Optimizing SPR-MS for isolation and characterization of low level impurities in complex, protein pharmaceutical samples

- Label-free interaction analysis satisfies characterization and quantification requirements with:
  - low sample demands
  - acute specificity and sensitivity
  - well-defined assay conditions and buffer compositions
- MS provides additional characterization information including identification of impurities

Background

The development of recombinant protein pharmaceuticals and advancements in analytical techniques and methodology has created significant interest in the isolation and characterization of the residual impurities found in drug candidates. Impurities can be either process-related (e.g. host cell proteins, DNA, media components) or product-related (e.g. protein aggregate), and they are generated as a result of the manufacturing process and product storage. Due to safety concerns, understanding the impurity profile of a biotherapeutic drug is a regulatory requirement. Therefore, impurities are characterized and continually monitored during drug development. Problematic impurities are eliminated or reduced to safe levels, and any trace amounts remaining are regularly monitored.

Many antibodies and IgG fusion protein therapeutics are purified over Protein A affinity columns. Protein A species are known to leach off the column during routine purification. Despite purification steps, residual Protein A may be present in a drug substance at picogram-to-nanogram per milligram of antibody. Protein A has the potential to cause adverse clinical effects, including sequestering and cross-linking of antibodies, and activation of the classical complement cascade, leading to pathologic immune reactions. To our knowledge, these adverse effects from trace levels of Protein A in protein pharmaceuticals have not been observed in human clinical studies.

Introduction

There are several examples in the literature of how the combination of Biacore™ systems based on surface plasmon resonance (SPR) technology and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been used to isolate and identify material from complex mixtures (Nedelkov and Nelson, 2001; Zhukov *et al.,* 2004). SPR-MS was applied in the example described here to detect and characterize the trace quantities of Protein A in preparations of purified monoclonal antibodies[1] intended for therapeutic use.

[1] Work carried out by Kristin Murray and colleagues at Wyeth Pharmaceuticals, Andover, MA, USA.

Acknowledgement

We are grateful to Kristin S. Murray, Jonathan M. Brooks, Dr. Keith A. Johnson, and Dr. Jason C. Rouse at Wyeth Pharamaceuticals, Andover, MA, USA for allowing us to use their data.

In a typical SPR-MS experiment, molecules are isolated based on their ability to specifically bind to an interaction partner immobilized on the surface of a sensor chip. The bound material then can be recovered in a nondestructive manner allowing for MS analysis of the sample. Acceptable recovery processes must satisfy four demands:

- the immobilized interaction partner should ideally recognize the molecule and its potential breakdown species within a complex sample
- the recovery solution used should completely dissociate the bound molecule and its related forms from its immobilized interaction partner in a nondestructive manner
- the recovery solution must be compatible with MS analysis; more specifically, it should not induce signal suppression or spectral artifacts that interfere with or obscure data
- the recovery buffer should not irreversibly damage the immobilized interaction partner on the sensor chip surface.

This Application Note describes how, with minimal sample manipulation, the interface between recovering sample from the sensor chip surface and subsequent analyses by MS was optimized. The principle objective of the work was to maintain the sensitivity and efficiency of the process enabling automated, routine analysis in a preparative pharmaceutical setting.

Methodology

General procedures

Biacore 3000 was used in all experiments described. MALDI-TOF MS analyses were carried out using a Bruker Reflex system.

Sensor chip surface preparation

Affinity purified polyclonal chicken anti-Protein A antibodies (Wyeth Research) were diluted to 20 µg/mL in 10 mM sodium acetate, pH 4.5 and immobilized on Sensor Chip CM5 using standard amine coupling procedures. Typical immobilization levels were 10000-15000 resonance units (RU), corresponding to approximately 10-15 ng/mm^2 of immobilized antibodies.

Digestion of recombinant Protein A

A wide range of polypeptide sizes was generated from Protein A by time-limited trypsin digestion and used as a mock purification sample.

Extracted Protein A sample preparation

Residual Protein A was extracted from antibody product after elution from the column but prior to interaction analysis. Samples were diluted 1:1 in 10 mM glycine containing 0.05% (v/v) Tween-20, pH 1.5. Samples were sonicated for 5 minutes and then incubated on a heating block at 85–95°C for 10 minutes. Samples were centrifuged at 10000 rpm through a 50,000 MW cutoff filter for 5–10 minutes and the flow-through material was characterized using SPR-MS.

Sample recovery

Capture and recovery experiments were performed at 25°C. 20 mM ammonium acetate, pH 7.8, was used as both the running and sample dilution buffer. Prior to sample injection, the instrument was primed twice with running buffer and the sensor chip surface was normalized using 70% (v/v) glycerol followed by another prime. Extracted samples containing residual levels of Protein A were injected over the prepared sensor chip surface at 10 µL/minute for 120 seconds. After sample injection, residual buffer components were removed using the DESORB command where 0.005% (v/v) octyl-glucoside and Milli-Q water were used as DESORB buffers 1 and 2 respectively. This was followed by a RINSE command with running buffer. Elution and recovery of bound Protein A was performed using the MS_RECOVER command that controls the passage of a series of liquid plugs and air segments over the sensor chip surface in the following sequence: wash buffer (running buffer), air segment, recovery solution, air segment, running buffer. Parameters integrated into the MS_RECOVER command enable accurate positioning of the recovery solution segment over the sensor chip surface, and allow the user to choose the rack location where the recovered sample is to be deposited.

The recovery solution was held over the surface for 20 seconds and 4 µL of recovered sample was deposited either onto a MALDI target or into a polypropylene vial. Finally, the auto sampler needle was washed with 70% (v/v) formic acid followed by an EXTRACLEAN command prior to starting the next cycle.

In this study, the recovery of bound Protein A using 0.1% (v/v) trifluoroacetic acid (TFA) yielded inconsistent mass spectra. A variety of recovery solutions were evaluated and the results are shown in Table 1. A 2 µg/mL solution of digested Protein A was added to running buffer and injected over all four flow cells at 10 µL/minute for 120 seconds. Four different recovery buffers: (a) 0.1% TFA, (b) 5 mM glycine in 0.1% TFA, (c) 5 mM phenylalanine in 0.1% TFA and (d) 5 mM glycine plus 5 mM phenylalanine in 0.1% TFA were injected into a single flow cell at 50 µL/minute for 6 seconds. Baseline report points were taken 15 seconds prior to sample injection and 8 seconds after the end of the sample injection. Relative RU levels of binding versus cycle number were plotted to visualize the reproducibility of binding.

Table 1. Recovery solutions were assessed for their ability to recover captured protein A from the antibody immobilized sensor surface and for their impact on the anti-protein A antibodies immobilized onto the sensor surface over multiple cycles. The impact to the sensor chip surface is an important consideration in assay repeatability. For MALDI-TOF analysis, 5 mM phenylalanine + 0.1% TFA was selected for its spectral reproducibility characteristics, a wide usable mass range, higher overall signal-to-noise ratio, improved overall signal magnitudes, and the increased proportions of high m/z polypeptides.

Recovery solution	Regeneration quality	Effect on anti-protein A sensor chip surface
0.1% (v/v) TFA	+	None
10 mM glycine HCl, pH 1.5	+	None
5 mM glycine + 0.1% TFA	+	None
5 mM phenylalanine + 0.1% TFA	**+**	**None**
5 mM glycine/5 mM phenylalanine + 0.1% TFA	+	None
1% (v/v) formic acid	+	Damage noted
10 mM phenylalanine + 0.1% TFA	+	Damage noted
0.1% (v/v) formic acid	+/-	None
1% (v/v) acetic acid	-	None
3% (v/v) 2,5-dihydroxybenzoic acid	-	None
1% (v/v) citric acid	-	None

Sample recovery

Basic conditions for microrecovery into a vial using standard IFC are listed in Table 2.

Table 2. Basic conditions for recovery.

Analyte recovery assay parameter	Recovery conditions
Running buffer	20 mM ammonium acetate
Wash buffers	0.005% octyl-glucoside, water
Sample contact times	120 seconds
Recovery solution	Tested recovery solutions
Recovery contact time	20 seconds
Flow rate	10 µl/minute
Recovery volume	3–7 µl

Sample handling for MALDI-TOF MS

For vial collection, 40 µL of recovered sample from 10 deposition cycles (see above) were vacuum centrifuged down to approximately 2 µL and this was transferred to the MALDI target. Deposition of a duplicate sample from 2 recovery cycles was programmed for direct placement onto the MALDI target. In both cases, 1 µL of a sinapinic acid matrix solution (6 mg in 500 µL of 33% (v/v) aqueous acetonitrile with 0.1% (v/v) TFA) was added to the dried sample on the MALDI target and mixed using a pipette. Samples were allowed to air dry prior to MALDI-TOF MS analysis.

Results

Assessment of recovery solutions

Although standard regeneration buffer (2.5 mM glycine acidified to pH 1.5 with HCl) efficiently eluted Protein A from the capture antibody, it was anticipated that polypeptide-chloride adduct ions would form during ionization and hinder MALDI and future electrospray ionization (ESI) MS evaluations. Glycine buffer was compared to phenylalanine and other aromatic amino acids (all acidified initially with nitric acid) as recovery solutions (Figure 1). In general, with 10 mM phenylalanine in the recovery solution, all peptides from the Protein A digest sample exhibited greater signal strengths and signal-to-noise ratios for an equal number of laser shots when compared to the other solutions. Moreover, the recovery of polypeptides at high m/z appeared with elevated signal proportions with 10 mM phenylalanine.

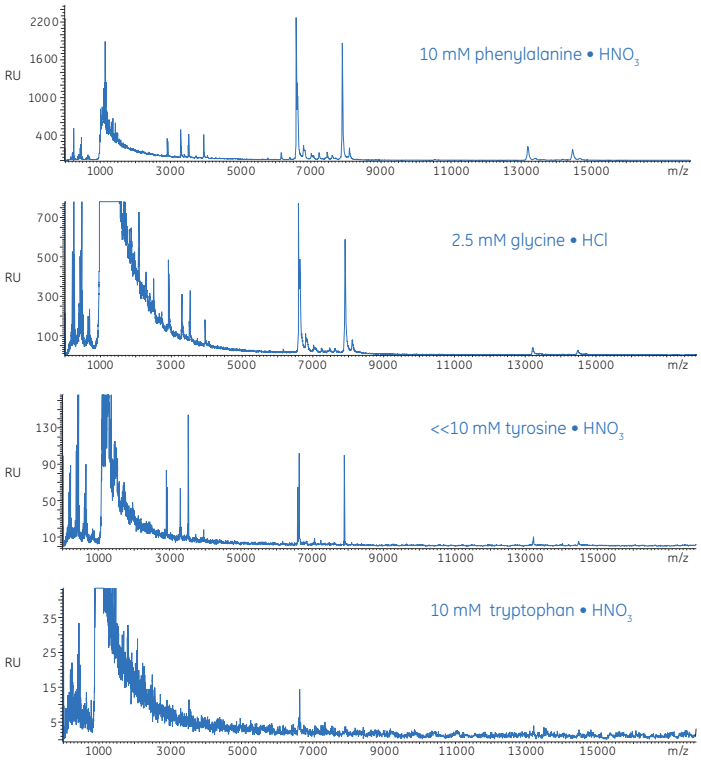

Figure 1. Comparison of glycine with phenylalanine and other aromatic amino acids as recovery solutions. Using 10 mM phenylalanine in the recovery solution, all peptides from the protein A digest sample yielded stronger signals and an improved signal-to-noise ratio for an equal number of laser shots when compared to the other buffers. The recovery of polypeptides at high m/z was improved in the presence of phenylalanine. Note the different scales of the Y-axes.

Importantly, it was confirmed that repeated injections of phenylalanine up to a concentration of 5 mM did not reduce the binding activity of the immobilized antibody. Although phenylalanine concentrations greater than 5 mM may moderately improve sensitivity in certain regions of the mass spectrum, the antibody surfaces were altered and captured less analyte following multiple regeneration cycles with the higher concentrations (data not shown).

MALDI-TOF MS spectra from specific SPR-MS applications were assessed after using 0.1% TFA to acidify the recovery solution. Recovery experiments that were performed with approximately 1.8 ng digested Protein A and 5 mM phenylalanine acidified with 0.1% TFA consistently yielded higher signals for all polypeptides. Moreover, greater proportions of polypeptide signals at high m/z were observed with 5 mM phenylalanine and 0.1% TFA as compared to the other recovery solutions tested. Signals from lower mass peptides, however, were similar and satisfactory for all recovery solutions tested. Additionally, the 5 mM phenylalanine acidified with 0.1% TFA recovery solution, produced more reproducible spectral distributions inter-experimentally. A summary of all recovery solutions evaluated is included in the Appendix (Tables 3–5) and may serve as a useful starting point in selecting solutions for SPR-MS applications.

Carry-over evaluation

Carry-over in recovery experiments was assessed. Digested Protein A samples were injected over underivatized, control sensor surfaces (activated with EDC/NHS and blocked with ethanolamine) to assess whether non-specific binding could be detected in the MS spectra of recovered samples. In addition, blank samples (running buffer) were substituted for digested Protein A on the antibody sensor surface in order to test whether any of the buffers or additives might affect the sensitivity of MS analyses.

These carry-over evaluations were performed sequentially, so any carry-over that might potentially affect successive recovery cycles in a multi-cycle recovery experiment could be assessed. During these evaluations, samples were injected and recovered to the MALDI target in the following recovery cycle sequence: extracted sample (digested Protein A), blank (running buffer), extracted sample (digested Protein A), blank (running buffer). This generated four spots on the MALDI target, two samples recovered from specific (active) sensor surfaces (antibody immobilized) and two recovered from non-specific control surfaces (activated and blocked only), with each spot containing the polypeptides from two cycles of recovered sample.

As expected, samples recovered after injections over the specific sensor chip surfaces produced mass spectra with high levels of Protein A polypeptides whereas samples recovered from blank (running buffer) injections resulted in minimal MALDI signals, with no indication of Protein A polypeptides (Figure 2). Spectra from samples recovered from the non-specific control surfaces where extracted sample (digested Protein A) and blank (running buffer) samples had been injected did reveal some low abundance, Protein A-related signals at m/z 3000. For the non-specific chip surface, buffer injections resulted in a high level of Protein A-related signals at m/z 3000 and sample injections resulted in low level Protein A-related signals across the m/z range. This indicated carry-over may be present in the tubing and more rigorous cleaning of the system between sample injections may be required.

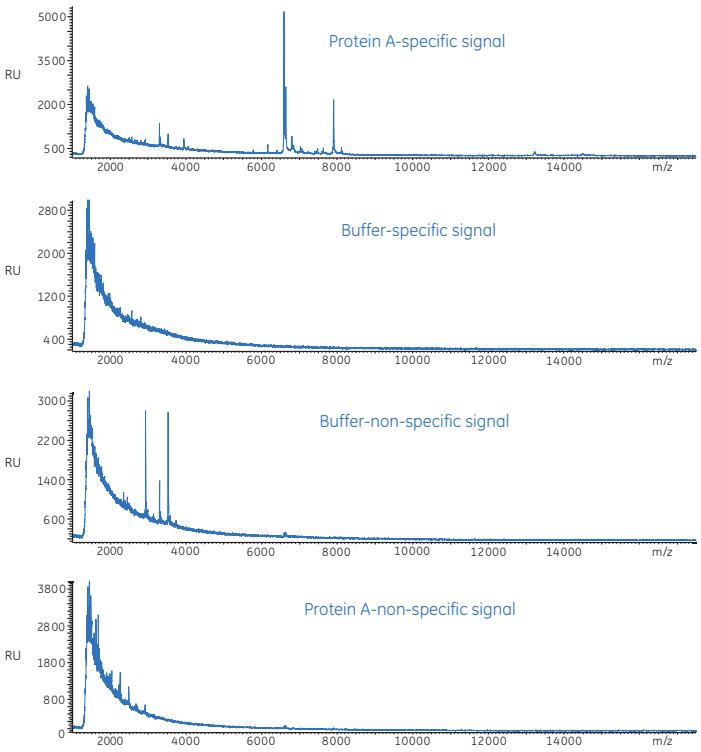

Figure 2. Mass spectra after recovery of sample following injection of buffer containing protein A or running buffer alone. Note the different scales of the Y-axes.

Collection to a vial or on-target deposition

Mass spectra of Protein A digested polypeptides that were recovered from sensor chips and collected in a vial were compared to spectra from samples spotted directly onto a MALDI target (Figure 3). Signal magnitudes and signal-to-noise ratios were improved after direct on-MALDI target deposition for all recovery solutions tested.

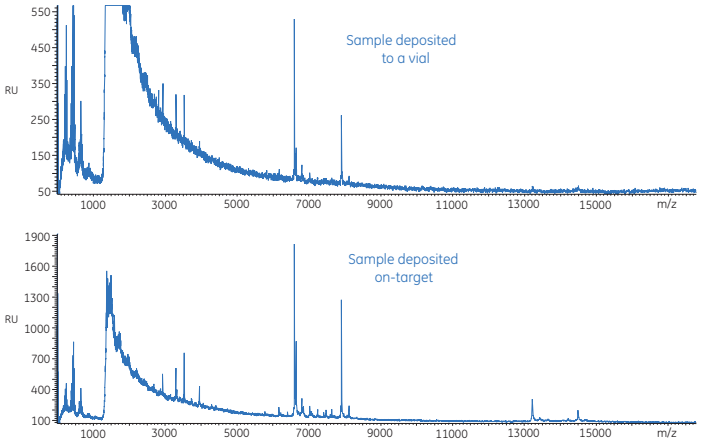

Figure 3. Mass spectra after collection into a vial compared with direct on-target deposition. Note the different scales of the Y-axes.

MALDI-TOF MS analysis of a monoclonal antibody sample

SPR-MS was used to isolate and characterize the Protein A-related species that may have leached from the affinity column during purification of a monoclonal antibody. In this example, the Protein A species that were present at very low concentrations in the monoclonal antibody purification sample were extracted, concentrated and then injected over anti-Protein A antibodies immobilized on Sensor Chip CM5 for capture. The Protein A species were subsequently eluted and recovered using 5 mM phenylalanine acidified with HNO_3, and then finally deposited directly onto a target for MALDI analysis. Figure 4 shows the MALDI-TOF MS spectrum before and after capture on a sensor chip surface, and as expected, two unique mass spectra of peptides were obtained.

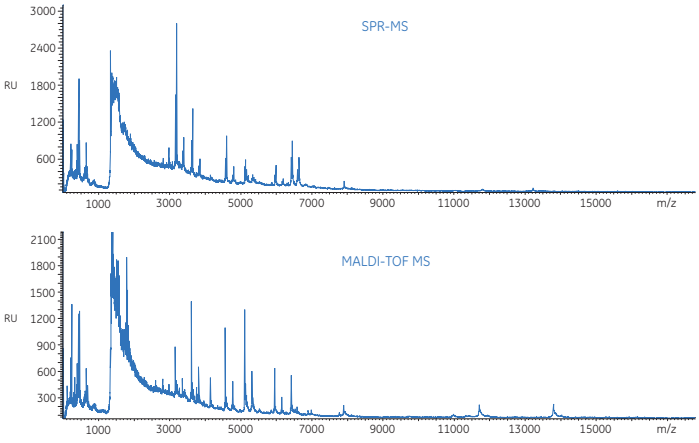

Figure 4. MALDI-TOF MS spectrum of a monoclonal antibody purification sample before and after capture on a sensor chip surface. Note the different scales of the Y-axes.

Discussion

A recovery protocol has been optimized for isolating and identifying minute quantities of residual Protein A species in a monoclonal antibody sample after affinity purification. The specific aim of Murray and co-workers was to define the conditions that allow the most efficient capture and transfer of digested Protein A from a Biacore sensor chip surface to a MALDI target while maintaining high quality mass spectral data in terms of complete mass coverage, sensitivity, and reproducibility. Their investigations included an empirical assessment of recovery solutions employed throughout the SPR phase of the technique, with particular attention paid to the effects of recovery (regeneration) solutions on data quality. Comparisons were also made between sample transfer to the MALDI target by direct deposition and intermediate transfer into a vial. Direct deposition involved less sample handling by the analyst, which is desirable when dealing with sample quantities in the sub-picomolar range due to the risk of sample loss and contamination with repeated manipulations. In this specific application, the direct deposition mode of recovered sample transfer enhanced MS signals for the larger polypeptides that were otherwise lost during sample concentration and transfer from polypropylene vials to the target.

Protein A is important for the separation and purification of monoclonal antibodies, some of which are intended for therapeutic applications. Despite the potential risks involved due to leached Protein A, it is likely to continue to be a popular means of purification due to its high specificity and purification efficiency. However, understanding the amount and types of impurities in the resulting antibody drug substance is essential. A robust automated system is required to characterize and quantify impurities in the purified product. The low sample demands, acute specificity and sensitivity, combined with well-defined assay conditions and buffer compositions for specific applications, of the Biacore system can satisfy either the characterization or quantification requirement. Combining SPR with MS provides additional characterization information, including the identification and profiling of impurities in a given product.

In principle, SPR-MS is adaptable to many biologically relevant impurities of therapeutic preparations, provided certain prerequisites are met. A primary prerequisite is the availability of an interaction partner that can be immobilized to a sensor surface and retain its ability to capture the molecule of interests and any derivatives. The characteristics of these solutions must be determined empirically and the effects on chip lifetime, sample carryover, MS sensitivity, signal-to-noise ratio, and reproducibility must be considered.

References

1. Analyte recovery in Biacore 3000: optimized functions for SPR-MS applications Technology Note 18: BR-9003-19 (2003) Nedelkov, D. and Nelson, R.W.
2. Analysis of human urine protein biomarkers via biomolecular interaction analysis mass spectrometry. Am J Kidney Dis 38: 481–7 (2001) Zhukov, A., Schürenberg, M., Jansson, Ö., Areskoug, D. and Buijs, J.
3. Integration of Surface Plasmon Resonance with Mass Spectrometry: Automated Ligand Fishing and Sample Preparation for MALDI MS Using a Biacore 3000 Biosensor. J Biomolecular Techniques 15:112–9 (2004)

Appendix

The following tables are summaries of empirical investigations aimed at selecting the best running, washing, and recovery solutions for the analysis of picomolar quantities of digested protein A in a monoclonal antibody preparation by SPR-MS.

Table 3. Running buffer evaluation. Running buffers were assessed both for their capacity to mediate binding of digested protein A to anti-protein A antibody immobilized on Sensor Chip CM5 and their MS compatibility. The most suitable running buffer was ammonium acetate, which gave an acceptable degree of protein A binding and did not adversely affect the performance characteristics of the MS. Ammonium acetate was evaluated in greater detail (Table 4).

Running buffer	Protein A bound to chip after injection of running buffer (% of most effective buffer)	Compatibility with MS
Sodium sulphate/sodium citrate/boric acid/sodium phosphate, pH 8–9	100 (715 RU)	no
Ammonium acetate	**77**	**yes**
Ammonium citrate	73	yes
Phosphate buffered saline (PBS) + Tween 20	73	no
Ammonium carbonate	64	yes
Sodium sulfate/sodium citrate	48	no
Ammonium formate	22	yes

Table 4. Ammonium acetate running buffer evaluation. Different concentrations of ammonium acetate were assessed as suitable running buffers. 20 mM ammonium acetate was selected as the running buffer of choice as higher salt concentrations were shown to interfere with the MS analysis. All tested buffers were compatible with MALDI-TOF MS.

Ammonium acetate buffer concentration	Protein A bound to chip after injection of running buffer (% of most effective buffer)
10 mM	83
15 mM	87
20 mM	**94**
25 mM	100 (630 RU)
50 mM	89
100 mM	87

Table 5. Wash solution evaluation. Wash buffers were selected based on lack of disruption of protein A:anti-protein A complexes on the sensor chip prior to microrecovery as well as for the potential compatibility with MS. The importance of compatibility of these buffers with MS is crucial since these have the potential of being carried over into the recovered sample.

Biacore washing solution	Protein A eluted from chip after injection of washing solution (multiples of response of least disruptive buffer)	Compatibility with MS
0.005–0.05% octyl glucoside	**1 (75 RU)**	**yes**
MilliQ water	**1.6**	**yes**
10 mM HCl	5.0	no
4 M urea	8.9	no
50 mM NaOH	15.7	no
6 M guanidine HCl	44.3	no

GE Healthcare
Biacore AB
Rapsgatan 7
754 50 Uppsala
Sweden

www.biacore.com

imagination at work

BR-9003-66 (05/2007)
28-9214-23 AA First published 09/2003