# Hierarchical Imprinting Using Crude Solid Phase Peptide Synthesis Products as Templates

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Molecularly imprinted polymers (MIPs) are gaining in importance as robust molecular recognition elements in analytical and separation sciences.[1] These are commonly synthesized by polymerizing complexes of functional monomers and a template to be imprinted with matrix-forming monomers to yield highly cross-linked polymers. Template removal results in a MIP containing nanometer-sized binding sites in addition to larger sized pores.[2] For guest molecules to access the host binding site they must penetrate pores whose sizes are difficult to control independently from the generation of the imprinted site.

One way to decouple these processes is to immobilize the template on the surface of porous, disposable solids that act as molds to create a desired porosity.[3,4] In this way, the pore system is controlled by the solid mold regardless of the conditions applied to generate the imprinted sites. In addition, the imprinted sites are confined to the pore wall surface of the resulting material. So far, the feasibility of this approach has been demonstrated in the imprinting of small molecules, that is, nucleotide bases[4] and small drugs.[3] However, the benefits of confining the sites to the pore wall surface have never been demonstrated. Particularly intriguing would be to use this concept for the development of affinity phases for the separation of biological macromolecules, for example, peptides and proteins[5] according to the epitope approach.[5b] A smaller peptide corresponding to a unique amino acid sequence of a target protein is here used as a template to generate a site that can subsequently selectively bind the larger target molecule.

By using the crude products resulting from solid-phase peptide synthesis as epitope templates, we here show that surface-confined sites for larger peptides can be prepared. The concept is demonstrated by first synthesising the peptide epitope on the surface of a porous silica support (Figure 1). The immobilized peptide is then used as a template for the generation of a hierarchically imprinted material.

With use of aminopropylsilica (APS) with an average pore size of 11.5 nm as a common support material, peptides were synthesized using standard Merrifield chemistry (Figure 1).[6] Thus, in the first step, BOC−Gly−OH was coupled through DCC-catalyzed amide bond formation. After deprotection, FMOC−Phe−OH was coupled to obtain the N-protected or, after deprotection, free dipeptide coupled through its carboxy terminus to the support surface. From the change in carbon and nitrogen content of the modified silica particles the coupling steps appeared to occur in high yield and could, aside from the amide characteristic bands in the IR spectra, be followed visually by fluorescence microscopy. Thus, coupling of FMOC−Phe−OH was accompanied by a strong particle fluorescence which disappeared completely upon deprotection. The area density of the final coupling products was found to be in the range 1−2 $\mu$mol/m$^2$. Assuming a random ligand distribution, this corresponds to an average distance between the ligands of 10−15 Å.

After the template synthesis, the pores of the immobilized amino acid or peptide templates were filled with a mixture of methacrylic acid (MAA) ethylene glycol dimethacrylate (EDMA) and azoinitiator (AIBN) (Figure 1). The particles were thereafter thermally cured at 60 °C. Dissolution of the silica mold by treatment with a solution of $NH_4HF_2$ (aq) resulted in organic polymer beads with a size and morphology reflecting those of the original silica mold.[7]

The polymers were subsequently assessed as stationary phases in chromatography. Our first concern was to what extent the template immobilization influences the selectivity and kinetic properties of the formed sites, compared to a material prepared from a nonimmobilized template. For this purpose we compared two materials (P(FMOC−Phe//Si) and P(FMOC−Phe−Si)) prepared using free FMOC−Phe−OH (FMOC−Phe//Si) or FMOC−Phe−OH coupled to APS−Si (FMOC−Phe−Si) as templates. As seen in Figure 2A, the polymer obtained using the latter template preferentially retained N-protected phenylalanine derivatives, including the dipeptide FMOC−Phe-Gly−OH, with ca. 5 times larger retention factors ($k'$) than those obtained using the polymer imprinted with soluble FMOC−Phe−OH as stationary phase. Meanwhile, phenylalanine derivatives containing free amino groups were retained similarly on both materials. In view of the similar template load used when preparing both materials, the enhanced retention

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(7) As an example, P(H−Phe-Gly−Si): specific surface area, 204 m$^2$/g; specific pore volume, 0.58 mL/g; average pore diameter, 5.4 nm.

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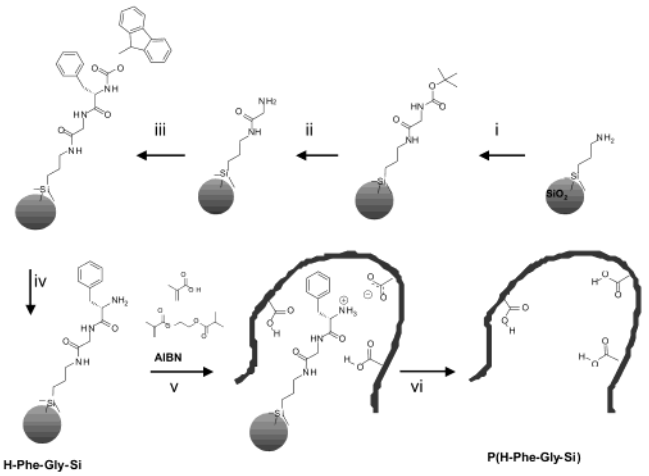


**Figure 1.** Solid-phase synthesis of peptide templates and preparation of corresponding hierarchically imprinted polymers. (i) BOC−Gly−OH, DCCI/HOBt, $CH_2Cl_2$, 25 °C, 12 h; (ii) $Me_3SiCl$ (1 M), $C_6H_5OH$ (3 M), $CH_2Cl_2$, 25 °C, 1 h; (iii) FMOC−Phe−OH, DCCI/HOBt, $CH_2Cl_2$, 25 °C, 12 h; (iv) piperidine (20%), DMF, 25 °C, 30 min. (v) (a) Pore filling with EDMA (83%), MAA (17%), and AIBN (1% w/w) by repeated vacuum-nitrogen purge cycles. (b) △ 60 °C, 24 h (vi) $(NH_4)HF_2$ (aq). 2 d.

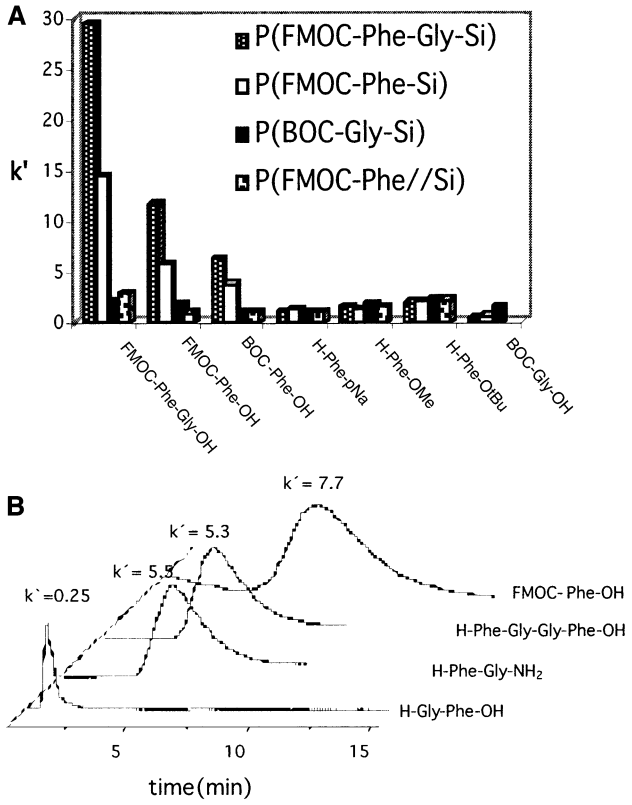


**Figure 2.** Retention factors ($k'$) of amino acid derivatives and peptides injected (10 $\mu$L of 1 mM stock solutions) on columns (50 × 5 mm) packed with hierarchically imprinted polymers as indicated using acetonitrile (A) or acetonitrile: water (95/5 v/v) containing 1% acetic acid (B) as mobile phases and a flow rate of 0.5 mL/min. (B) shows the elution profiles obtained using P(FMOC−Phe-Gly−Si) as the stationary phase. The void marker acetone eluted as a Gaussian band ($N$ = 2000/m).

factors are most likely due to a higher accessibility of the surface-confined binding sites. This behavior contrasted with the observed enantioselectivity of the materials; here, P(FMOC−Phe//Si) exhibited a higher separation factor for FMOC−Phe−OH ($\alpha = k'_L/k'_D$ = 1.5) than the surface-imprinted material P(FMOC−Phe−Si) ($\alpha$ = 1.1).

This supports the previous hypothesis that embedded less accessible sites exhibit higher structural fidelity than surface-exposed sites of higher accessibility.[8]

We thereafter focused on the dipeptide-imprinted materials. As seen in Figure 2A, FMOC−Phe-Gly−OH is ca. 2 times more strongly retained on P(FMOC−Phe-Gly−Si) than on P(FMOC−Phe−Si) and ca. 15 times more strongly than on P(BOC−Gly−Si). However, similar relative retentions were seen for FMOC−Phe−OH on the three materials. This may be the result of different surface densities of templated sites, which is in agreement with a somewhat lower ligand area density obtained for FMOC−Phe−Si. The marginal contribution of Gly to the observed recognition is indicated by the weak retention of BOC−Gly−OH on all materials and the behavior of H−Gly-Phe−OH in aqueous mobile phases (vide infra); however, although weakly retained, $k'$ = 1.6, BOC−Gly−OH was more than 2 times more retained on P(BOC−Gly−Si) than on P(FMOC−Phe−Si).

The retention behavior in aqueous mobile phases is crucial for the application of these phases to biological samples. We thus added water to the mobile phase in increments of 5% and compared the retention of different peptides on the dipeptide-imprinted materials (P(FMOC−Phe-Gly−Si) and P(H−Phe-Gly−Si)), using the glycine-imprinted materials (P(BOC−Gly−Si and P(H−Gly−Si)) as controls. With 5% water a pronounced selectivity for peptides containing the imprinted dipeptide motif is seen. This also included larger peptides containing the H−Phe-Gly motif as the N-terminus (Figure 2B). Thus, on P(FMOC−Phe-Gly−Si) H−Phe-Gly-Gly-Phe−OH was well-retained, similarly to H−Phe-Gly−$NH_2$, whereas on the glycine-imprinted phases only weak retentions were observed ($k' < 1$). The retentions observed for the two peptides on the complement P(H−Phe-Gly−Si) were slightly lower ($k'$ = 4.4), although this phase exhibitied some preference for the unprotected peptides over their protected counterparts. This contrasted with the retentions observed on P(FMOC−Phe-Gly−Si) where the partially complementary solute FMOC−Phe−OH showed the highest retention (Figure 2B). Additional strong evidence for the presence of peptide discriminating sites is provided by the retention behavior of the dipeptide H−Gly-Phe−OH with the inverse amino acid sequence. Contrary to the other dipeptides, this is most strongly retained on the materials imprinted with H−Gly−Si ($k'$ = 1.2) and BOC−Gly−Si ($k'$ = 1.3). The question remained to what extent even larger peptides containing the H−Phe-Gly− N-terminus would be recognized by the dipeptide-imprinted polymers. For this purpose we investigated the heptadecapeptide nociceptin (H−FGGFTGARKSARKLANQ−OH), an endogenous opioid receptor agonist. Also, this peptide was ca. 2 times more strongly retained on the dipeptide N-

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terminus complement P(H−Phe-Gly−Si) than on P(H−Gly−Si) in this mobile phase.

In conclusion, the use of solid-phase synthesis products as templates offers a facile route to imprinted materials exhibiting affinity binding sites for peptides exceeding the template in size. Importantly, the retention and imprinting factors far exceed those observed using the conventional bulk imprinted materials.[5b]

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**Supporting Information Available:** Procedure for synthesis and evaluation of the polymers and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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