MAA through two salicoyl groups connected to the lysine amino groups and to the MAA carboxylic acid group (**14**). The lability of this linker would result in a high recovery of template with all resulting carboxylic acid groups associated with templated sites. Intramolecular hydrogen bonding between the amide and ester group of the linker would position the amino group at a hydrogen-bond distance from the carboxylic acid group after cleavage of the template. Furthermore, it was anticipated that



Scheme 3. Synthesis of peptide-selective polymers according to A) Andersson et al.[22] or B) Klein et al.[11] based on noncovalent or covalent linkages, respectively, of the MAA groups to the template prior to polymerization. Association constants K_a and site densities n obtained from batch equilibrium rebinding experiments using A) Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH, **13**) or B) **15** are given. Solvents: A) EtOH/20 mM sodium citrate (pH 4.5) 10/90 (v/v), B) acetonitrile/water 4/1 (v/v); a) 1. divinylbenzene (DVB), initiator, 2. UV, polymerization, 3. NaOH, aq. MeOH, 4. addition of Lys-Trp-Asp.

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the aryl group of the linker would create the space necessary for template removal and rebinding. Finally the covalent strategy to recognize the lysine group was combined with the addition of 2-vinylpyridine to complement the carboxylic acid groups of the aspartic acid residue. The resulting monomer assemblies were copolymerized with an excess of divinylbenzene (DVB) by UV initiation, and the template was cleaved from the polymer by refluxing the polymer in methanolic aqueous NaOH, resulting in 58% template removal. The polymer was then tested in a batch equilibrium rebinding experiment and compared to a control polymer prepared by replacing the template with an equimolar amount of MAA. The results demonstrated that this strategy templated the peptide. Particularly noteworthy was the amount of bound template in comparison with structurally similar peptides in aqueous/organic solvents. However, in view of the binding to the control polymer at the applied concentrations the results should be interpreted with caution. The amount adsorbed nonspecifically to the control polymer—although much lower than the amount adsorbed to the peptide-imprinted polymer—correlated to some extent with the amount adsorbed to the imprinted polymer.

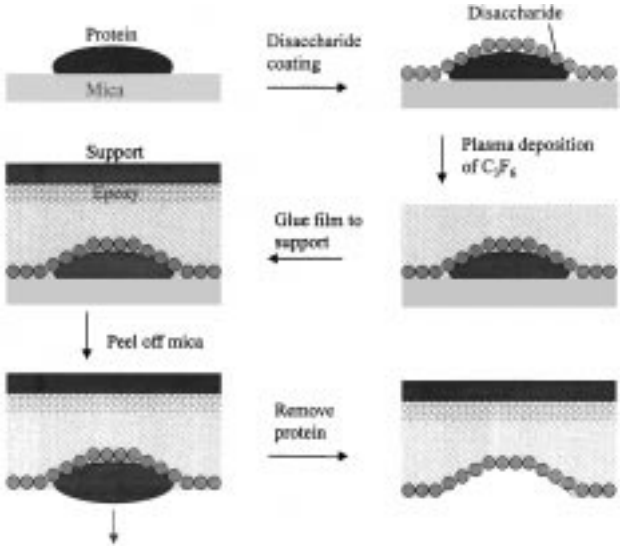
It is interesting to compare the covalent imprinting strategy with the noncovalent one described above. Although the polymers synthesized by the covalent strategy exhibit pronounced recognition of the template peptide, it is unclear whether they contain sites capable of rebinding the template with the affinities shown by the materials prepared by the noncovalent strategy. This may be decisive for the applications, analytical or preparative, in which the respective materials will be used.

Molecular Recognition of Proteins

The design of stable molecular recognition elements for proteins is, due to the complexity of these macromolecules, a difficult task, but attractive in view of the great need for such tools in biotechnology for down-stream processing, in therapeutics for drug delivery, for bioanalysis in sensors, and in diagnostics.[5] Affinity separations of proteins can be achieved using antibodies, enzymes, or receptors capable of recognizing protein epitopes. However, the limited stability of these recognition elements precludes the development of robust and cheap methods to recognize or separate this class of compounds. The recent development of stable cross-linked peptide-recognition elements prepared by screening of phage peptide libraries may be an exception in this regard. Otherwise separation of proteins using nonbiological affinity techniques is based on the presence of certain functional groups. Thus protein surface thiol groups, imidazoles, diols in glycoproteins, or hydrophobic side-chain amino acids can be targeted using ligands immobilized on solid supports.[5] The level of discrimination that can be achieved is limited to the amount of surface-exposed functional groups, and these techniques cannot be used to differentiate between closely related proteins. In this regard, molecular imprinting would allow the functional groups to be spatially distributed at positions complementary to the distribution of the surface functional groups of the protein. However, the structure and function of proteins are sensitive to solvent, temperature, pH, and ionic strength. New imprinting techniques considering these factors need to be developed.

Previous examples of the preparation of protein-imprinted gels or coatings[33] are all limited with respect to selectivity, binding capacity, site accessibility, or robustness.

However, an interesting approach to confine the imprinted sites to biocompatible hydrophilic surfaces was recently published by the group of Ratner.[13] They adsorbed the template protein (plasma proteins: bovine serume albumine (BSA), immunoglobuline G (IgG), ribonuclease (RNase), or lysozyme (LSZ)) to a mica surface. This substrate was chosen because it is atomically flat and because proteins are adsorbed with minimal denaturation and conformational changes. Thus only the template protein causes changes in surface topography. A 1–5 nm layer of a nonreducing disaccharide was then spin-coated on the surface followed by plasma deposition of a 10–30 nm layer of a fluoropolymer (Scheme 4). The



Scheme 4. Protein surface imprinting technique based on carbohydrate–protein interactions.

resulting film was epoxyglued to glass, the mica sheet peeled off, and the protein removed by treatment of the surface in aqueous NaOH/NaOCl. This resulted in a sugar-coated surface containing templated pits. Through the use of surface analytical techniques sensitive to elemental composition (ESCA) and mass distribution (TOF-SIMS), it could be shown that the protein was effectively removed after this treatment. The resulting surfaces, when examined with atomic force mictroscopy (AFM), revealed pits of approximately the size and shape of the protein used as template. Furthermore, in competitive adsorption experiments the surfaces exhibited a preference for the protein used as template. Even though all the templated surfaces adsorbed all proteins nonspecifically, the protein used as template was only displaced at elevated concentrations of competing protein (Table 2). Since only one procedure was used for the synthesis of all templated surfaces, this technique may become generally applicable to the imprinting of proteins.

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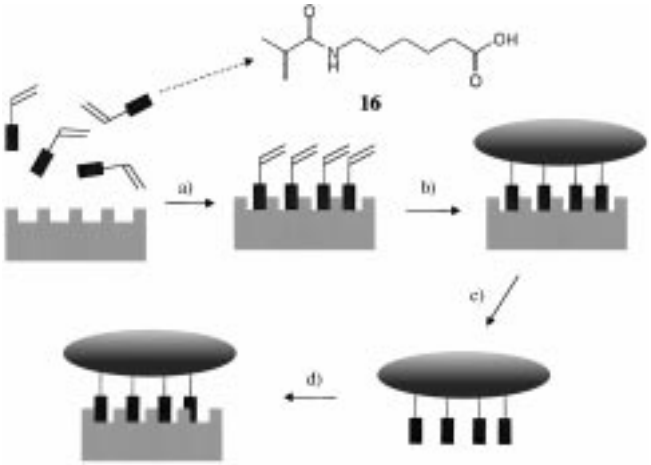
Table 2. Results from competitive adsorption experiments showing the ratio of competing protein to the substrate radiolabelled protein at which the binding of the substrate was reduced by 50%.

Substrate	Displacer	Displacer:substrate at 50% displacement			
		BSA imprinted	IgG imprinted	LSZ imprinted	RNase imprinted
BSA	IgG	30.2	3.1	–	–
IgG	BSA	0.9	3.4	–	–
RNase	LSZ	–	–	0.2	4.0
LSZ	RNase	–	–	31.0	1.2

Imprinting of Crystals for Structure-Directed Nucleations

Nature uses proteinaceous templates to direct the crystallization of inorganic materials into structures that deviate from the spontaneously formed structure.[34] Mimicking these templates may lead to a new class of biomaterials with superior properties.[35] In a recent report the group of Vulfson showed that imprinting of inorganic crystals resulted in polymeric templates capable of directing crystallizations into structures that would not form in solution.[15] Thus a network copolymer of divinylbenzene (DVB) and 6-methacryloylamidohexanoic acid (**16**) was prepared by free radical polymerization in the presence of crystals of calcite (rhombohedral, ca. 4 μm) suspended in chloroform (Scheme 5). Additionally two control polymers were prepared, one in the absence of crystals and one in the presence of crystals but without the functional monomer. Otherwise the control polymers were



Scheme 5. Process for the imprinting of crystals and subsequent use of the polymer for directing crystal nucleation of $CaCO_3$. a) Incubation in chloroform, b) DVB, polymerization, c) HCl, aq. MeOH, template removal, d) supersaturated $CaCO_3$ solution.

prepared identically to the crystal-imprinted polymer. After polymerization the polymer monolith was ground to give particles about 100 μm in size, and the template crystal removed by washing the polymers with acidified aqueous methanol. After this treatment no polymer-bound calcite could be detected by scanning electron microscopy (SEM), X-ray powder diffraction, or IR spectroscopy, indicating that the wash procedure effectively removed the crystals.

Nucleation experiments were then performed by mixing aqueous solutions of Na_2CO_3 (0.8 mM) and $CaCl_2$ (1.0 mM) and allowing nucleation to take place over 24 hours. The polymer surfaces were then washed with water, and the surfaces investigated by SEM. The surface of the imprinted polymer contained considerably more calcite crystals than the surface of the control polymers. Moreover, their morphology was different from the crystal formed spontaneously in solution and resembled the original template morphology. Powder X-ray diffraction experiments confirmed that the crystals contained more than 96% calcite. Interestingly, even under conditions favoring the formation of the alternative structure aragonite (95 °C), calcite was still formed at the imprinted sites. By potentiometric titrations it was shown that the number of accessible carboxylic acid groups in the imprinted polymer (39 $\mu mol\,g^{-1}$) was much higher than in the control polymer (10 $\mu mol\,g^{-1}$), but lower than the theoretical number (79 $\mu mol\,g^{-1}$). Furthermore, it was shown that the crystals adsorbed a large amount of the acid functional monomer in chloroform. It can then be assumed that the templated sites are enriched in accessible carboxylic acid groups.

To what extent are accessible and densely packed acid groups capable of the same structural control? In a previous contribution by Tremel and co-workers it was observed that self-assembled monolayers of ω-carboxylic and sulfonic acid functionalized alkanethiols on gold can exert a similar level of structural control.[36] Unfortunately in the work of Vulfson et al. aragonite imprinted polymers did not enhance the nucleation of aragonite, which would have provided additional strong evidence for the proposed mechanism.

In the context of the crystal imprinting concept, some similarities are seen with a recently revived proposal[37] for the mechanism of recognition exhibited by noncovalently imprinted polymers.[38] This suggests that template remaining in the polymer after template removal is capable of acting as a nucleation site for the template in the rebinding experiment. Thus the actual binding sites are complementary to small clusters of the template. This is an interesting theory that can explain the adsorption behavior in some systems (note especially the use of cholesterol monomers to imprint cholesterol (**11**)),[30] particularly at low site occupancy. However, in contrast, the commonly observed bi- or tri-Langmuir adsorption isotherms[39] indicate a true receptor behavior (for example, in the case of **8**).[23]

Rapid Synthesis and Screening of Large Groups of Polymers

A key to the development of new imprinted polymers is the identification and optimization of the main factors affecting the structure and molecular recognition properties of the materials. These factors can be the type and the relative amounts of functional monomer and cross-linking monomer, the polymerization initiator, the temperature, the pressure, or the solvent of polymerization.[16] The presently used monolith procedure—that is, synthesis of the polymer as a block that needs to be crushed and sieved—is time-consuming, and a careful optimization of all the factors affecting the material's properties is therefore not feasible. One way around this problem, as suggested in two recent reports by the groups of Takeuchi[9] and Sellergren,[10] is to scale down the batch size to

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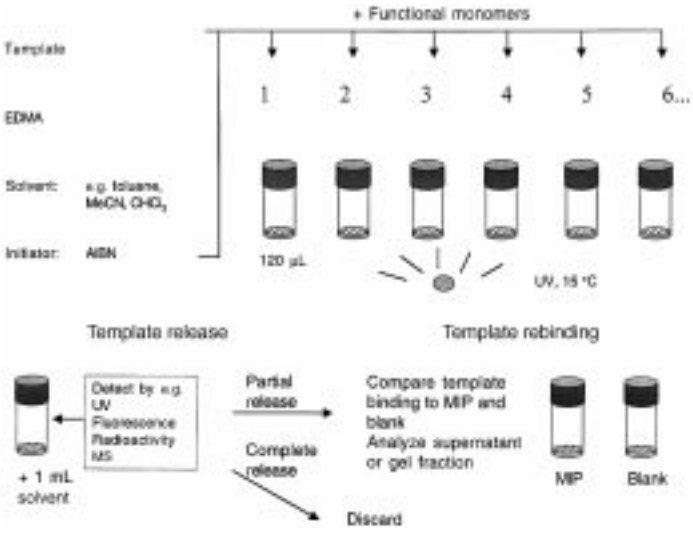
about 50 mg and perform the synthesis in small vials adopted for automated handling of samples (Scheme 6). Since this batch size leaves the polymer as a layer on the bottom of the vials, intralayer diffusion of template is fast enough for the polymer to be analyzed in situ without workup. This significantly speeds up the synthesis and analysis of the materials. By automation of this procedure with simple equipment for sample handling, it is estimated that over 100 materials can be processed and analyzed in parallel within 24 hours. Based on the previous examples the method is



Scheme 6. Combinatorial imprinting technique suitable for automation.

reproducible and has been further validated by comparing the selectivity of the small-scale batch with a corresponding normal-scale batch of materials. Therefore, a rational synthetic approach for MIPs may soon be a reality.

Final Remarks

Of the above-discussed systems, the imprinting of small molecules has, to date, reached the highest level of maturation. Nevertheless, some limitations still hamper real applications of the materials. One of these concerns the recognition of templates at either the low or high end of the polarity scale. In the former case the problem is mainly related to the lack of monomers that provide sufficiently stable monomer–template assemblies. In the latter case, poor solubility in commonly used organic monomer–solvent systems can be expected. This is true for a number of important target biomolecules. Further problems are the low yield of high-affinity binding sites and the resulting nonspecific binding, poor site accessibility, and a lack of practical and economic methods for manufacturing the materials. Finally, even if these problems can be solved, a challenging competition with alternative biological and synthetic recognition elements can be expected. The ultimate test of the usefulness of the imprinted materials will be whether they can be synthesized with comparable recognition properties at a lower cost and in a shorter time than their counterparts.

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