Artificial Receptors

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Imprinted Polymers Displaying High Affinity for Sulfated Protein Fragments**

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One of the most important challenges in protein science is the comprehensive mapping of post-translational modifications (PTMs) in qualitative, quantitative, as well as dynamic terms. These efforts are driven by the important role of PTMs in the regulation of cellular processes and the observation that several PTMs directly correlate with pathogenic conditions.[1]

A PTM of underestimated importance is tyrosine sulfation, with known targets being membrane proteins, for example, involved in blood coagulation, cell adhesion, and hormone regulation.[2,3] The analysis of this modification is complicated by the lability of sulfotyrosine and isobaric masses of phospho- and sulfotyrosine.[4,5] Sulfotyrosine readily decomposes at elevated temperature and at low pH values, which has precluded it from being analyzed by chemical sequencing experiments. Hence, the scarcity of reports of this PTM can be attributed to an analytical problem rather than to a low abundance and importance of this PTM. One approach to address the problem is to develop selective enrichment techniques, which could effectively fractionate the various modified proteins or protein fragments.[5] Sulfotyrosine antibodies or more recently antibody fragments generated by in vitro display technologies have proven promising in this regard.[6] However, these are biological receptors, which suffer from inherent problems related to robustness and cost.

We previously reported that phosphotyrosine molecularly imprinted polymers (MIPs) can be used to selectively enrich phosphotyrosine peptides found at trace levels from proteolytic digests.[7] The robustness, reproducibility, and low cost of these synthetic polymer receptors are particularly attractive as alternatives to their biologically derived counterparts. As a continuation of our efforts to develop imprinted polymers for affinity-based enrichments in proteomics, we demonstrate herein that MIPs can be used to selectively enrich sulfopeptides in a strongly pH-dependent manner.

A common motif in neutral hosts for complexing oxyanions are 1,3-disubstituted ureas.[8–10] Acting as a twofold donor to the acceptor, stable cyclic hydrogen bonds are formed with the stability decreasing in the order of decreasing acceptor basicity (i.e. phosphate dianion > carboxylate ≈ phosphate monoanion > sulfonate/sulfate).[11] As a first step in our evaluation of hosts for complexing sulfated biomolecules, we decided to investigate host monomer **1** (see Figure 1) with respect to its complex stability with three model anions in the form of tetrabutylammonium (TBA) salts of phenylphosphonic acid (PPA), benzoic acid (BA), and phenylsulfonic acid (PSA). As monoanion guests, we anticipated these to crudely mimic the phenylphosphate or phenylsulfate side chain of the targets. The complex stoichiometries between **1** and the model anions were determined by Job's method of continuous variation and the binding parameters determined by ^{1}H NMR spectroscopy titration or by isothermal titration calorimetry (ITC; see Supporting Information). After having confirmed the 1:1 stoichiometry, a 1:1 binding model was used to determine the respective association constants (K). In agreement with our predictions, the oxourea monomer formed the most stable complex with the benzoate anion ($K = 8820\,\text{M}^{-1}$), followed by the phosphomonoanion ($K = 1926\,\text{M}^{-1}$), whereas the complex with the sulfonate was too weak to be detected (Supporting Information, Table S1 and Figure S1).

Thereafter, the imprinted polymers were prepared (**P1** and **P2**, Figure 1) and characterized (see Supporting Information and Table S2–S3) using the urea host monomer **1** in a 2:1 (pTyr) and 1:1 (sTyr) stoichiometric ratio to the template Fmoc-pTyr-OEt in its dianionic form and Fmoc-sTyr-OEt in its monoanionic form; two different cross-linking monomers were used, EGDMA (hydrophobic) and PETA (hydrophilic). Nonimprinted polymers ($\mathbf{P_N1}$ and $\mathbf{P_N2}$) were prepared identically to the imprinted polymers but omitting the template. Methacrylamide (MAAM) or acrylamide (AA) were added as supplementary monomers to provide additional hydrogen-bond stabilization. Imprinting effects were investigated by chromatography using the crushed polymer monoliths as the stationary phases. Thus, Fmoc-protected amino acids were injected onto the columns in an acetonitrile-rich mobile phase buffered with either triethylamine (TEA) or trifluoroacetic acid (TFA; Figure 2). Basic conditions will promote deprotonation of the template, potentially allowing it to bind to the MIP through stable quadruple hydrogen bonds.[12] **P1** and **P2** exhibited strong affinity for Fmoc-pTyr-OH in this mobile phase (Supporting Information, Figure S2)

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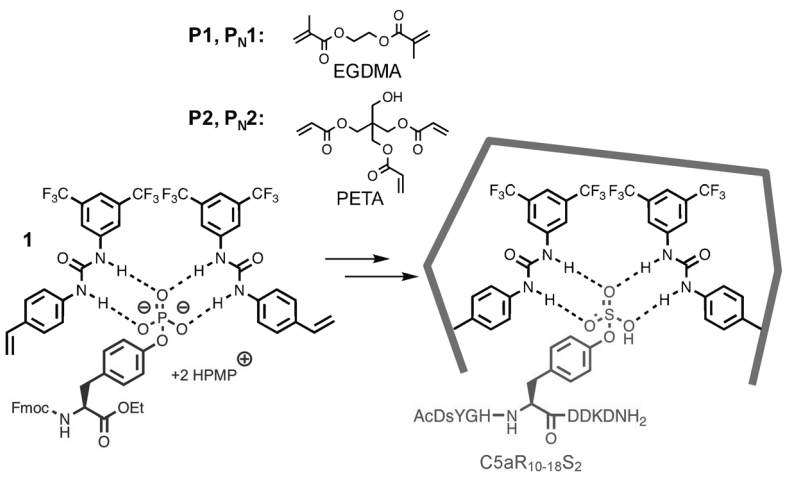

Figure 1. Possible prepolymerization complex of monomer **1** and Fmoc-pTyr-OEt and recognition of an sTyr containing peptide by the resulting MIP. Fmoc = fluorenylmethyloxycarbonyl group; HPMP = 1,2,2,6,6-pentamethylpiperidinium.

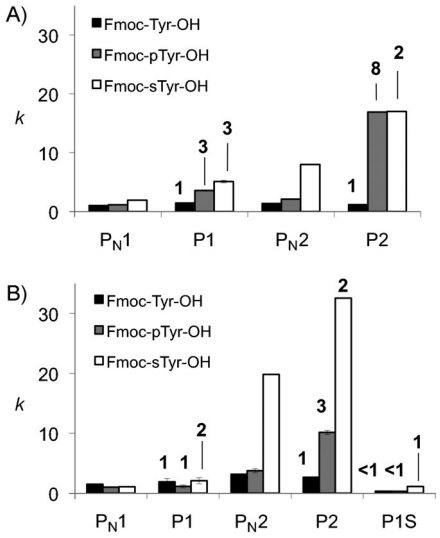

Figure 2. Retention factors (*k*) for the indicated solutes on pTyr- (**P1** and **P2**), sTyr-imprinted (**P1S**) polymers, and nonimprinted control polymers (**P_N1** and **P_N2**). The mobile phase was A) MeCN:water 95:5 (0.1% TFA) and B) MeCN:water 50:50 (0.1% TFA). The imprinting factors (k_{MIP}/k_{NIP}) rounded to a single digit are shown above the MIP data bars.

with nearly 70% of the injected analyte remaining on the column. Meanwhile, the other amino acids were not retained and **P_N1** and **P_N2** exhibited no affinity for any of the analytes. These results can be explained as follows: the phosphomonoester exists under these conditions in the dianionic form (Supporting Information, Table S4), which corresponds to the ionization state of the templated species. In addition, this anion is the most basic of those tested, thus exhibiting the highest affinity to the urea-group donors in the MIP host.[12] On the other hand, sulfate is a strongly hydrated anion[15] (Supporting Information, Table S4) and is therefore not retained by **P1** and **P2** in basic media. Furthermore, the sTyr analytes were only weakly retained by the sTyr imprinted polymer **P1S** in spite of it being programmed to bind this analyte (Figure 2B).

Considerably more interesting was the retention behavior of the sTyr analyte in mobile phases containing TFA as acidic modifier (Figure 2 and Figure S3). In agreement with our previous report, the acidic conditions led to a strong enhancement in the retention of pTyr over Tyr. We previously explained this behavior in terms of the relative strengths of the acids involved (Supporting Information, Table S4). The pH of a 0.1% TFA solution is not sufficiently low to fully protonate the phosphate groups, which still carry one oxygen-localized partial negative charge, a potent hydrogen bond acceptor, for interacting with the donor N–Hs of the binding site.[12] At the same time, the analyte is less strongly hydrated in this state (i.e. it should cost less energy for it to transfer from the bulk solvent to the imprinted site). The type of cross-linking monomer strongly influenced this behavior. Hence MIPs prepared using the hydrophilic cross-linking monomer PETA (**P2**) displayed much enhanced retentivity and selectivity than those prepared using the hydrophobic monomer EGDMA (**P1**). Apart from the more hydrophilic character of the PETA cross-linker, it also leads to higher imprinting factors, notably for the pTyr template. This reflects an enhanced fidelity of the imprinted site, possibly involving an active participation of the matrix OH groups in interacting with the guest. Also striking was the "turning on" of retention of the sTyr analytes in presence of TFA. This led to *k*-values of Fmoc-sTyr-OH exceeding that of Fmoc-pTyr-OH (notably on **P2**) and contrasted with the weak retention in the presence of base (TEA; Supporting Information, Figure S2). This interesting retention behavior is also linked to the protonation state of the functional groups (Supporting Information, Table S4). As in the case of pTyr, the TFA-buffered mobile phase should render sTyr less charged and hence less strongly hydrated.

Comparing a water-poor (Figure 2A) with a water-rich (Figure 2B) mobile phase revealed a strong influence of the hydrophilicity of the polymer on the retention and selectivity of the MIPs for the anions. Whereas the EGDMA based polymers displayed a decrease in retention when changing to a water-rich mobile phase, the hydrophilic polymers (**P2** and **P_N2**) on the contrary retained the analytes more strongly in this mobile phase. This suggests that the PETA scaffold is better adapted to the analysis of aqueous biological samples.

For practical applications, the sTyr selective MIPs should also display affinity for sulfated peptides and proteins. The sulfopeptide affinity must prevail when the MIP is challenged with complex biological samples containing an excess of not only unmodified but also phosphorylated protein fragments, the latter offering a strong competition for binding to the MIP host.

To test this possibility, the synthetic sulfated peptide C5aR$_{10-18}$S$_2$[13] (Figure 1) was chosen. C5aR is a G protein-coupled receptor involved in the inflammatory response in the presence the proinflammatory agent C5a. The two sTyr

residues (sTyr-11 and sTyr-14) are located near the N-terminus and are essential for high affinity binding of C5a by C5aR. This sequence is hence a target for inflammation inhibitors.[14]

First we incubated polymers **P1** and $\mathbf{P_N1}$ with $C5aR_{10\text{-}18}S_2$ in MeCN:water 80:20 (0.1% TFA) under equilibrium conditions and measured the specific binding to each of the polymers by C18 HPLC quantification of the nonbound peptide. A strong imprinting effect was seen, with more than 80% of the peptide bound to **P1** (ca. 0.8 $\mu mol\,g^{-1}$), whereas $\mathbf{P_N1}$ showed only minimal affinity for the peptide (Supporting Information, Figure S4). We then employed a simple on/off solid-phase extraction (SPE) protocol comprised of one loading step and one elution step, followed by reverse-phase HPLC analysis of the eluted fractions. Figures S5–S6 show the recovery of the model peptide after SPE on **P1** and **P2** using an optimized extraction protocol. Also, in this test **P2** performed better, offering a near quantitative recovery of the peptide at lower sample loads—significantly exceeding the recoveries obtained by the other polymers.

By increasing sample complexity, **P2** and $\mathbf{P_N2}$ were then subjected to two stringent tests. In the first, an artificial peptide mixture was prepared containing $C5aR_{10\text{-}18}S_2$, its desulfated parent peptide, and methylated peptides, resulting from hydrolytic treatment of C5aR in acidified methanol (2% TFA in MeOH; Supporting Information, Table S5). The mixture was loaded onto the **P2** or $\mathbf{P_N2}$ columns and SPE performed as described in Figure 3A. From the chromatograms shown in this Figure, we conclude that the sTyr peptide was selectively retained by **P2** with minimal retention of the non-sulfated peptides and that it could be cleanly eluted from the column (recovery approximately 40%). It is noteworthy that mass spectrometry, in contrast to the MIP sorbents, cannot be used to distinguish the sulfated peptide from its nonsulfated counterpart (Supporting Information, Table S5). In this context, the non-imprinted polymer failed to retain any of the peptides tested.

In a final test, we loaded an equimolar mixture of the monophosphorylated peptide pYY-ZAP70 (see Supporting Information for the sequence) and $C5aR_{10\text{-}18}S_2$ on **P2** and $\mathbf{P_N2}$ and performed SPE according to two different methods. In the first method, the SPE consisted of only a loading and an elution step (SPE1), whereas the second method had, in addition, an intermediate aqueous wash step (SPE2). Figure 3B shows the chromatograms of the elution fractions and Figure 3C the corresponding MALDI-TOF mass spectra. First we observed that the non-optimized load/elute SPE1 (red traces) resulted in a approximately 40% total peptide recovery with both peptides present in roughly equal amounts (see Supporting Information). This suggests that the peptides do not compete for the binding site under these conditions. In the load–wash–elute SPE2 (blue traces), on the other hand, the elution fraction contained pure sulfopeptide whereas the phosphorylated peptide had been removed in the wash step. The recoveries based on peak areas were approximately 20% for $C5aR_{10\text{-}18}S_2$ and less than 1% for pYY-ZAP70.[16]

Hence, MIPs combined with different SPE methods may be used to fractionate peptides according to the nature and/or number of phospho- or sulfotyrosines (Figure 4). We attribute this unique behavior to the presence of neutral binding sites in the MIP, presenting urea based hydrogen-bond donors in a complementary arrangement to the sulfo- or phosphotyrosine anion acceptors.

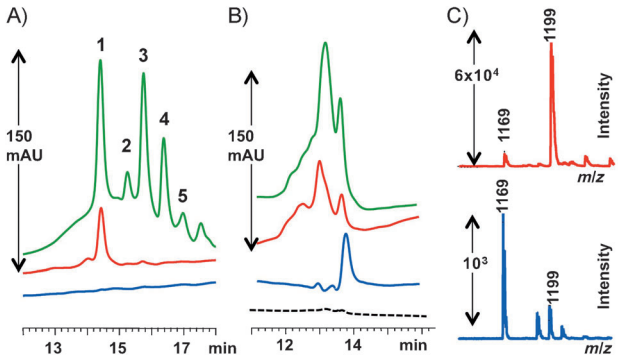

Figure 3. Reversed phase HPLC chromatogram of a mixture of A) $C5aR_{10\text{-}18}S_2$ and its hydrolysis and esterification products (green; see Table S5: 1 = Ac-DsYGHsYDDKD ($C5aR_{10\text{-}18}S_2$); 2 = Ac-DYGHYDDKD; 3 and 4 = Ac-DYGHYDDKD(Me); 5 = Ac-DYGHYDDKD$(Me)_2$) and the elution fraction after SPE on **P2** (red) and $\mathbf{P_N2}$ (blue). SPE conditions: loading: MeCN (0.1% TFA); washing: MeCN:water 95:5 (0.1% TFA); elution: MeOH (0.1% TFA). B) An equimolar mixture of $C5aR_{10\text{-}18}S_2$ and pYY-ZAP70 (GADDSpYYTAR; green) and the elution fraction after SPE on **P2** (SPE1 = red; SPE2 = blue) and $\mathbf{P_N2}$ (black dashes). SPE1 conditions: loading: MeCN; elution: MeOH (0.1% TFA). SPE2: loading: MeCN; washing: MeCN:water 90:10 (0.1% TFA); elution: MeOH (0.1% TFA). C) MALDI-TOF MS spectra of the **P2** elution fractions after SPE1 (red) and SPE2 (blue). The signal at m/z 1199 is assigned to pYY-ZAP70 and m/z 1169 is desulfated $C5aR_{10\text{-}18}S_2$.

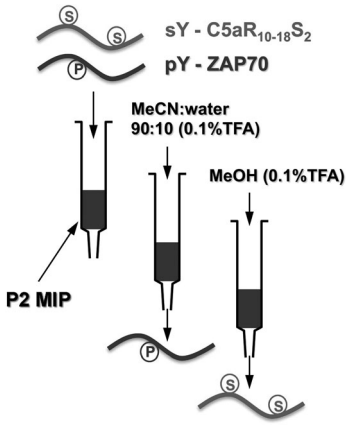

Figure 4. Principle of selective fractionation of sulfopeptides and phosphopeptides by stepwise elution from an MIP affinity column.

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[16] As indicated in Figure S5, higher overall recoveries can be expected at lower sample loads.

