temperature coefficients, Rietveld refinement: R_{wp}: 0.0849, R_{bragg}: 0.0435, GOF: 3.585. Neutron diffraction data: Space group $R\bar{3}m$, $a=2.92471(2)$, $b=24.0746(4)$ Å, $V=178.342(3)$ Å^3, $Z=3$; ROTAX/ISIS, Vanadium tube, time-of-flight (TOF) measurement, 3 detector banks; detector bank A: 1355 data points; detector bank B: 2129 data points; detector bank C: 2016 data points, 1006 observed reflections, 2 refined atomic parameters, 3 refined temperature coefficients, Rietveld refinement: $R_{wp}=0.0283$, $\chi^2=3.619$. Single-crystal X-ray refinement: Space group $R\bar{3}m$, $a=2.9193(4)$, $b=24.031(4)$ Å, $V=177.36(5)$ Å^3, $Z=3$; Bruker AXS Smart CCD 1000, $Mo_{K\alpha}$, measurement range: $2.54^\circ<\theta<34.49^\circ$, 131 reflections, refinement with SHELXL 97-2: $R_1=0.0132$, $R_{wp}=0.0126$, Further details on the crystal structure investigation may be obtained from the Fachinformationszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany (fax: (+49) 7247-808-666; e-mail: crysdata@fiz-karlsruhe.de), on quoting the depository number CSD-412279.

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A Mass Spectrometry Based Direct-Binding Assay for Screening Binding Partners of Proteins**

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Recent advances in genomics and proteomics have enabled the discovery of more drug targets than ever before. The majority of these drug targets are proteins. One of the most challenging areas of drug development is the search for novel receptor–ligand pairs and enzyme inhibitors,[1, 2] as many novel proteins have no known binding partners. During the drug discovery process, the binding partners of a receptor or protein must be found in order to determine possible ways to address its pathological pathway.

Ligand fishing is a widely used process in which proteins are screened against a multitude of compounds to search for the binding partners of the proteins. The most common methods for ligand fishing are those based on direct binding and one of the most versatile direct-binding assays involves the use of biosensors based on surface plasma resonance (SPR).[3–5] However, a major drawback of SPR is that once a sample is identified as having a possible binding partner, other conventional biochemical techniques, for example, HPLC and mass spectrometry,[6] need to be applied to identify the new ligand.

Herein we describe a novel on-probe direct-binding assay to screen the binding partners of a target protein. Contrary to the case in SPR, the captured binding partners in this direct-binding assay are directly analyzed on-probe by means of mass spectrometry. The target protein is first immobilized on a porous silicon probe. A sample that contains possible binding partners is then incubated with the probe, and the binding partners are captured by the immobilized protein. The captured binding partner is then identified by means of on-probe laser desorption/ionization time-of-flight (TOF) mass spectrometric analysis.

Unlike other mass spectrometry based assays in which biologically active probes are used for rapid analyses of a sample,[7–12] this new assay involves desorption/ionization on silicon (DIOS)[13–15] instead of matrix-assisted laser desorption/ionization (MALDI) in the ionization process. The use of DIOS is based on two considerations. First, the majority of the drug candidates are low-mass molecules. In MALDI, the matrix is essential to desorption/ionization, but the use of matrix also generates strong matrix-related background noise in the low-mass region, which obscures or even suppresses the analytical signals of other low-mass molecules. In contrast, a matrix is not required for DIOS and laser desorption/ionization is performed on porous silicon, therefore eliminating the matrix-related background problem. DIOS is very effective for analysis of low-mass molecules. Second, DIOS involves the use of porous silicon as a probe on which proteins can easily be immobilized. Furthermore, the porous silicon probe can be generated routinely through electrochemical anodization[15, 16] or chemical etching[17–19] of flat crystalline silicon. The porous silicon surface can be regenerated easily.

A silicon wafer was galvanostatically etched and oxidized. The preparation of a good porous silicon probe is essential for the success of this assay, as only well-etched silicon wafers produce good ion signals by laser desorption/ionization without the use of matrix. We have a porous silicon probe for the routine generation of good mass spectra of a variety of drugs, including atropine, propanolol, atenolol, alprenolol, metoprolol, warfarin, sulpride, naproxen, and ketoprofen. Figure 1 shows the mass spectrum[20] of naproxen obtained when using a) MALDI with 2,5-dihydroxybenzoic acid (DHB) as matrix and b) the DIOS technique. Clearly, a number of strong matrix-related peaks were present in the mass spectrum when a DHB matrix was used (Figure 1a). In contrast, the only major peak in the DIOS spectrum (Figure 1b) corresponds to naproxen and, importantly, no fragmentation of naproxen was observed in DIOS. We have also been able to produce good mass spectra of a variety of peptides of less than 500 Da by means of DIOS. Furthermore, we have successfully used this method to detect the products of the derivatization of cyclodextrin.

Because many drug molecules are good binding partners of bovine serum albumin (BSA), we tested this new direct binding assay with BSA. BSA was immobilized on the porous silicon probe with 2,4,6-trichloro-1,3,5-triazine as the activated agent. The experimental procedure for the immobilization

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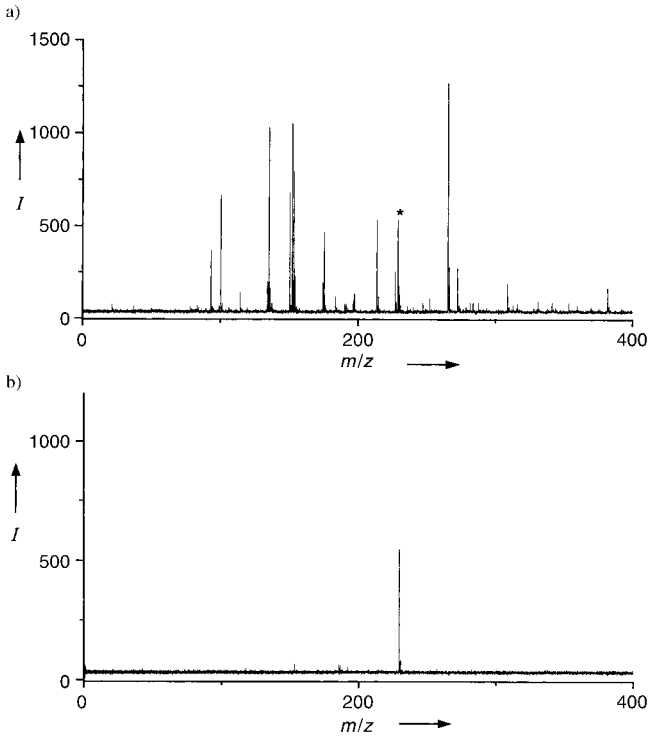

Figure 1. Mass spectra of naproxen ($M = 230.26$): a) MALDI with DHB as matrix (* = naproxen) and b) DIOS (sample deposited on porous silicon).

of BSA on porous silicon to obtain the biologically active BSA surface has been reported previously.[21] The experimental protocol for preparing the probe is outlined in Figure 2: generation of porous silicon, covalent linking of BSA, and washing the treated probe with suitable solvents such as ethanol (10%).

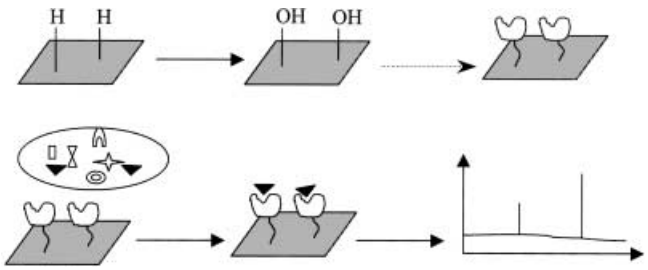

Figure 2. Experimental protocol for the mass spectrometry based direct-binding assay on protein-immobilized porous silicon wafers.

A solution (2 μL) of ketoprofen (6.0 mg mL^{-1}) and sulpride (5.0 mg mL^{-1}) was incubated with the immobilized BSA probe for about 2 min. Thereafter, the probe was washed with ethanol (20%, 20 μL) to remove the molecules that were not captured by the immobilized BSA. Finally, the probe was inserted into the mass spectrometer for analysis. Figure 3a shows a typical mass spectrum obtained by using this procedure. The only signal observed corresponds to ketoprofen and no signal for sulpride was present (Figure 3a), which clearly shows that ketoprofen was captured by the immobilized BSA, whereas sulpride was not. This result, in turn, reveals that ketoprofen is a good binding partner of BSA, whereas sulpride is not.

For comparison, the probe without the immobilized BSA was also investigated. The same sample used to measure the mass spectrum in Figure 3a was deposited on the probe, but the probe was not washed with ethanol. The signals of both ketoprofen and sulpride were observed (Figure 3b). However, when the probe without the immobilized BSA was washed with ethanol (20%, 20 μL), the signals from both ketoprofen and sulpride were no longer observable in the mass spectrum (Figure 3c). This comparison experiment further confirms the fact that the immobilized probe captures only the binding partner of the target protein.

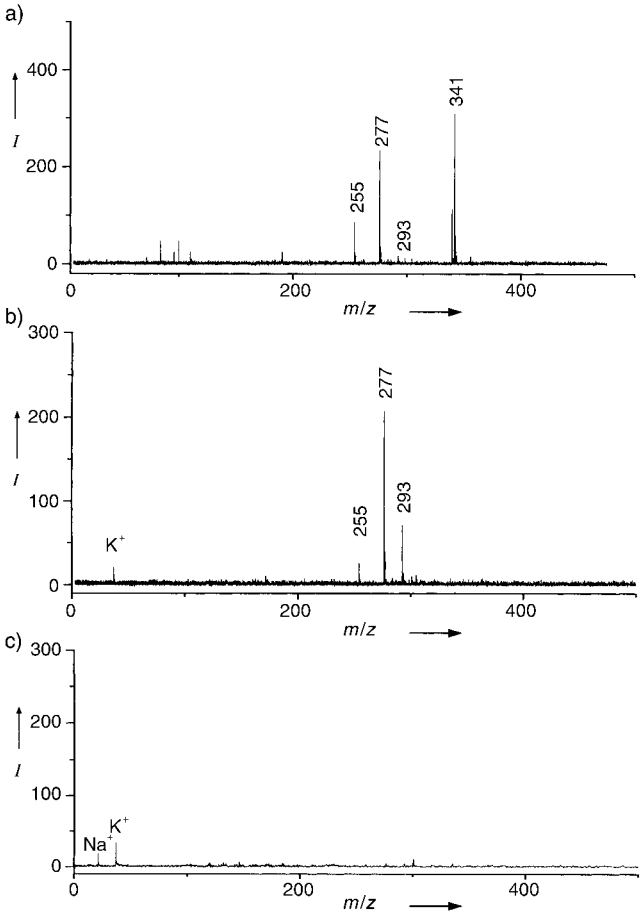

Figure 3. Mass spectra of ketoprofen and sulpride on a BSA-immobilized affinity probe: a) ketoprofen (the peaks at $m/z = 255$, 277, 293 indicate $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, respectively) and sulpride ($M = 341$) deposited on freshly etched porous silicon, b) ketoprofen and sulpride deposited on the probe (not immobilized with BSA), and c) ketoprofen and sulpride deposited on freshly etched porous silicon, and washed with ethanol (20%, 20 μL).

This new assay can also be used to probe the relative binding affinity of different ligands to a target protein. For example, we applied this assay to analyses of mixtures of propanolol (8.9 mg mL^{-1}) and atenolol (4.6 mg mL^{-1}). When the sample was incubated with the BSA-immobilized probe, followed by washing with ethanol (10%, 10 μL), a strong signal for each of the molecules was observed in the mass spectrum (Figure 4a). This suggests that both propanol and

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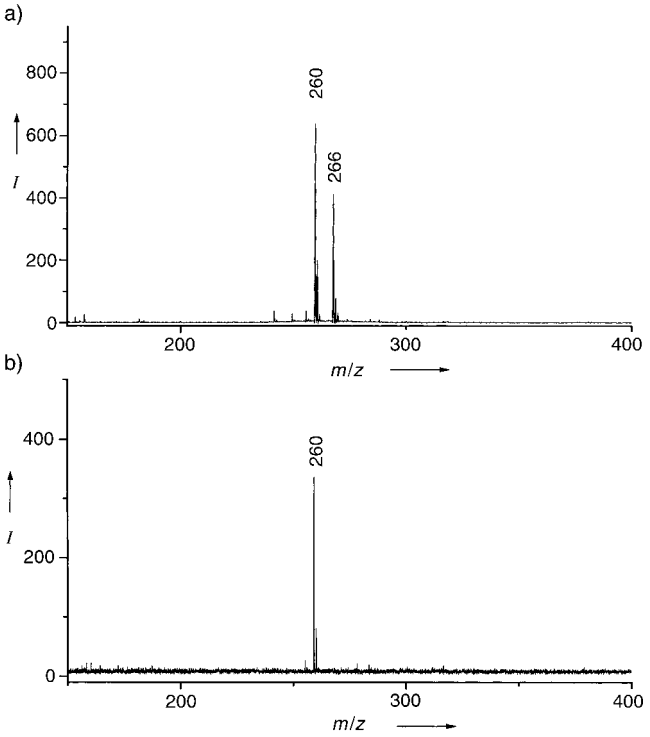

Figure 4. Mass spectra of propranolol and atenolol deposited on the BSA-immobilized affinity probe and washed with a) ethanol (10%, 10 μL), and b) ethanol (10%, 30 μL).

atenolol were captured by BSA under these conditions. However, when the probe was washed with 30 μL rather than 10 μL of ethanol (10%), the peak of atenolol ($M = 267$) disappeared from the spectra, whereas the peak of propanolol was still present (Figure 4b). This may reflect the fact that propanolol has a stronger affinity for BSA than does atenolol. To validate this argument, we studied the binding affinity of these two drugs for BSA by using a chromatographic column packed with BSA-immobilized silica. We found that propanolol was retained more strongly than atenolol in the column, thus confirming that propanolol has a stronger affinity for BSA than does atenolol.

In summary, we have presented a novel direct binding assay for fishing the binding partner of a target protein. In this assay, the porous silicon probe not only captures the binding partner of the immobilized protein, but also serves as the substrate for direct laser desorption/ionization, thereby eliminating the matrix-related background problems. As a result, the captured molecules can be directly identified on probe by means of mass spectrometry. This assay does not require fluorescence tags, radioactive markers, or even prior purification. The major application of this new assay is in the drug discovery process. The strength of this assay may be particularly useful in high-throughput screening of lead-drug candidates. For example, this assay can be used to screen large compound libraries. It can also be used to fish the active ingredients present in natural products such as Chinese medicines.

The directed binding assay presented herein may not be limited to the study of protein targets and may be modified to study the binding partners of other biomolecules, including DNA, RNA, antibodies, lipids, peptides, and whole cells. This assay can be upgraded to a chip-based assay in which a number of proteins or other biomolecules are immobilized on the same probe to search for lead drug candidates for multiple targets.

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