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Mass Spectrometry after Capture and Small-Volume Elution of Analyte from a Surface Plasmon Resonance Biosensor

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The identification of binding partners of proteins by mass spectrometry following specific capture on a biosensor surface is a promising tool for proteomics research and the identification and characterization of protein−protein interactions. Previous approaches include the direct ionization of analyte from the biosensor chip on a matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOFMS) apparatus and the on-chip digestion followed by elution, chromatographic concentration of the fragments, and electrospray mass spectrometry. In the present paper, using the small-volume microfluidic sample manipulation technique with oscillatory flow reported recently (Abrantes et al. *Anal. Chem.* 2001, 73, 2828−2835), analyte is shown to be eluted from the sensor surface into a small volume of buffer that promotes dissociation from the capture surface and delivery to the mass spectrometer. Both the incubation of the sensor surface with the sample and the recovery of analyte can be achieved with a few microliters and conducted until steady-state is attained. Because the procedure is nondestructive for the sensor surface, multiple cycles of capture and elution allow the transfer and concentration of analyte into the elution buffer. The eluted analyte can be studied directly by MALDI-TOFMS, or subjected to proteolytic digestion for protein identification. Transfer into the elution buffer and MALDI-TOFMS detection was achieved from 5 μL of starting samples containing <50 fmol of analyte. Examples are presented for the specific detection and recovery of a protein from a complex mixture of cytosolic proteins.

Advances in proteomics have made available the sequences of a large number of proteins, and the development of high-throughput structural determination methods is underway.[1] For an understanding of the structure and function of expressed proteins and their relationship within regulatory pathways, the identification and characterization of protein−protein interactions will be a crucial step. Likewise, pharmaceutical drug discovery has spawned significant interest in identifying protein interactions. For the biophysical characterization of protein binding partners in vitro, mass spectrometry is a superb tool. The unambiguous identification and sequence determination of proteins from only femtomoles of molecules in a mixture can be obtained. Typically, either MALDI-TOFMS[2,3] or HPLC−MS/MS using electrospray ionization[4−6] is applied to PAGE-separated proteins that have been eluted from affinity chromatographic columns or after immunoprecipitation.

New possibilities for the study of protein interactions have emerged with the introduction of optical biosensors. In this technique, a series of association and dissociation experiments between an immobilized species and analyte in solution are performed, and from the observed surface-binding kinetics and steady-state, affinity and kinetic constants can be determined.[7] The combination of MS with the biosensor as an affinity−chromatographic matrix (termed BIA-MS) is very attractive, because complementary information can be obtained in both methods, such as the specificity of the interaction and the identity of the binding partners from MS as well as the information about affinity and kinetics from the biosensor[8−16] In addition, the use of biosensors as an affinity purification step can be superior to immunoprecipitation or conventional affinity chromatography with

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respect to the real-time information on the extent and quality of the capture process.[13]

In principle, commercial biosensors and MS are compatible concerning the amount of material studied; usually in the order of 100 fmol of analyte is captured in biosensor experiments. Several groups have described that a sufficient amount of analyte can be recovered from a biosensor surface for MS, mostly using a surface plasmon resonance (SPR) biosensor surface from Biacore. However, a key problem in interfacing MS with biosensors is the strategy for analyte recovery, because with the commercial optical biosensors (both with the microfluidics-based surface plasmon resonance biosensor from Biacore and the cuvette-based resonant mirror system from Affinity Sensors), the analyte is rinsed off the surface in a relatively large volume of buffer, yielding sample concentrations too dilute for optimal application of mass spectrometry.

To address this problem, the deposition of the MALDI matrix directly onto the surface of a Biacore sensor chip after analyte capture was described by Krone et al.[8] (for a review, see ref 16). This detection is very sensitive and effective but has the drawback that the sensor chip has to be manually removed from the biosensor instrument. A second approach was described by Sonksen and colleagues in which analyte was eluted from the sensor surface in a small volume; formed by an arrangement of different buffers separated by air bubbles, which is aspirated into a pipet tip; injected manually into the SPR instrument, Biacore X; and recovered in a second pipet tip from the outlet of the biosensor.[12] Both of these procedures have the drawback that they are not readily amenable to automation. More recently, electrospray tandem mass spectrometry (ESI-MS/MS) was applied to analyte eluted after on-chip digestion and recovery in a reversed-phase microcapillary column.[9] An important advantage of this method is amenability to high-throughput screening by use of the HPLC−MS/MS interface. But as with the direct deposition of the MALDI matrix, there is a potential limitation in that the biosensor surface may not easily be reused after the MS experiment for the study of other analytes. In addition, as pointed out recently by Natsume et al., full automation of BIA-MS with high throughput is currently limited by the absence of integrated microfluidics control.[14]

Recently, a new method for the computer-controlled manipulation of microliter-sized volumes within the microfluidics of the Biacore surface plasmon resonance biosensor was described.[17] Using a computer-controlled syringe pump with stepping motor, small solution plugs separated by air bubbles can be precisely delivered to the sensor surface in a serial fashion. This application of an oscillatory flow pattern leads to an efficient mixing of the sample plugs and provides for high mass transfer of solutes to the sensor surface. Use of an oscillatory flow, in contrast to unidirectional flow, significantly reduces the sample volume requirements and at the same time extends the contact time of solution plugs with the sensor surface, leading to more efficient binding. Extended observation time for the biosensor experiments can lead to better detection limits and improved thermodynamic and kinetic characterization of the protein interaction.[17]

In the present paper, the same principle is used for recovering the analyte into a small volume of elution buffer. This is suitable for MS, and there are two potential advantages: First, this procedure relies on computer-controlled microfluidics manipulation and can be automated. Second, this is a nondestructive procedure and several cycles of loading and elution can be performed with the same sample and elution buffer plugs such that the limiting factor for the number of eluted analyte molecules can be the depletion of the analyte from the original sample instead of the binding capacity of the surface. To demonstrate the principle of interfacing MS to the biosensor with the external microfluidics control, in the following the specific capture, recovery and MS analysis of a model protein from a solution, with an excess of an unrelated protein, or in a complex mixture of cytosolic proteins is described.

EXPERIMENTAL SECTION

Immobilization and Preliminary Binding Experiments. A surface plasmon resonance biosensor Biacore X (Biacore, Piscataway, NJ) was used in the conventional injection mode for the immobilization and characterization of the interaction. A monoclonal antibody to lysozyme was covalently attached to a carboxymethylated dextran chip CM5 (Biacore) by standard amine coupling.[18] In short, using a flow rate of 5 μL/min, a 35-μL injection of a mixture of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysuccinimide was employed to activate the surface, followed by injection of the antibody diluted into 10 mM sodium acetate buffer pH 4.5 at a concentration of 50 μg/mL. A total of 17 ng of protein was immobilized to both flow channels, and the surface was deactivated by 35 μL of 1 M ethanolamine, pH 9.0. Binding experiments were performed at 25 °C using the standard running buffer (HBS/EP) consisting of 0.01 M HEPES, pH 7.4; 0.15 M NaCl; 3 mM EDTA; and 0.005% polysorbate 20 (v/v). After a 5-μL injection of analyte (lysozyme) at 7.0 μM, a signal of $\sim$ 3000 RU, or 3.0 ng/mm^2, was observed. For the elution of the analyte, 15 μL of 10 mM glycine−HCl, pH 2.0, was injected over both flow channels. This regeneration was found not to cause irreversible changes in the immobilized antibody, and typically, at least 20−30 elutions were applied to the chip during the course of several days without loss of binding capacity.

The standard amine coupling immobilization technique was also used to covalently attach a polyclonal antibody of horse myoglobin to a carboxymethylated dextran chip CM5 (Biacore). A total of 36 ng of protein was immobilized to both flow channels. After a 10-μL injection of the analyte (horse myoglobin) at 0.59 μM, a signal of $\sim$1421 RU, or 1.4 ng/mm^2, was observed. For the elution of the analyte, 5 μL of 10 mM glycine-HCl, pH 2.0, was injected over both flow channels.

Microfluidics Control for the BIA-MS Experiments. The Biacore X instrument was operated in the external injection mode where the biosensor functions only as a passive sensor. The microfluidics was extended as described previously.[17] In brief, the inlet of the microfluidics was connected to a syringe pump equipped with a three-way valve (Gilson 402, Gilson Inc., Middleton, WI), which was externally computer-controlled using custom

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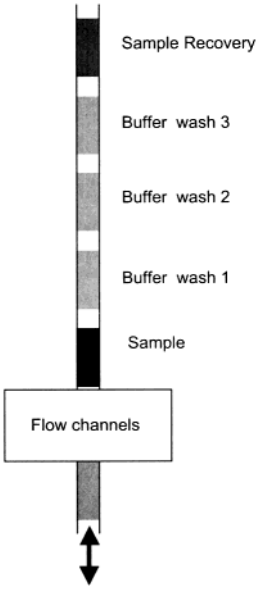

Figure 1. Schematic representation of the microfluidics injection method. A series of 5-μL volumes of sample or buffer were aspirated at a flow rate of 20 μL/min into the microfluidics by using an external syringe pump. For separation, 2-μL air bubbles were used. The configuration shown was then transported to a position symmetrical above both sensor surfaces, and an oscillatory flow pattern was applied using an amplitude of 1 μL at a flow rate of 20 μL/min.

written software. On the basis of the elementary commands recognized by the Gilson 402 pump, a set of medium-level functions was implemented in an object-oriented C++ program for the operation of the valve and the syringe. These were assembled in higher-level functions that automate the sample handling process described below.

The outlet of the connector block of the microfluidics was modified to accept a zero dead-volume HPLC union, and Teflon tubing was connected as a new inlet. Aspiration of buffer or air was accomplished by lowering the plunger of the syringe pump, and in this fashion, a sequence of air and buffer plugs can be generated in the Teflon tubing. The relative position of each of the flow channels can be precisely measured by observing an air/buffer interface (in the present case, this was determined to be 41.3 μL), and the sample (or buffer) plugs can be transported through the microfluidic cartridge to the sensor surface with submicroliter precision. Previously, transient air bubbles have been shown not to significantly disturb the biosensor signal. After the sample/buffer was centered across the sensor surfaces, the solution could be efficiently mixed, and a high mass transfer rate to the sensor surface could be achieved by an application of an oscillatory flow pattern. With sample/buffer plugs on the order of a few microliters, as compared to the volume of the flow channel with the sensor surface, <100 nL, most of the sample is in the microfluidic channels surrounding the sensor surfaces, and the sensor surface is covered with the sample/buffer during the oscillatory flow at all times. Because the oscillatory flow produces no net movement, each sample/buffer plug can be in contact with the sensor surface for a virtually unlimited time. For details, see ref 17. Several plugs of sample and buffer were generated in series (see Figure 1), and after incubation of the sensor surface using one plug, the arrangement can be translated to the next plug in either direction.

This microfluidics configuration was used in the following way (unless noted otherwise): First, the entire system was rinsed with HBS/EP buffer, then a sequence of 5-μL volumes, each of analyte sample, HBS/EP buffer, a high-salt buffer (10 mM sodium acetate, pH 5.5, 1 M NaCl), HBS/EP buffer, and elution buffer (10 mM glycine, pH 2.0) was aspirated into the Teflon tubing, each of which separated from the next by 2 μL of air (Figure 1). A flow rate of 20 μL/min was used. The sample plug was transported to a position where the plug was centered over both sensor surfaces, and an oscillatory flow pattern at an amplitude of 1 μL at a flow rate of 20 μL/min. After the surface binding signal observed from the Biacore X had reached steady-state, the oscillatory flow was stopped, and the surface was rinsed with HBS/EP buffer by further aspiration of 7 μL, followed by a wash with high-salt/low pH buffer, a second HBS/EP buffer, and finally, the elution in the glycine buffer, pH 2.0. The final sample was sandwiched between air bubbles and delivered through HPLC tubing.

Mass Spectrometry. Mass spectrometry analysis was performed using a PerSeptive BioSystems Voyager Elite DE-STR (PE Biosystems, Framingham, MA) MALDI-TOF instrument. The instrument was operated in linear mode at a 25 kV accelerating voltage and a 350-ns ion extraction delay. Recovery sample volumes recovered from the Biacore X were manually applied to the plate as follows: a 1.0-μL aliquot of matrix, a saturated solution of sinapinic acid (Sigma, St. Louis, MO) in CH_3CN/0.1% trifluoroacetic acid (Fluka, Milwaukee, WI), was applied to the plate and allowed to dry. Five 1-μL aliquots of recovered glycine solutions were applied and allowed to dry. Finally, an additional 1 μL of saturated sinapinic acid matrix was applied over the sample. MALDI-TOFMS negative control experiments of 5 μL with 70 nM lysozyme in the presence of a cytosolic kidney proteins mixture were prepared in water instead of HBS/EP buffer to eliminate potentially interfering salts and detergents. Cytosolic proteins were collected from the supernatant of lysed canine kidney cell cultures.

In the proteolytic digestion experiments of the recovery solutions, proteins were subjected to digestion using trypsin (Promega, Madison, WI), as previously described.[19] The extracted peptides were spotted using a 10-fold dilution of saturated solutions of α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in CH_3CN/0.1% trifluoroacetic acid using the PerSeptive BioSystems Voyager Elite DE-STR.

RESULTS AND DISCUSSION

Our first goal was to specifically capture the analyte on the functionalized sensor surface; to elute the analyte into a 5-μL volume of 10 mM glycine, pH 2.0; to recover the solution; and to identify the analyte by MALDI-TOFMS. As an analyte, 0.070 μM lysozyme was used in the presence of an 85-fold molar excess of an unrelated protein (horse myoglobin) dissolved in 5 μL HBS/EP. This buffer contains detergent and a high concentration of salt, as commonly used for biosensor experiments. In a negative control experiment with a nonfunctionalized sensor surface, it was found that a simple sequential application of sample plug followed by the elution buffer plug and separated by a small volume of air did lead to a small MS signal from the analyte. Interestingly, there

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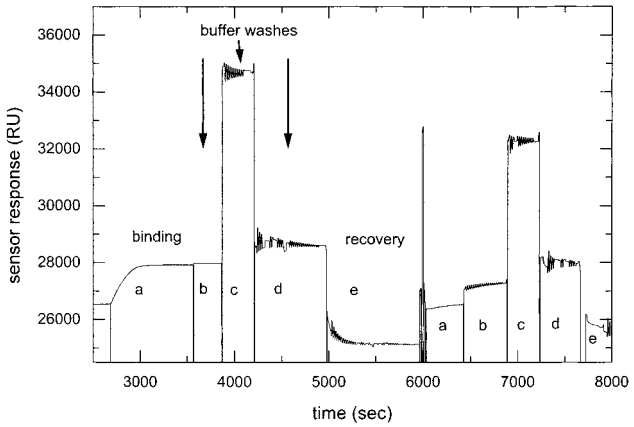

Figure 2. Sensorgram of a capture and recovery experiment using the microfluidic configuration shown in Figure 1. Large dips in the response signal correspond to air passing over flow channels. A 5-μL portion of sample (70 nM of lysozyme analyte in the presence of 5.9 μM horse myoglobin dissolved in HBS/EP buffer) was incubated until steady-state binding was achieved (a). This was followed by a buffer wash with HBS/EP (b), a wash with 10 mM sodium acetate, pH 5.5, and 1M NaCl (c), and a second HBS/EP wash (d). Recovery of the captured analyte was observed in area e. This was followed by a second cycle a to e of capture and recovery using the same solutions.

was no bulk mixing of the sample and the elution buffer plug, as judged by the absence of detergent or detrimental amounts of salt in the elution buffer and the absence of a signal from the horse myoglobin in 85-fold excess. (If the detergent would be transferred into the elution sample, Natsume and co-workers[9] have shown that substitution of polysorbate 20 by *n*-octyl glycopyranoside can prevent its detrimental effect on the MS signal.) Further, a lysozyme MS signal was detected in a mock experiment when aspirating the sample into the microfluidics only, without exposing the sensor surface to the samples. This shows that nonspecific adsorption of the lysozyme analyte to the sensor surface and to the tubing or microfluidics took place. In contrast, no nonspecific adsorption of the myoglobin was observed. This suggests that depending on the nature of the analyte, washing steps may be required to remove nonspecifically bound material from the microfluidics. Therefore, for the experiments using lysozyme as the analyte after incubation of the sensor surface with the sample plug, a washing sequence was introduced, consisting of a brief HBS/EP wash, a low-pH and high-salt wash (10 mM sodium acetate, pH 5.5, 1 M NaCl), and a second HBS/EP wash before exposing the sensor surface to the elution buffer. This could be conveniently achieved by aspirating the corresponding sequence of solutions into the microfluidics and by computer-controlled microfluidics manipulation (See Experimental Section and Figure 1). In control experiments using the conventional biosensor injection method, it was verified that these washes have no effect on the immobilized sites or the formed complex. In this configuration, MS could not detect proteins in the elution buffer when using a nonfunctionalized sensor surface.

Figure 2 shows the signal obtained in an experiment with the sensor surface functionalized with capture antibody. The sample consisted of a mixture of 5.9 μM horse myoglobin and 0.070 μM lysozyme. In the first phase (a), the typical binding kinetics during the adsorption of analyte to the sensor surface is observed. The application of the oscillatory flow to the 5-μL sample plug allows the surface binding to extend until steady-state is achieved. This is followed by a brief HBS/EP wash (b), during which no significant dissociation occurs as a result of a low intrinsic off-rate constant for this interaction, and by rebinding enhanced due to the high immobilization density.[20] Next, the incubation with the high-salt buffer (c) results in a higher signal, mainly because of the refractive index contribution of the salt. A second HBS/EP wash (d) is followed by the elution buffer (e), during which the dissociation of the analyte can be observed. Part of the analyte is released very rapidly, and dissociation cannot be distinguished from the change in bulk refractive index, but it was found that a significant amount of analyte elutes more slowly, possibly originating from the portions of the immobilization matrix closer to the sensor surface. The elution can also be conducted until a steady-state signal is obtained.

Because most biosensor experiments for the characterization of kinetic or affinity constants of molecular interactions are conducted in a series of cycles with binding and dissociation followed by surface regeneration,[7] reversible regeneration conditions are typically known as part of the biosensor experimental protocol.[18] Conditions can be found making use of the real-time binding signal. Frequently, this consists of the application of glycine or other low-pH buffers that cause the release of the analyte but do not destroy the immobilized sites. Commonly, several dozen cycles of binding and regeneration can be conducted without loss of binding capacity, frequently with a stability of the immobilized site of several days or more. This was also true in the present case in which a single chip was reused for several experiments during the course of a few days. Typical regeneration buffers are 10 mM glycine or HCl, pH 2.0, which are compatible with MALDI-TOF. To study the possibility of accommodating different elution buffers, 0.1% trifluoroacetic acid (pH 2.0) was tested. Like 10 mM glycine, this resulted in good recovery of analyte without compromising the activity of the immobilized antibody (data not shown). However, in the experiments shown, 10 mM glycine, pH 2.0, was used as elution buffer.

The knowledge of reversible regeneration conditions allows a natural extension of the BIA-MS experiment also to take advantage of multiple binding/elution cycles. Therefore, a second cycle of binding, washing, and elution is appended in order to demonstrate the principle of collecting more analyte for MS detection (although this would not be required in the present case for a good MS signal—see Figure 2). It is noteworthy that the second binding phase (a) shows a considerably slower kinetics, most likely due to depletion of analyte from our original 5-μL sample plug. With the conversion constant of ~1 pg of surface-bound material/1 RU binding signal,[21] the amount of analyte bound in both cycles would be 224 fmol, which is comparable to the total of 0.35 pmol contained in the 5-μL sample. As described previously,[17] the conversion constant has some uncertainty, and 1 RU may correspond to more than 1 pg of material (in particular, considering the inhomogeneous sensitivity and the swelling of the dextran matrix at high loading concentrations[20,22]). More precise information can be obtained from the kinetic traces observed in real time

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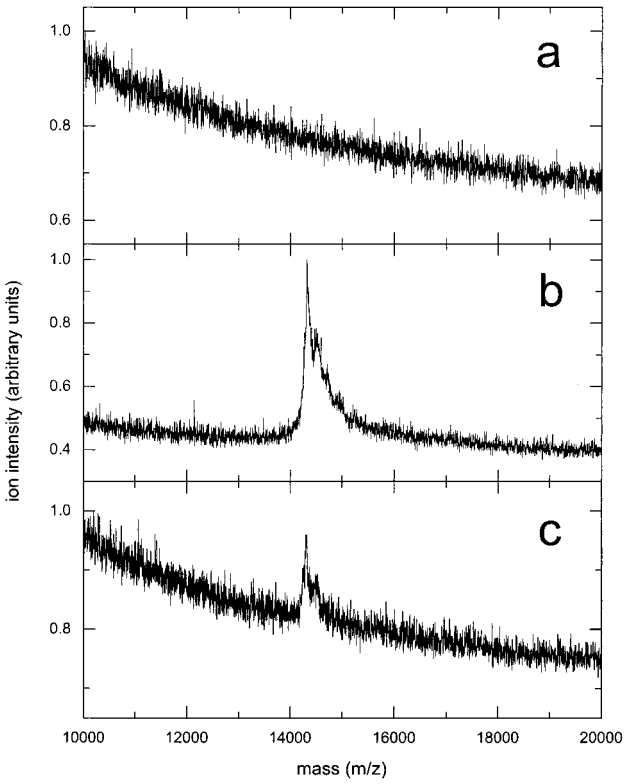

Figure 3. Mass spectra of the solution recovered from an experiment, as shown in Figure 2. Lysozyme was used as the analyte (350 fmol) in an 85-fold excess of myoglobin injected over a sensor surface that was (a) nonfunctionalized, (b) functionalized with anti-lysozyme antibody, and (c) 35 fmol of lysozyme analyte in the starting sample (5 μL) with an 850-fold excess of unrelated protein (horse myoglobin) injected over a functionalized sensor surface.

during binding. Since the binding of the lysozyme is mass-transport-limited, the initial binding rate is proportional to the analyte concentration. With the rates of the second sample application only 10.5% of the first, it can be concluded that ~90% of the analyte molecules in the original sample were captured to the surface in the first cycle. Obviously, without changes in the method, a larger sample volume could be applied, if required.

Figure 3 shows the MALDI-TOF mass spectrum obtained after application of the recovered elution buffer to the MALDI plate (Figure 3b) and the corresponding results from the control experiment with the nonfunctionalized surface (Figure 3a). A clear peak at the m/z for a single charged lysozyme (14 300) was observed, with no signal visible at m/z 16 900 for myoglobin that was in an 85-fold excess in the solution.

To explore the sensitivity of this approach, the analyte concentration in our 5-μL sample was lowered by a factor of 10 (7.0 nM, 35 fmol) while maintaining the myoglobin concentration at 5.9 μM, thereby having the myoglobin in an 850-fold excess. The biosensor capture was performed under identical conditions, which now led to a total of 550 RU bound, suggesting within the uncertainty of the conversion constant that virtually all analyte was bound (data not shown). (This was due to both the high affinity of the antibody that was used and the long contact time enabled by the oscillatory flow technique.) As shown in Figure 3c, the resulting mass spectrum shows a small but clearly visible peak at the correct value of m/z. Again, no myoglobin was

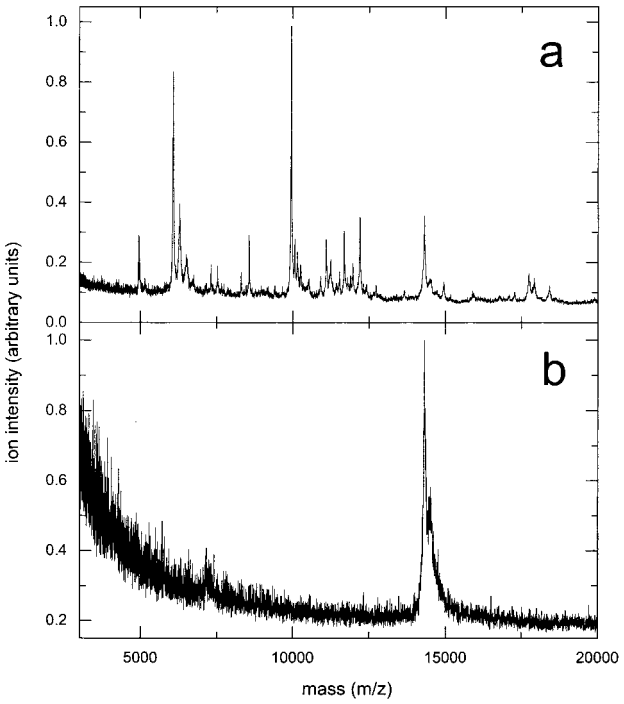

Figure 4. Mass spectra of a 5-μL sample with 70 nM lysozyme in the presence of cytosol protein mixture: (a) sample prepared with water and spotted directly on the MALDI plate and (b) sample prepared in HBS-EP and recovered after biosensor capture.

observed. This demonstrates the great potential of this approach with regard to sensitivity and specificity.

Next, we examined in more detail the specificity of the method by placing a 0.070 μM lysozyme analyte into a mixture of cytosolic proteins from kidney cells. Figure 4 shows the MS spectra obtained both for the original protein mixture and the single peak at m/z 14 300 obtained after biosensor capture and elution, as described above. This confirms that the stringent wash conditions were effective in preventing nonspecific binding to the flow channels or microfluidics to contaminate the recovered sample.

The problem of binding specificity was studied further by using another experimental model system that is less favorable to affinity purification. This was achieved by using a polyclonal antibody to horse myoglobin as capture antibody, which showed faster dissociation of the antigen. When 5 μL of 60 nM horse myoglobin was injected using the computer-controlled microfluidics method described above but without buffer washes between sample and recovery solution, the recovered protein could be correctly identified by MALDI-TOFMS (data not shown). As can be expected, under this condition, no myoglobin was detected in control experiments with a nonfunctionalized sensor surface (see above). However, addition of 115-fold molar excess of lysozyme as unrelated protein in our sample required additional buffer washes because of the high degree of nonspecific binding of this highly charged protein. Because of the more rapid dissociation of horse myoglobin from the immobilized polyclonal antibody, analyte was not detected when these washing steps were added. This demonstrates that the familiar need for a balance between the stringency of washes to remove nonspecific binding and the character and affinity of the specific interaction under study, which is well-known from affinity purification, also applies to the biosensor-based analyte isolation.

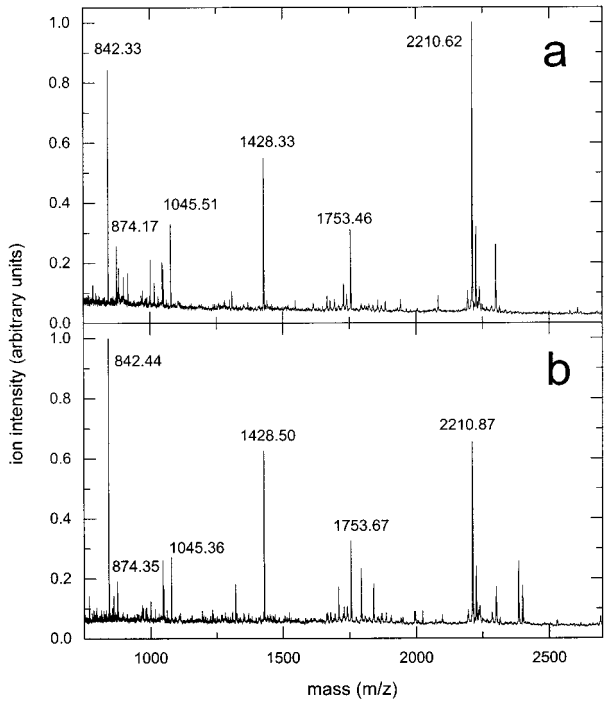

Figure 5. Mass spectra of tryptic digest of lysozyme (0.35 pmol in 5 μL) recovered after biosensor capture (a) and the same experiment in the presence of an 85-fold excess of myoglobin (b).

An important approach in protein identification is the isolation and purification followed by proteolytic digestion of protein and identification by mass spectrometry. This approach was applied using the Biacore X and our microfluidics configuration. Lysozyme (0.070 μM) in the presence of an 85-fold excess of horse myoglobin was captured (2200 RU, corresponding to ~200 fmol), and the recovery solution was subjected to enzymatic digestion. From previous analysis of the tryptic digest at picomole quantities, and from the theoretical MS-digest fit of the amino acid sequence of lysozyme from Protein Prospector at NIH, there are four principal peptide ions that should be present (606.37, 1045.54, 1428.65, and 1753.84 Da). In our mass spectrum (Figure 5), three of these characteristic ions (1045.36, 1428.50, and 1753.67 Da) were observed. We attribute the remaining ions to autodigestion of trypsin (842 and 2211 Da) and protonation of peptides from lysozyme variants. No additional peptide ions were observed from the unrelated protein that was in 85-fold excess in our original sample.

CONCLUSIONS

The present paper demonstrates the principle of a new method for the interfacing of optical biosensing with mass spectrometry (BIA-MS). By adopting an improved technique for the microfluidic sample manipulation with oscillatory flow, which was originally introduced to improve detection efficiency of the biosensor and conditions for the kinetic and thermodynamic characterization of protein interactions,[17] the coupling of BIA and MS was achieved with several useful features: a small (fully recoverable) sample volume is required, all capture and elution processes can be conducted to steady-state by virtue of an oscillatory flow principle, and multiple cycles of analyte capture from the sample can be used to collect analyte in a small volume of elution buffer, which is suitable for MS. The identification of the captured analyte by digestion was demonstrated. Importantly, the method is nondestructive for the biosensor surface and has the potential to be fully automated. Overall, a very efficient transfer of analyte from the sample to the MS is accomplished, and the detection of <50 fmol of analyte starting material was achieved.

We anticipate that the most critical point for the application in practice will be related to the specificity and efficiency of the biosensor capture. Both can be very high, as judged by the absence of signal from unrelated cytosolic proteins. Although the binding capacity is theoretically not the limiting factor with the present method capable of multiple surface loading, the success of the method will nevertheless be dependent on the affinity and dissociation kinetics of the captured molecule. In that regard, it is useful to consider that the hydrogel of the biosensor is equivalent to an affinity chromatographic matrix.[23] It is obvious, therefore, that problems similar to those well-known for other affinity purification techniques will occur. One important question is the stringency of washes that may be required for the removal of nonspecifically bound protein. Obviously, this will depend strongly on the nature of the specific interaction (its affinity, dissociation kinetics, and stability in different chemical environments), as well as on the nature of the nonspecific interactions. The two examples in the present paper highlight two different cases with high and medium affinity and with background proteins that exhibit different degrees of nonspecific binding, and the examples show how to apply washes if necessary. It is apparent that if the affinity of the capture molecule is not very high and if the dissociation rate constant is fast, shorter washing steps may be required, and nonspecific binding will become more difficult to remove. This will likely require experimental optimization for any particular capture/analyte system under study. Further, in many cases, nonspecific binding of known unrelated molecules may be tolerable.[15] However, these limitations are not deficiencies of the method presented in this paper, but rather are fundamental problems in affinity purification.

Despite these similarities in the affinity purification methods (and beyond differences in the surface chemistry) there is an implication of the difference in physical dimensions between affinity chromatography and biosensor affinity isolation for elution of specifically and nonspecifically bound material. The difference in the size of the matrix and the lower total binding capacity result in a significantly reduced retention, which should generally require less-stringent conditions for elution (from both nonspecific and the specific sites). In typical biosensor experiments, virtually fully reversible regeneration conditions are known from prior determination of the kinetic and affinity constant of the interacting molecules, and several of the buffers common in biosensing are compatible with MALDI-TOF (such as the 10 mM glycine, pH 2.0, used in the presented examples). However, alternatively, the ability to use trifluoroacetic acid as eluent may simplify further preparative steps of MS.

It is beyond the scope of the present paper to address fundamental issues common to any immunoprecipitation- or affinity-chromatography-based separation method (discussions in

(23) Winzor, D. J. *J. Biochem. Biophys. Methods* **2001**, *49*, 99−121.

the context of biosenors can be found in ref 24). It is a strength of combining MS with biosensors that these possible problems can be identified and optimized by real-time monitoring of the surface binding. Other research groups have demonstrated previously that these problems can be successfully addressed and that the use of the SPR biosensor surface for affinity purification prior to MS is a very powerful tool.[12,14,15] The present paper describes a new technique for the sample handling using a modified microfluidics control, which significantly simplifies the interface between the biosensor and MS. This should prove to be a very effective technique for isolating, identifying, and characterizing protein−protein interactions.

(24) *J. Biochem. Biophys. Methods* **2001**, *49*, 1−3.

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