Development of an integrated system for activity-based profiling of matrix metallo-proteases
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CHAPTER 5

Activity-based analysis of MMP-12 with an automated system comprising inhibitor affinity extraction, on-line digestion, reversed-phase trapping, and nanoLC-MS analysis

An integrated system for activity-based analysis of matrix metallo-proteases (MMPs) was developed. Selective enrichment of spiked MMP-12 in buffer and urine samples was performed with an affinity sorbent carrying the immobilized, high-affinity TAPI-2 inhibitor as a stationary phase packed in a cartridge. The enrichment was followed by on-line digestion in an immobilized, acetylated trypsin reactor. Hyphenation of the sample pre-treatment steps to a nanoLC-MS system was achieved by loading tryptic MMP-12 peptides on a reversed-phase trap column, followed by back-flush elution to a 50-µm reversed-phase silica-based monolith capillary column coupled to a nanoESI-ion trap for MS detection and identification. The use of non-ionic surfactants in the sample pre-treatment steps resulted in better sensitivity. The completely automated method is able to analyse a sample every 75 min. Spiking of MMP-12 at 4 pmol into 500 µL urine resulted in selective detection of tryptic MMP-12 peptides with high intensities. At sub-pmol MMP-12 levels the signal dropped strongly, but some tryptic MMP-12 peptides were still detected at 0.25 pmol in 100 µL injections (from buffer) with good signal-to-noise ratios. Further research is needed to improve sensitivity and validate the automated system.
5.1 Introduction

A critical issue in proteomics is the high sample complexity (related to the heterogeneous physico-chemical nature of proteins and the wide dynamic concentration range), which often outweighs the resolving power of classical separation methods such as two-dimensional gel electrophoresis [1]. The additional labour-intensive nature and low reproducibility of this method has driven the development of alternative separation methodologies which are amenable to automation like multi-dimensional chromatography (shotgun proteomics) [2]. By increasing the peak and loading capacity, multidimensional approaches increase the number and dynamic range of peptides that can be analyzed in complex biological samples. Separation methods using different physical properties of peptides have been combined with varying degrees of success [3].

The development of immobilized trypsin reactors on various support materials (see Chapter 4) has allowed further automation and increased reproducibility by integration of the protein digestion step with chromatographic separation and mass spectrometric analysis. Despite a higher number of identified proteins in complex samples, as investigated in a study that compared different digestion protocols, the use of immobilized trypsin reactors for shotgun proteomics has been minimal [4].

In considering strategies for detection of low-abundance proteins in complex biological samples, the use of affinity sorbents for enrichment (described in Chapter 1) prior to on-line digestion can be envisaged for several reasons. Besides selective enrichment, resulting in increased sensitivity for targeted proteins, additional information is obtained through the selective nature of the interaction on which the enrichment is based. Importantly, affinity sorbents can also be implemented in automated protein analysis platforms, where they can be coupled to a trypsin reactor, thus reducing the need for manual sample preparation steps. In most integrated systems where affinity enrichment is followed by on-line digestion in a trypsin reactor and LC-MS analysis, only single proteins were monitored, related to the use of immobilized antibodies in the enrichment step [5–7]. Another mode of enrichment prior to on-line digestion which has been described is size exclusion chromatography [8,9].

For the activity-based analysis of MMPs in complex samples, there have been no automated methods described yet. A recently developed chemical proteomics approach, relying on labelling with activity-based probes (ABPs) to profile active MMPs in biological samples, was developed by Saghatelian et al., and does allow family-wide MMP analysis based on activity [10]. A disadvantage of this
approach is the need for a gel-based separation step after labelling of the samples. As an alternative for this ABP approach, this chapter describes the development of an automated method to detect active MMPs on a family-wide scale using immobilized MMP inhibitors (see Chapter 2 and 3). A major objective of this research was to eliminate manual sample handling steps. The method is based on the enrichment of active MMPs by inhibitor affinity chromatography, digestion on an acetylated-immobilized trypsin reactor (see Chapter 4), capturing of peptides on a reversed-phase trap column, and nanoLC-MS/(MS) analysis.

5.2 Materials and methods

5.2.1 Materials

Unless mentioned otherwise, all chemicals were from the same suppliers as mentioned in Chapters 2, 3 and 4. Polyethyleneglycol-400-monoctylether (PEG400-C₈) and polyethylene glycol-600-monoctylether (PEG600-C₈) were from Acros Organics (Geel, Belgium), dodecyl-β-D-maltoside (DDM) was from MP Biomedicals (Aurora, OH, USA) and octylglucopyranoside (OGP) was from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC Supra-Gradient grade) was from Biosolve B.V. (Valkenswaard, The Netherlands) Seph-PLG-NHOH and Seph-TAPI-2 affinity cartridges (2 mm i.d. x 10 mm length) with a ligand density of 5 mmol/L were prepared as described in chapter 3. AANHS modified immobilized trypsin reactors were prepared as described in chapter 4. Ultra-pure water, produced by an Elga purification system, was used for all buffer and mobile phase preparations, which were all filtered (0.22 µm pore size) before use.

5.2.2 Elution of MMP-12 from the inhibitor affinity cartridge

A 2-mm Seph-PLG-NHOH cartridge was equilibrated with 500 µL of extraction buffer (ExB; 25 mmol/L Tris, pH 7.4, 0.1 mol/L NaCl, 10 mmol/L CaCl₂) at a flow rate of 50 µL/min using a syringe pump set-up (described in chapter 3) at ambient temperature. A sample of 14 µg/mL MMP-12 (100 µL) in ExB (but with 0.05% (w/v) Brij-35 included) was loaded on the cartridge at 50 µL/min, followed by a washing step with 100 µL ExB (without Brij-35) at the same flow rate.
rate. Captured MMP-12 was eluted with 25 mmol/L Tris, 10 mmol/L EDTA, pH 8.2 at 20 µL/min and collected in 7 fractions of 15 µL (fraction collection was started after discarding the systems dead volume of 16 µL). A second elution step was applied at 10 µL/min with the same buffer, but with 1.1% (w/v) added SDS, and 3 fractions of 10 µL were collected. Collected fractions were analyzed by SDS-PAGE (described in Chapter 2) after addition of SDS-PAGE loading buffer (4x concentrated).

5.2.3 Elution buffer compatibility with on-line digestion

A 2-mm immobilized trypsin digestion reactor (Sepharose support, in-house trypsin immobilization and AANHS modification, see Chapter 4), was equilibrated at 50 µL/min, with 600 µL digestion buffer (25 mmol/L Tris, pH 8.2, 1 mmol/L CaCl$_2$) using the syringe pump set-up at ambient temperature. A 400 µL sample of 4 µmol/L cytochrome c in digestion buffer was pumped through the reactor at 50 µL/min. After washing the reactor with 600 µL of digestion buffer, digestion of cytochrome c was repeated but now in elution buffer (25 mM Tris, 10 mM of EDTA, pH 8.2). The flow-through of the cytochrome c digestions with and without EDTA was analysed by LC-MS as described in Chapter 4 (injections of 2.5 µL).

5.2.4 On-line coupling of extraction-digestion to LC-MS

The integrated system consisted of an SPE workstation (Prospekt II, Spark-Holland, Emmen, NL), comprising a Triathlon autosampler (with sample cooling to 4 °C), an automated cartridge exchanger (ACE) unit, and a micro high-pressure dispenser (µ-HPD, flow rate range: 12-1000 µL/min). Experiments with the integrated system were performed with 2-mm i.d. Seph-PLG-NHOH cartridges, 2-mm i.d. AANHS modified Poroszyme trypsin cartridges (see Chapter 4), a Vydac C$_8$ trapping cartridge (10 mm, 1 mm i.d., 5 µm, 300 Å pore size) and injection of 100 µL samples. The composition of the buffers which were connected to the inlet of the µ-HPD was as follows: extraction buffer (ExB) 25 mmol/L Tris, pH 7.4, 10 mmol/L CaCl$_2$, 0.1 mol/L NaCl; elution digestion buffer (EDB) 25 mmol/L Tris, 5 mmol/L EDTA, pH 8.2, 3% (v/v) ACN; trypsin equilibration buffer (TEB) 25 mmol/L Tris, 1 mmol/L CaCl$_2$, 3% (v/v) ACN; wash buffer (WB) 25 mmol/L Tris, pH 8.2, 45% (v/v) ACN. ExB was used to load and wash the injection loop of the autosampler. Samples were prepared in ExB containing 0.05% (w/v) Brij-35.
A schematic overview of the integrated system and the Prospekt II method steps are presented and discussed in more detail in the Results and Discussion section of this chapter.

The SPE platform was coupled to an Agilent 1100 capillary HPLC system equipped with a Vydac C$_8$ analytical column (250 mm, 1 mm i.d., 5 µm, 300 Å pore size), coupled on-line to an SL ion trap mass spectrometer (Agilent). After loading and washing of the Vydac C$_8$ trap column (10 mm, 1 mm i.d., 5 µm, 300 Å pore size), the LC-MS analysis is automatically started with an external start signal from the Prospekt II system. Peptides were backflush-eluted from the trap column in a linear gradient (0.875% ACN/min) from 3 to 52% ACN with 0.1% formic acid at a flow rate of 50 µL/min. Trap and analytical column are subsequently washed by increasing the ACN to 80% in 2 min, followed by a 2-min wash at a constant ACN level of 80%. Equilibration is performed by a decrease of organic modifier from 80% to 3% in 1 min, followed by a 10-min equilibration at 3% ACN. ESI settings: capillary voltage, 3900 V; nebulizer pressure, 40 psi; dry gas (N$_2$) flow, temperature, 7.5 L/min, 335 °C. MS smart parameter settings: target mass 1000 m/z, compound stability 70%, trap drive level 85%, optimize wide.

A series of injections with varying MMP-12 amounts (0 - 40 pmol) in ExB (in 100 µL) was performed to test the integrated system signal response. A 10-pmol MMP-12 injection was repeated three times to study the repeatability. The impact of the pH during the elution-digestion step was investigated by injecting 10 pmol MMP-12 at different EDB pH values.

### 5.2.5 Effect of surfactants in ExB and EDB

The effect of Brij-35 addition in ExB and EDB (connected to the inlet of the µ-HPD pump) was investigated with three 25-pmol MMP-12 injections. First, MMP-12 was injected without Brij-35, the second injection was done with 0.01% Brij-35 added to ExB and the third with 0.01% Brij-35 added to ExB and 0.005% Brij-35 added to EDB.

PEG400-C$_8$, PEG600-C$_8$, DDM, and OGP were other surfactants that were included (at concentrations of 0.005% in both ExB and EDB) and tested with blank injections (only ExB) and 4 pmol MMP-12 injections.

A series of MMP-12 injections was done with a combination of the surfactants PEG400-C$_8$, PEG600-C$_8$, and DDM at equal concentrations in ExB and EDB. The total concentration (added individual concentrations) of this surfactant cocktail was 0.005% in both buffers.
5.2.6 EDB composition and elution of MMP-12

100 µL samples containing 10 µg/mL MMP-12 (53 pmol) were extracted on 2 mm i.d. Seph-PLG-NHOH and Seph-TAPI-2 cartridges using the syringe pump set-up as described before. Fractions of 30 µL were collected in the elution step. SDS-PAGE analysis of 3 elution fractions was performed as described before, but only half of the eluted fractions were analysed with SDS-PAGE. Cartridge washing after sample loading was performed with ExB (with 0.005% surfactant cocktail included). EDB composition was as follows; 30 mmol/L Tris, 5 mmol/L EDTA pH 8.2, 3% (v/v) acetonitrile and 0.005% (w/v) surfactant cocktail. MMP-12 elution from Seph-TAPI-2 affinity cartridge was also tested with EDB as described above but at a pH of 8.7 and with varying EDTA concentrations.

5.2.7 On-line coupling of extraction-digestion to nanoLC-MS

The set-up of the integrated system was modified to allow coupling of the extraction-digestion steps with nanoLC-MS analysis. The standard ESI interface was changed into an in-house modified nanoelectrospray ion source, identical to that described by Rieux et al., to minimise post-column dead volumes [11]. A silica-based reversed-phase (C18) monolith, 560 mm x 0.05 mm (Merck) prepared in a capillary was used as an analytical column. The nanoelectrospray tip (gold-coated nanoelectrospray emitter, 50-µm fused-silica capillary tapered to 10 µm i.d., from Nanoseparations) and analytical column were butt-connected using a teflon sleeve of 360-µm i.d. [11]. Tryptic MMP-12 peptides eluting from the digestion reactor were loaded on a C18 trap column (Zorbax 300 SB C18, 5 µm particle diameter (dp), 5 mm 0.3 mm, Agilent), which was mounted on a six-port micro switching valve. The nanopump was also connected to the micro switching valve, in order to direct the flow through the trap column and elute the analytes onto the monolithic (analytical) column in the backflush mode. When loading of the trap column starts, the LC-MS method is started with an external start signal from the Prospekt II system. After loading, the trap column is washed for 2 min using a stand alone pump (ISCO model 2350 from Teledyne ISCO Inc.), with a solution of 0.1% formic acid in water:ACN (98:2) at 25 µL/min. After washing, the trap column was switched on-line with the analytical column to analyse the peptides by backflush elution. Gradient elution was performed with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as mobile phase B. Gradient elution was performed according to the following scheme: 10% B for 3 min,
linear increase to 18% B in 10.5 min, linear increase to 39% B in 9 min, linear increase to 50% B in 2 min, linear increase to 95% B in 2.5 min, wash at 95% for 7.5 min, return to initial conditions (10% B) in 3.5 min, column equilibration at 10% B for 12 min. The flow rate of the nanoLC system was set at 0.4 µL/min and held constant by a dynamic nanoflow splitter and flow meter.

Nanoelectrospray settings: capillary voltage, 1500 V; dry gas (N\textsubscript{2}) flow and temperature, 5 L/min, 280 °C. MS smart parameter settings: target mass 1000 m/z, compound stability 70%, trap drive level 100%, optimized wide.

The integrated system set-up (including the Prospekt system), is given in Figure 5.12 and is discussed in more detail in the Results and Discussion section. 2-mm i.d. Seph-TAPI-2 cartridges were used in the enrichment step of all experiments. The Prospekt II autosampler was equipped with 100 or 500 µL injection loops.

The Prospekt buffers WB and TEB (connected to the inlet of the µHPD) were the same as described before, but the composition of ExB was modified by the addition of 0.005% surfactant cocktail (equal concentrations of DDM, PEG400-C\textsubscript{8} and PEG600-C\textsubscript{8}). EDB composition was modified into 25 mmol/L Tris, 1 mM EDTA pH 8.7, 3% ACN, 0.005% surfactant cocktail.

5.2.8 Effect of surfactants in ExB and EDB on MMP-12 peptide signals in nanoLC-MS analysis

The effect of the surfactant cocktail concentration in ExB was investigated doing triplicate MMP-12 injections of 2.5 pmol (100 µL injection volume) at each surfactant cocktail concentration. The surfactant cocktail concentration in EDB was kept constant at 0.001% throughout the whole experiment.

The effect of the surfactant cocktail concentration in EDB was investigated doing triplicate MMP-12 injections of 2 pmol (100 µL injection volume) at each surfactant cocktail concentration. The surfactant cocktail concentration in ExB was kept constant at 0.005% throughout the whole experiment. At each new surfactant concentration a blank injection was performed before the 3 MMP-12 injections.

5.2.9 Non-specific adsorption in the digestion reactor and the effect of surfactants

The non-specific adsorption at the peptide level in the digestion reactor was investigated, with 200 fmole injections of a cytochrome c digest (see Chapter 4;
obtained with acetylated solution phase trypsin, 75 min digestion time). To this end, two Prospekt II methods were made for injection of the digest either directly on the nanoLC-MS system, or through the trypsin reactor. The affinity SPE cartridge from the integrated system (see Figure 5.12) was bypassed by connecting the injection valve of the auto sampler directly to valve 2, where the trypsin reactor was mounted. An injection loop of 30 µL was used in these experiments. Cytochrome c samples were prepared in TEB. Different surfactant cocktail concentrations (from 0 to 0.015%) were used in the samples and the surfactant cocktail was composed of PEG400-C₈ and DDM at equal concentrations. The Prospekt methods are not given in detail but the following steps were used. After purging the lines with WB, with the cartridges off-line, the injection loop is filled with sample. For the direct injection method, the lines are then purged with TEB and in the next step the sample is injected on the trap column, followed by 2 min washing with the stand-alone pump and nanoLC-MS analysis. For the injection method through the trypsin reactor, the method continues with washing the trypsin reactor with WB and subsequently with TEB. The sample is then injected on the trap column through the trypsin reactor and the method continues like the direct injection method. All injections were done in duplicate.

5.2.10 Spiking of MMP-12 in urine samples

Urine from a glomerulonephritis patient was thawed on ice and 1/5 volume of cold ExB (with 0.125% Brij-35) was added, followed by centrifugation for 10 min (4 °C, 11300g). MMP-12 was spiked into the urine supernatant at different concentrations and 100 µL (5 pmol MMP-12) and 500 µL (4 pmol MMP-12) samples were injected into the integrated system with nanoLC-MS analysis. Both ExB and EDB contained 0.005% surfactant cocktail (DDM and PEG400-C₈). Urine samples were also injected on the integrated system equipped with a control cartridge with immobilized ethanolamine instead of the affinity SPE cartridge.
5.3 Results and discussion

5.3.1 Coupling of inhibitor affinity extraction to the trypsin digestion reactor

For the development of an integrated MMP profiling system, the inhibitor affinity cartridge (developed in Chapter 2 and 3) must be coupled on-line to the chemically modified trypsin digestion reactor (see Chapter 4). It was hypothesized that two factors would be of primary importance for optimal on-line digestion of the enriched MMPs. First, the volume in which MMPs elute from the inhibitor affinity cartridge is of importance, because for (on-line) digestion it is critical to keep the enriched MMPs as concentrated as possible to avoid digestion rates being limited by low substrate concentration. Second, the activity of the digestion reactor should not be decreased under the conditions of the elution buffer.

Thus far, the development of inhibitor affinity cartridges has mainly focused on capturing MMPs and not on their elution from the cartridge. Elution was mostly performed using SDS in the elution buffer, which is no problem when the final analysis is performed by SDS-PAGE but is less suited when trypsin digestion followed by LC-MS is the goal. For this reason, an elution buffer with EDTA as an MMP dissociating agent was tested with MMP-12. EDTA is thought to remove Zn\(^{2+}\) and Ca\(^{2+}\) from MMP-12 (due to its chelating properties), resulting in loss of affinity for the immobilized inhibitor.

![Figure 5.1: Elution of MMP-12 from a Seph-PLG-NHOH affinity cartridge (2 mm i.d. x 10 mm), analyzed by SDS-PAGE. 100 µL of 14 µg/mL MMP-12 in ExB was loaded at 50 µL/min, followed by washing with 100 µL ExB at the same flow rate. Fractions 1-7 (15 µL) were collected after elution with 25 mmol/L Tris, 10 mM EDTA, pH 8.2, and fractions 8-10 with the same buffer, with 1% SDS included.](image)

SDS-PAGE analysis (see Figure 5.1) of the elution fractions of MMP-12 captured on a Seph-PLG-NHOH cartridge, revealed that nearly complete elution of
MMP-12 was achieved in 60 µL (first 4 fractions).

Figure 5.1 shows also that elution has only a short delay, because eluted MMP-12 is already present in the first collected fraction. It was anticipated that losses of MMP-12 could occur during elution due to inactivation of the enzyme by EDTA and the surfactant-free environment. To investigate whether losses occur in the cartridge, a second elution step with 1% SDS included in the elution buffer was performed. Figure 5.1 shows, that hardly any MMP-12 was detected in the eluted fractions (8-10), indicating that elution of MMP-12 with EDTA from the cartridge is almost complete.

Before coupling the inhibitor affinity extraction cartridge to the trypsin reactor to obtain a peptide map of enriched MMP-12, the effect of the elution buffer on the activity of immobilized, acetylated trypsin was investigated. The chromatograms in Figure 5.2 show that the cytochrome c digestion patterns without and with 10 mM EDTA in the elution buffer are nearly identical.

![Chromatograms showing the effect of EDTA on trypsin activity](image)

**Figure 5.2**: Effect of EDTA on the activity of an AANHS modified trypsin reactor (2 mm i.d. x 10 mm, 31 µL bed volume), assessed with cytochrome c digestions (4 µmol/L, flow rate 50 µL/min), analyzed by LC-MS. For both digestions, the reactor was equilibrated with CaCl₂-containing digestion buffer.

No undigested cytochrome c could be detected in either of the digestions. Incomplete cytochrome c digestion was, however, obtained when the digestion reactor was incubated with the elution buffer, rather than equilibrated with the CaCl₂-containing digestion buffer, before injection of the cytochrome c sample (data not shown). Apparently, Ca²⁺ in the trypsin structure is critical for ac-
tivity of immobilized, AANHS-modified trypsin but depletion from the trypsin structure by EDTA is probably so slow that digestion is not affected when the cytochrome c sample with EDTA enters the digestion reactor. Interestingly, the loss of digestion reactor activity, by incubation with EDTA prior to applying the cytochrome c sample, is reversible, since a cytochrome c sample that is applied after equilibrating the reactor with digestion buffer (with CaCl$_2$), is again completely digested. The implication of these results for the development of the integrated system is that EDTA can be used to elute enriched MMPs directly to the digestion reactor without any buffer exchange steps.

Initial attempts to couple both cartridges and perform both sample pre-treatment steps automatically were performed using the SPE platform in almost the same configuration as described in Chapter 3 (Figure 3.3). Addition of the trypsin reactor to a second six-port switching valve allowed both cartridges to be switched into the liquid flow, independent of each other. By using the tubing configuration from the autosampler to a third six-port switching valve of the ACE unit, the autosampler can also be used to collect fractions from the digestion reactor. An injection of 100 µL sample, containing 100 pmol MMP-12 in ExB, was loaded on the inhibitor affinity cartridge and eluted to the trypsin reactor, followed by collection of 5 fractions (of 40 µL each), which were analyzed by LC-MS. Though several tryptic MMP-12 peptides could be detected, they were distributed over all 4 fractions and their intensity was relatively low (data not shown), despite the high quantity of MMP-12. Though further optimization is required, this initial experiment showed that it is feasible to couple the extraction and digestion cartridges, resulting in a MMP-12 peptide map.

5.3.2 On-line coupling of extraction-digestion to LC-MS

To test whether a better MMP-12 peptide response could be reached when the two automated sample pre-treatment steps are coupled on-line to LC-MS analysis, the integrated system as outlined in Figure 5.3 was tested. All cartridges can be switched on- and off-line independent of each other by valve switching. Ports 3 and 6 of valve-1 are connected to a clamp, in which the affinity SPE cartridge can be automatically introduced, allowing automated extraction with a series of cartridges. To avoid contamination of the mass spectrometer all surfactants were omitted from the µHPD buffers. Only the injected sample still contained Brij-35 to keep MMPs active and prevent non-specific adsorption.
Figure 5.3: Design of an integrated system for automated, activity-dependent MMP extraction, digestion, trapping and capillary LC-MS analysis. Autosampler (AS) injection loop = 100 µL. Injection valve; - - - fill loop, — inject. Valve 1; - - - purge, — equilibrate, load, wash, elute. Valve 2; - - - purge, — equilibrate, load, wash. Valve 3; — trap, - - - backflush elution, equilibrate. ExB = extraction buffer, (for compositions of buffers, see Materials and Methods section) EDB = elution digestion buffer, WB = wash buffer, TEB = trypsin equilibration buffer, µHPD = micro high pressure dispenser, ACE = automated cartridge exchanger.

The complete Prospekt II method is detailed in Table 5.1 and starts with cleaning of the lines with WB (step 1), with the cartridges excluded from the flow path. This step is followed by washing of the affinity SPE cartridge with WB (step 2) and washing the affinity SPE cartridge and the trypsin reactor with WB (step 3). In step 4, the lines are purged with ExB and the injection loop is washed with ExB. Then the injection loop is filled with sample and the affinity SPE cartridge is equilibrated with ExB (step 5). In step 6, the sample is loaded on the affinity SPE cartridge and the effluent is sent to waste (other cartridges still off-line). During this step, the SPE-cartridge is also washed with ExB to remove excess of surfactant from the sample. When the MMPs are captured on the affinity SPE cartridge, the trypsin cartridge is prepared for digestion in the following steps (7+8) by equilibration with trypsin equilibration buffer (TEB).
### Table 5.1: Prospekt II method for automated MMP extraction, digestion, trapping and LC-MS analysis.

With all cartridges off-line, the lines are purged with EDB (step 9) and MMPs are eluted from the affinity SPE cartridge to the trypsin reactor (step 10). The C₈ trap column is still off-line in this step to avoid backpressure (generated by flow over the C₈ trap column) on the Sepharose support in the affinity SPE cartridge. Keeping the trap column off-line was possible due to the delay of the tryptic MMP-12 peptides, caused by system dead volumes, and delay of the elution and digestion steps. In step 11, the trap column is switched on-line to start trapping the tryptic MMP peptides. Prior to this, the trap column has been equilibrated with 3% acetonitrile, 0.1% formic acid delivered by the HPLC pumps (50 µL/min). In step 12, the affinity SPE cartridge is switched off-line after elution with 80 µL of EDB of the captured MMPs, which is sufficient to reach complete
elution (see Figure 5.1). During this step trapping of tryptic MMP-12 peptides eluting from the digestion reactor continues. Loading the trap column is done with TEB to supply immobilized trypsin again with calcium and to minimize exposure to EDTA. The trypsin reactor is further equilibrated with TEB in step 13 and the C_8 trap column is switched out of the μHPD and into the HPLC flow path to wash it with 3% acetonitrile, 0.1% formic acid for 3 min. After washing, the LC-MS run is started with an external signal from the Prospekt II system, by backflush elution of the trap column to the analytical column. The Prospekt II system is starting with the following sample, while the LC-MS run finishes the analysis.

5.3.3 MMP-12 peptide signals and elution-digestion pH

Figure 5.4: Analysis of 40 pmol MMP-12 using the integrated system (see Fig 5.3). Peaks indicated with * were tryptic MMP-12 peptides with 2 missed cleavages or less. The broad peak at the end of the chromatogram is undigested MMP-12.

Figure 5.4 shows that with a 40 pmol MMP-12 injection, a nearly complete peptide map could be obtained with this integrated system reaching a sequence coverage of 93%. The absence of disulfide bridges in the catalytic domain of MMP-12 likely contributes to this high sequence coverage, since no reduction/alkylation was performed. Besides the tryptic MMP-12 peptides (10 out of 13 detected peptides have no missed cleavages), a broad peak of undigested MMP-12 was detected around 49 min. The experimentally determined mass of 18797 Da corresponds within 2 Da to the theoretical mass based on the primary sequence of the catalytic domain (100F-268N: numbering of amino acids is according to entry P39900 in the Swiss-Prot database). The three calcium and the two zinc atoms, present
in active MMP-12 [12] have thus been removed, most likely due to chelating by EDTA, and the elution conditions from the reversed-phase column. Strangely, only fully digested peptides or completely undigested MMP-12 was detected but no digestion intermediates with 3 or more missed cleavages. This could be an indication that the first step in the digestion reaction is rate limiting and that MMP-12 will be rapidly digested further once this slow first step has occurred.

One advantage of performing on-line LC-MS analysis after extraction and digestion, is illustrated by the fact that more hydrophobic tryptic peptides are detected (compared to the off-line LC-MS analysis of 100 pmol MMP-12), resulting in a higher sequence coverage. These peptides are probably lost due to non-specific adsorption or precipitation after fraction collection, given their longer exposure to larger surface areas prior to off-line LC-MS analysis. Another advantage of the integrated system is the fact that the whole sample can be trapped on the trap column after passing the digestion reactor (whereas the collected fractions can only be partially injected, due to vial hold-up volumes). This resulted in higher signal intensities obtained with the integrated system, although a lower amount of injected MMP-12 was used.

The injection of 10 pmol MMP-12 was repeated three times (within 1 day, in the same injection series) to investigate the system repeatability. The extracted ion chromatograms (peak areas) of the three peptides with the best responses (19-36, 143-150 and 3-11) had an RSD of 16, 8 and 9%, respectively.

**Figure 5.5:** Response of tryptic peptides of MMP-12 versus injected amount. Peak areas of extracted ion chromatogram (EIC) signals were obtained with a series of MMP-12 injections from 0-40 pmol on the integrated system (see Figure 5.3). Peptides are indicated by their positions in the primary sequence.
Despite the improvements of the MMP-12 analysis by integrating the sample pre-treatment steps with LC-MS analysis, the signal intensities are still relatively low. An injection series with varying amounts of MMP-12 gave insight in the peptide signal responses. For good method sensitivity the signal should be linearly dependent on the injected amount. Figure 5.5 shows that this is not the case and MMP-12 peptide signals drop strongly at lower injected MMP-12 levels (other peptides give equal responses). At 10 pmol injected MMP-12, the peptide signals have almost disappeared completely in the noise and background peaks when examining the total ion chromatogram (TIC). At 4 pmol of injected MMP-12 most peptides cannot be detected anymore, even in the extracted ion chromatogram (EIC).

Several mechanisms could contribute to the poor response at lower MMP-12 levels. One factor that may be important is the nature of the digestion kinetics, which is limited by the substrate concentration (in this case MMP-12). The signal drop is, however, more pronounced for more hydrophobic peptides, which is an indication that digestion kinetics alone is not responsible for the nonlinear response. Lower solubility (giving rise to more non-specific adsorption) of more hydrophobic peptides is thus another effect which may play a role. Unfavourable conditions for MMP-12 during and after elution caused by destabilization of the protein structure due to the EDTA mediated removal of cations and the absence of surfactants in the buffers, causing non-specific adsorption or even precipitation, could be another potential factor. The overall method recovery may thus be improved by use of surfactants in ExB and EDB, thus ensuring better solubility of captured and eluted MMP-12 by making it less susceptible to non-specific adsorption. After digestion, the surfactants may also prevent non-specific adsorption of hydrophobic peptides.

Another potential parameter to optimize is the pH of the elution digestion buffer (EDB) which was studied with 20 pmol MMP-12 injections. Figure 5.6 shows the signals of the 5 peptides, at three different EDB pH values. A clear pH effect was found, with the curve shape indicating that the pH optimum is even higher than 8.2. Apparently the pH optimum of acetylated, immobilized trypsin is shifted to more alkaline conditions. This is in agreement with the shifted pH optimum which was found for acetylated trypsin in solution [13]. The relatively strong dependence on pH during digestion is another indication that the digestion is a critical step in the integrated system which may require further optimization.
5.3. Results and discussion

Figure 5.6: Effect of elution digestion buffer (EDB) pH on MMP-12 peptide signal intensity. Peak areas of extracted ion chromatogram (EIC) signals were obtained with 20 pmol MMP-12 injections on the integrated system. Peptides are indicated by their positions in the primary sequence.

5.3.4 Effect of surfactants in ExB and EDB

The previous experiments gave some indication that the overall integrated system performance (in terms of sensitivity) may be negatively influenced by non-specific adsorption and/or precipitation of eluted MMP-12 (intact and/or digested). To investigate whether the use of surfactants may be able to prevent this, thereby reducing sample loss and improving the overall system performance, the use of Brij-35 in the enrichment and digestion steps was investigated.

Brij-35 has already been reported to be of crucial importance for maintaining activity during purification of MMP-13 from buffy coats [14]. Because the solubilizing effect may be maximal during the steps prior to digestion, Brij-35 was added to ExB in one experiment, while in another experiment it was used in both ExB and EDB. Both were compared with MMP-12 injected without added Brij-35 in these buffers. Figure 5.7 shows that Brij-35 addition results in undesired background peaks, which are interfering with signals of the tryptic MMP-12 peptides in the TIC. Many of these background peaks had a mass difference of 44 Da, corresponding to the mass of a polyoxyethylene monomer. These polyoxyethylene chains are probably present as degradation products or contaminants in Brij-35. Because the hydrophilic polyoxyethylene chain in Brij-35 has a highly heterogeneous character (variable number of monomers), the background peaks were found over a wide elution range.

Figure 5.7 also shows that, despite the high background peaks, some MMP-12 signals increase strongly upon using Brij-35 in ExB and EDB. Especially the hydrophobic peptides (with retention times of 42 and 44 min) show a dramatic sig-
nal increase upon Brij-35 addition, which indicates an improved recovery of these peptides. Although most other peptides co-elute with background peaks, it can be seen that some signals are also increased in intensity by Brij-35 addition. By integrating the EIC signals, the surfactant effect can be measured for all peptides.

![Figure 5.7](image)

**Figure 5.7:** Effect of Brij-35 on background and MMP-12 peptide signals. All injections were done with 25 pmol MMP-12. TICs are plotted on the same scale. Peaks labeled with * are MMP-12 peptide signals.

![Figure 5.8](image)

**Figure 5.8:** Effect of Brij-35 on EIC peptide signals (25 pmol MMP-12 injections). The normalized peptide signals are given for 7 tryptic MMP-12 peptides with increasing (from left to right) hydrophobicity (according to elution order from the C8 column). Normalization is done independently for each peptide based on the highest EIC signal. Peptides are indicated by their amino acid numbers in the legend.
Figure 5.8 shows that surfactant addition results in increased signals for all peptides. This is an indication of better MMP-12 recovery from the affinity-SPE cartridge or a higher digestion efficiency. The fact that the more hydrophobic peptides are increased the most in signal intensity is a confirmation that non-specific adsorption at the peptide level plays an important role and can be suppressed through surfactant use.

**Figure 5.9:** Interference of different surfactants added to the ExB and EDB at 0.005% with LC-MS analysis.

**Figure 5.10:** Effect of surfactants (in ExB and EDB) MMP-12 peptide signals, tested with 4 pmol MMP-12 injections. The normalized peptide signals are given for 7 tryptic MMP-12 peptides (indicated by amino acid numbers in the legend). Normalization is done independently for each peptide, with the highest EIC signal.
As alternative for Brij-35, other non-ionic surfactants were compared with Brij-35 for interference with LC-MS analysis and impact on MMP-12 peptide signal intensity. Two Brij-35 resembling surfactants with shorter aliphatic and PEG chains PEG400-C₈, PEG600-C₈, and two alkylglycosides (octylglucopyranoside (OGP) and dodecyl-β-D-maltoside (DDM)) were tested. Figure 5.9 shows that the degree of interference of these surfactants with LC-MS analysis is the lowest for DDM, followed by PEG400-C₈ and PEG600-C₈.

The impact of these surfactants on MMP-12 peptide signal intensities was tested with 4 pmol MMP-12 injections. Figure 5.10 shows that the new surfactants are as good as, or even better than Brij-35 in improving MMP-12 peptide signals. To improve signal intensity further, the effect of a surfactant cocktail ((DDM, PEG400-C₈ and PEG600-C₈) at equal concentrations) in both ExB and EDB was tested with MMP-12 injected at different levels (0, 4, 10 and 20 pmol) as compared to PEG400-C₈ alone added to both buffers at the same total concentration (data not shown). At all MMP-12 levels, but most strongly at 4 pmol, peptide signal intensities obtained with the "surfactant cocktail" were significantly higher than similar injections with a single surfactant in ExB and EDB.

![Graph](image)

**Figure 5.11:** Response of tryptic MMP-12 peptides. Peak areas of extracted ion chromatogram (EIC) signals were obtained with a series of MMP-12 injections with 0.0033% (total concentration) surfactant cocktail consisting of DDM, PEG400-C₈ and PEG600-C₈ in ExB and EDB. Peptides are indicated by their positions in the primary sequence of MMP-12.

Figure 5.11 shows the EIC peptide signals of various MMP-12 peptides with different hydropathicity, obtained with the surfactant cocktail. Compared to the same peptides plotted in Figure 5.5, the use of the surfactants clearly resulted
in better sensitivity for all peptides, though the response is still not linear. At 4 pmol MMP-12 injections, peptides 38-49 and 53-66 (the most hydrophobic ones of the 5 selected peptides) were not detected at all without the use of surfactants but gave good signals with the addition of the surfactant cocktail to ExB and EDB.

5.3.5 EDB composition and elution of MMP-12

With the use of the surfactant cocktail, the initial sensitivity and linearity problems were reduced, but a higher sensitivity is probably still needed to profile low levels of active endogenous MMPs in biological samples [15]. The yield of the on-line digestion step may be a further limiting factor in obtaining good peptide responses at lower MMP-12 levels (due to substrate-limited enzyme kinetics). Higher digestion yields (by improving digestion kinetics) may be obtained by using EDB with a higher pH (see Figure 5.6) and lower EDTA concentrations. The effect of these parameters on MMP-12 elution was evaluated with off-line experiments with both Seph-PLG-NHOH and Seph-TAPI-2 cartridges. SDS-PAGE analysis of the eluted fractions (data not shown) showed that the previously optimized EDB (30 mmol/L Tris, 5 mmol/L EDTA pH 8.2, 3% (v/v) ACN, 0.005% (w/v) surfactant cocktail) eluted MMP-12 completely in 60 µL from both Seph-PLH-NHOH and Seph-TAPI-2 cartridges. Despite the stronger interaction with the high affinity inhibitor TAPI-2 (see Chapter 3), elution of MMP-12 is also effectuated in 60 µL, which is important for effective on-line digestion. Increasing the EDB pH to 8.7 did not affect elution from the TAPI-2 cartridge, which indicates an increased EDB pH can be used for improved digestion kinetics (see Figure 5.6). Though the silica support of the trap column may be damaged under alkaline conditions, it was anticipated that this would not be problematic because the contact is only short and the trap column is washed directly with the acidic HPLC solutions after loading.

Since trypsin is a Ca$^{2+}$-dependent enzyme, it was evaluated whether MMP-12 could be eluted with lower EDTA concentrations in order to preserve trypsin activity (data not shown). Different EDTA concentrations (0, 0.2, 1 and 5 mmol/L) were tested for elution of MMP-12 from a Seph-TAPI-2 cartridge (EDB pH 8.7). Both 5 and 1 mmol/L EDTA in EDB resulted in complete MMP-12 elution in 60 µL. At 0.2 mmol/L EDTA, elution was delayed but still complete. The use of EDB without EDTA did not result in detectable elution of MMP-12 in the first 90 µL. This shows that the EDB pH can be increased to 8.7 and the EDTA concen-
tration can be reduced to 1 mmol/L without affecting MMP-12 elution. Whether the higher-affinity Seph-TAPI-2 extraction cartridge in combination with a further optimized EDB results in a further increase of the overall performance of the integrated MMP profiling system is further investigated in the following section.

### 5.3.6 On-line coupling of extraction-digestion to nanoLC-MS

![Diagram of the integrated system](image)

**Figure 5.12:** Design of the integrated system for automated MMP extraction, digestion, trapping and nanoLC-MS analysis. Autosampler (AS) injection loop = 100 μL or 500 μL. Injection valve; - - - fill loop, — inject. Valve 1; - - - purge, — equilibrate, load, wash, elute. Valve 2; - - - purge, — equilibrate, load, wash. Valve 3; — load C\textsubscript{18} trap, - - - wash C\textsubscript{18} trap. μValve; - - - load and wash C\textsubscript{18} trap, — backflush elute. ExB = extraction buffer, EDB = elution digestion buffer, WB = wash buffer, TEB = trypsin equilibration buffer, μHPD = micro high pressure dispenser, ACE = automated cartridge exchanger.

Though the yield of the extraction and digestion steps on the Prospekt II system is essential to develop a sensitive method, the sensitivity of the LC-MS step itself is also an important factor. To achieve a higher sensitivity at this level, the reversed phase trap column and analytical column were scaled down to dimensions that

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allowed flow rates in the nL/min range, thereby decreasing the chromatographic
dilution of the eluted MMP-12 peptides and increasing ionization efficiency [16].
In order to couple the nanoLC-MS system to the extraction-digestion steps, the
set-up of the integrated system was modified as shown in Figure 5.12.
The modified ESI interface, with the spray tip butt-connected to the analytical
column to minimise post-column dead volumes, does not allow washing of the
trap column with the nano HPLC pump, with the trap effluent directed to waste.
Therefore, a stand-alone HPLC pump was used to wash the trap column after
loading, with the effluent going to waste. Related to the use of this pump, valve 3
of the ACE was needed to switch between trap column loading by the \( \mu \)HPD, and
washing with the stand-alone pump. An advantage of the extra valve is the sep-
aration between the ”dirty” sample preparation steps (extraction and digestion)
and nanoLC-MS, which is very sensitive to contaminants and particles possibly
blocking capillaries or the spray tip. This set-up also allows for fast line purging
at high flow rates, because the effluent is directly going to waste and does not
need to be pumped through low i.d. tubing (connected to the \( \mu \)-switching valve),
which would generate too much backpressure for the \( \mu \)HPD pump. A potential
disadvantage of the extra valve is the exposure of the sample to a larger surface
area with the risk of increased sample loss due to non-specific adsorption.
Due to the modified system design, the Prospekt II method was also adapted at
several points, resulting in the method presented in Table 5.2. Though most steps
remained essentially the same, the most important modifications are explained in
the following paragraphs.
For the injection of large volume samples (500 \( \mu \)L), an extra step was included
(step 4) to wash the affinity-SPE cartridge thoroughly after sample loading with
the injection loop switched off-line in the load position. Though the \( \mu \)-HPD is
starting to pump with TEB during elution-digestion in step 9, the tubings are
still filled with EDB (from purging in step 8) and a volume of only 50 \( \mu \)L EDB is
thus used to elute MMP-12 from the inhibitor affinity cartridge to the digestion
reactor. Because less EDB is used, the exposure of the trypsin reactor to EDTA
is decreased and less surfactant is introduced into the nanoLC-MS system. Ex-
periments with and without additional pumping of EDB in the elution-digestion
step, showed no significant difference in the EIC peptide intensities (data not
shown), indicating that 50 \( \mu \)L EDB is sufficient to elute MMP-12. The backpres-
sure generated during loading of the trap column (\( \approx \)15 bar) is the reason why the
affinity-SPE cartridge is switched off-line before the start of the peptide trapping
step. To prevent peptides going to waste in step 9 before loading of the trap
column, a dead volume of 40 \( \mu \)L is created after the trypsin reactor by using a
0.5-mm-i.d. tubing, to delay the tryptic peptides. The volume, loaded on the trap column in step 10 was also reduced from 220 µL to 120 µL, because fraction collection experiments and off-line nanoLC-MS analysis revealed that 95% of the MMP-12 peptides eluted from the digestion reactor in this volume (data not shown). Compared to the previous method (Table 5.1), the volumes of all purging, equilibration and washing steps were increased to minimise carry-over.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Injection valve</th>
<th>Valve 1</th>
<th>Valve 2</th>
<th>Valve 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Purge lines (500 µL ExB, 1 mL/min), exchange affinity SPE cartridge, wash AS loop and needle with 2 mL ExB</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>2</td>
<td>Equilibrate affinity cartridge (500 µL ExB, 100 µL/min), load injection loop with sample</td>
<td>Load</td>
<td>6-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>3</td>
<td>Inject sample and wash affinity cartridge (1000 µL ExB, 40 µL/min)</td>
<td>Inject</td>
<td>6-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>4</td>
<td>Wash affinity SPE cartridge (250 µL ExB, 40 µL/min)</td>
<td>Load</td>
<td>6-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>5</td>
<td>Purge lines (250 µL WB, 1 mL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>6</td>
<td>Wash trypsin reactor (500 µL WB, 200 µL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>6-1</td>
<td>1-2</td>
</tr>
<tr>
<td>7</td>
<td>Equilibrate trypsin reactor (400 µL TEB, 200 µL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>6-1</td>
<td>1-2</td>
</tr>
<tr>
<td>8</td>
<td>Purge lines (250 µL EDB, 1 mL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>9</td>
<td>Elute from affinity SPE cartridge to trypsin reactor (100 µL TEB, 12 µL/min)</td>
<td>Load</td>
<td>6-1</td>
<td>6-1</td>
<td>1-2</td>
</tr>
<tr>
<td>10</td>
<td>Load C18 trap (120 µL TEB, 20 µL/min), start LC-MS run</td>
<td>Load</td>
<td>1-2</td>
<td>6-1</td>
<td>6-1</td>
</tr>
<tr>
<td>11</td>
<td>Wash C18 trap with external HPLC pump, purge lines (250 µL EDB, 1 mL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>12</td>
<td>Wash affinity cartridge (400 µL EDB, 100 µL/min)</td>
<td>Load</td>
<td>6-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>13</td>
<td>Purge lines and injection loop (2 mL WB, 1 mL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>14</td>
<td>Purge lines (50 µL WB, 100 µL/min)</td>
<td>Load</td>
<td>1-2</td>
<td>1-2</td>
<td>6-1</td>
</tr>
<tr>
<td>15</td>
<td>Wash affinity SPE cartridge (400 µL WB, 100 µL/min)</td>
<td>Load</td>
<td>6-1</td>
<td>1-2</td>
<td>1-2</td>
</tr>
</tbody>
</table>

Table 5.2: Prospekt II method for automated MMP extraction, digestion, trapping and nanoLC-MS analysis.

5.3.7 Effect of surfactants in ExB and EDB on MMP-12 peptide signals in nanoLC-MS analysis

With the use of the nanoLC-MS system, the bed volume (0.35 µL) of the reversed phase trap column was reduced by a factor 22, resulting in a decreased trapping
capacity. Overloading of the trap column with surfactants could therefore be a risk and may abolish the positive surfactant effect on MMP-12 peptide signals. The surfactant effect may also be different due to other method modifications such as the different affinity SPE cartridge (Seph-TAPI-2) and the change in EDB composition.

The effect of the surfactant cocktail concentration in ExB on MMP-12 peptide signals was studied with 2.5 pmol MMP-12 injections (triplicates) at varying surfactant concentrations (data not shown). A clear effect of the surfactant concentration in ExB was, however, not observed and its composition was consequently not changed. These experiments were performed with an EDB containing 0.001% surfactant cocktail.

![Figure 5.13: Effect of surfactant cocktail in EDB on peptide signals, investigated with 2 pmol MMP-12 injections on the integrated nanoLC-MS system (see Fig 5.12). The surfactant cocktail concentration in ExB was 0.005%. Presented values are means of peak area EIC signals of triplicate injections and error bars indicate standard deviation. Peptides are indicated by their amino acid numbers in the legend.](image)

Figure 5.13 shows that an increasing surfactant cocktail concentration in EDB has a positive impact on the EIC signals of three plotted MMP-12 peptides. For other peptides, there also is a positive surfactant effect on the signal intensity. Apparently, overloading of the trap column does not play a role at these levels of surfactants and 0.005% of surfactant cocktail was used in both buffers for further experiments. Though the background peaks (probably originating from buffer impurities or the trypsin reactor) were increased in intensity compared with the previous set-up (see Figure 5.3; 1-mm i.d. reversed phase trap and analytical column), these did not interfere with analysis of MMP-12 peptides. Importantly,
the EIC signals of most tryptic MMP-12 peptides were at least a factor 10 higher compared to the best response obtained injecting 4 pmol MMP-12. This shows the improved overall sensitivity due to the introduced system modifications.

5.3.8 Non-specific adsorption in the digestion reactor and the effect of surfactants

The contribution of non-specific adsorption of peptides in the trypsin reactor (as a potential limiting factor in detecting low levels of MMP-12) was investigated by injecting 200 fmol of a cytochrome c digest, either directly or through the trypsin reactor on the nanoLC-MS system. A digest was chosen instead of the whole protein to study non-specific adsorption specifically, without having to consider the digestion kinetics itself. Both types of injections were done with increasing concentrations of surfactants in the sample to study the effect on non-specific adsorption and to evaluate the risk of overloading the trap column.

Figure 5.14 shows that non-specific adsorption in the trypsin reactor does not play a significant role for the two peptides shown in the right graphs, since their signals are very similar when the digest is directly injected on the nanoLC-MS system or through the trypsin reactor. Because these two peptides have either a low (indicated by a short retention time, p40-53), or a high hydrophobicity (p56-72), hydrophobicity apparently does not contribute to non-specific adsorption in the digestion reactor. The signals of the peptides shown in the left graphs are, however, decreased upon injection through the digestion reactor. The only difference with the nearly identical peptides in the right graphs is one extra lysine residue (due to a missed cleavage), which renders these peptides more basic. Increased adsorption of these peptides to immobilized trypsin could be explained by the neutralization of positive charges on lysine upon trypsin acetylation, making trypsin more acidic (with 10 acetylated lysine groups the estimated pI is 5.1), relative to native trypsin (estimated pI of 8.7). At the slightly basic digestion pH, the acetylated trypsin reactor could thus display cation-exchanger properties, resulting in a decreased recovery of basic peptides. As potential trypsin substrates, (be it with trypsin resistant cleavage sites; see Chapter 4) these 1 MC peptides may also bind to the active site, resulting in a decreased recovery.
5.3. Results and discussion

Figure 5.14: Non-specific adsorption in the digestion reactor and the effect of surfactants. Peak areas of EIC signals were obtained by direct injection (direct inject) of 200 fmol cytochrome c digest, or injection via the trypsin reactor (tryp inject) on the nanoLC-MS system. Duplicate injections (plotted intensities are averages) were done with various surfactant cocktail concentrations in the sample. Peptides are indicated by their amino acid numbers and the number of missed cleavages (MC) is given together with the retention time (RT, in minutes) on the analytical C$_{18}$ column.

For the two most hydrophobic peptides (p56-73 and p56-72), the positive effect of the surfactant in the sample on the EIC peptide signals is very strong. Both peptides could not be detected without surfactant added to the sample. Without surfactant, both peptides were apparently lost due to non-specific adsorption to surfaces at these low levels. These losses could occur in the sample vial and at equipment surfaces like needle, tubing and valves. With moderate levels of surfactants (0.005%) these peptides give very good signals, indicating a strong solubilizing effect. Towards higher surfactant levels all peptide signals are decreasing. This is probably related to overloading of the trap column with surfactants, causing displacement of the peptides. The surfactant overloading effect on the cytochrome c digest sample (volume 30 µL) starts above 0.005%, while with the integrated nanoLC-MS system there was an increasing effect on MMP-12 peptide signals up to 0.01% (added to EDB, 50 µL plug, see Fig. 5.13). So,
despite being at the limit of trap overloading conditions, higher MMP-12 peptide signals are observed. This means that the positive effect of surfactants on digestion and/or non-specific adsorption of MMP-12 (before digestion) outweighs the negative effect caused by trap overloading. The optimal surfactant concentration for extraction and digestion is, however, probably too high for the capacity of the trap column.

This indicates that a reversed-phase trap column, directly after extraction-digestion, is not the best trapping mode. The use of relatively high surfactant levels will also pollute the mass spectrometer. Since the surfactants used are non-ionic and peptides are charged, an ion-exchange trapping step would allow easy removal of surfactants, before transfer to the nanoLC-MS. A potential problem with this approach is that the EDB is neither basic nor acidic enough for efficient trapping with either anion- or cation exchange resins, hence making it difficult to work on-line without the use of another solvent in a make-up flow to modify the pH after digestion. The option to include an ion exchange step, either on- or off-line, to allow the use of high surfactant levels during the extraction and digestion steps is a subject of further research.

5.3.9 Spiking of MMP-12 in urine samples

MMP-12 was spiked into urine from a glomerulonephritis patient to study sample matrix effects on MMP-12 peptide responses. Urine was chosen as a model sample, because of the role of MMPs in bladder cancer and renal diseases and their detection in urine samples from patients with these diseases [17–21]. PEG600-C₈ was omitted from the surfactant cocktail which is used in ExB and EDB, because this surfactant gave the highest background peaks in nanoLC-MS analysis, which were interfering with MMP-12 peptides. The overall surfactant concentration was maintained.

Figure 5.15 shows that MMP-12 can be selectively detected in a urine sample matrix. The two upper traces show that the MMP-12 peptide signals are somewhat lower when spiked in urine compared to ExB. The difference, however, is relatively low and could also be caused by analytical variation, which has not been studied yet with the integrated system using nanoLC-MS analysis. The most abundant background peaks in the upper trace are trypsin autolysis products. These tend to decrease during analysis of a sample series as illustrated by the middle trace, which was injected a few runs later.
Figure 5.15: Integrated nanoLC-MS system (see Figure 5.12) analysis of urine samples spiked with MMP-12. Peaks indicated with * were tryptic MMP-12 peptides with 2 missed cleavages or less. Surfactant cocktail was used in the Prospekt buffers ExB and EDB at 0.005%, and was composed of equal concentrations DDM and PEG400-C₈.

The lowest trace in Figure 5.15 represents an analysis of 500 µL urine, spiked with 4 pmol MMP-12. The high intensity for some peptides confirms that nMol/L concentrations of MMP-12 can be selectively detected from large volume samples. The injections of samples of 1 mL or more can be envisaged, since the strong capturing power of the TAPI-2 affinity cartridge (see Chapter 3) allows large-volume samples to be effectively enriched without breakthrough of active MMPs. With a further improvement of system sensitivity, the effective enrichment from large volume samples may allow future, activity-based analysis of MMPs in bronchoalveolar lavage (BAL) fluid to study the role of MMPs, and MMP-12 in particular, in the development of emphysema. Increased MMP-12 gene expression, induced by cigarette smoke condensates [22] and increased levels of MMP-12 in BAL fluid from chronic obstructive pulmonary disease patients [23] suggest a role for this MMP in the disease mechanism.

A control cartridge having immobilized ethanolamine instead of the TAPI-2 metalloprotease inhibitor was used to study whether the background peaks (see lower trace in Figure 5.15) originating from urine proteins or peptides were enriched in
a specific way, through interaction with the inhibitor. Most peaks were however also present in the analysis with the ethanolamine control cartridge (data not shown). This means that interaction likely occurred with the Sepharose support rather than with the immobilized inhibitor. Unfortunately, no endogenous urine proteins, enriched through specific interaction with TAPI-2, have been identified yet.

Carry-over of MMP-12 peptides was investigated by performing blank runs after injections of MMP-12 (data not shown) for most peptides carry-over was absent. For the most hydrophobic peptides (eluting around 32 min in Figure 5.15) there is, however, a considerable carry-over of around 10%, when 4 or 5 pmol of MMP-12 were injected. Because only hydrophobic peptides suffer from carry-over, this is probably due to non-specific peptide adsorption. Also of importance is the observed carry-over of proteins or peptides by binding to the Sepharose support itself. This has not been investigated yet but there are indications that this carry-over is considerable, emphasizing that the search for stationary phases with low non-specific protein or peptide binding is not finished. The capacity of the automated cartridge exchanger of the Prospekt II system to use a new cartridge for each analysis will likely also be instrumental in avoiding carry-over. The inclusion of higher surfactant levels in ExB, which is used to load and wash the sample on the affinity-SPE cartridge, could also reduce carry-over by reducing non-specific binding to the Sepharose support. Non-ionic surfactants are often used to suppress non-specific binding during chromatographic protein purification steps [24]. Despite the remaining challenges, a significant advantage of the integrated system, compared to off-line procedures is speed of analysis. The total time of the Prospekt II method with 500 µL sample injection is only 75 minutes. Because the Prospekt II system and the nanoLC-MS system operate in a parallel mode, the Prospekt II system can already start with the pre-treatment steps of the next sample while the LC-MS system is still busy analyzing the previous sample. One sample can be analyzed every 75 min in this way. Although the detection limit of the system was not determined systematically, several sub-picomole MMP-12 injections (data not shown) indicate that the detection limit (with respect to the EIC signals) will be below 500 fmol. For some MMP-12 peptides, EIC peaks with a good signal-to-noise ratio have even been observed with 250 fmol MMP-12 injections. Compared to the activity-based probe method developed by Saghatelian et al., who were able to detect as little as 45 fmol MMP-2 (by in-gel fluorescence scanning), spiked into the mouse kidney proteome (total volume of 15 µL), the integrated system needs further optimization in order to reach such absolute sensitivities [10]. The fact that these authors did not detect endogenous active
MMPs (although metallo-proteases from other families were detected) in different biological samples is an indication that the levels of active MMPs in biological samples can be very low and increasing the sensitivity of the integrated system is needed. A clear advantage of the SPE system is the strong enrichment from larger volumes of biological samples, resulting in improved concentration sensitivity. Despite method improvements with respect to peptide responses at lower levels of injected MMP-12, the strong drop of peptide signal towards lower MMP-12 levels is still a limiting factor for achieving sensitivities with detection limits in the low fmol per mL region. This is most likely related to the nature of the digestion kinetics, which becomes slower at lower protein substrate concentrations. The option to use a trypsin reactor with larger bed volumes or to perform stopped-flow digestions could increase the yield of the digestion process at low MMP levels, but also increase the risk of sample loss due to non-specific adsorption. The use of elevated digestion temperatures of 37 °C, to speed up digestion kinetics, is another option which may be worthwhile to study. Trypsin reactors, based on monolithic or membrane supports with low diffusional transport limitations [25–27] may contribute further to a higher yield of the on-line digestion reaction even at low protein levels.

5.4 Conclusion

A fully automated system was developed for the activity-based analysis of MMPs in complex samples. Activity-based enrichment of MMPs with an inhibitor affinity cartridge is followed by on-line digestion in an immobilized acetylated trypsin reactor. Hyphenation of the sample pre-treatment steps to a nanoLC-MS system was achieved by loading tryptic MMP-12 peptides on a reversed-phase trapping column, followed by back-flush elution onto a 50-µm reversed-phase silica-based monolith capillary column and nanoESI-ion trap MS detection. The system was developed using MMP-12 as a model enzyme. Initially, a capillary LC-MS system was used to develop the integrated system and the feasibility of the integrated system to detect active MMP-12, be it at high levels, was demonstrated. Establishment of the integrated system simplified the sample pre-treatment procedure compared to off-line enrichment and in-gel or in-solution digestion steps. Reduction of sample losses due to reduced surface exposure was illustrated by the increased sequence coverage of MMP-12 with the integrated system (compared to fraction collection after coupled extraction-digestion). It was shown that the use of non-ionic surfactants in the sample pre-treatment steps resulted in better sensitivity towards MMP-12, probably because on-line digestion efficiency is improved.
and non-specific adsorption is suppressed. Further improvement of the sensitivity was achieved by coupling the sample pretreatment steps to nanoLC-MS analysis. With the final system design the completely automated method is able to analyze one sample every 75 min. Spiking of MMP-12 at 4 pmol into 500-µL urine samples demonstrated selective detection of tryptic MMP-12 peptides with high intensities. At sub-picomole MMP-12 levels, the signal response dropped strongly, but some tryptic MMP-12 peptides were still detected at 0.25 pmol injections (from buffer) with good signal-to-noise ratios. The strong signal drop towards lower levels of MMP-12 is an indication that the digestion kinetics remain a limiting factor. For future detection of endogenous active MMPs in biological samples, further research is required to improve the sensitivity of the method.
References


Matrix metallo-proteases constitute a family of extracellular zinc-dependent endopeptidases that are involved in degradation of extracellular matrix (ECM) components and other bioactive non-ECM molecules. A plethora of studies have implicated important roles for MMPs in many diseases (including cancer and chronic inflammatory diseases) and normal biological processes. Our understanding of the complex roles of MMPs is, however, still limited, which is illustrated by the poor performance of broad-spectrum MMP inhibitors in clinical trials. The regulation of MMP activity by post-translational mechanisms is a complicating factor in MMP biology and pathology, and diminishes the effectiveness of conventional "expression-based" proteomic methods to study MMPs. The ability to profile MMPs based on functionally related activities would greatly facilitate research about their involvement in pathological processes.

This thesis describes the development of a liquid chromatography-mass spectrometry-based integrated system for the selective detection of active MMPs in complex biological samples. Throughout the thesis, purified recombinant MMP-12 (catalytic domain) is used as a model enzyme.

Chapter 2 demonstrates the feasibility of activity-based MMP-12 enrichment through batch extractions with an immobilized inhibitor affinity sorbent. A broad-range MMP inhibitor (PLG-NHOH) was immobilized on a Sepharose support with a ligand density of 9.8 mmol/L. The functionality of the inhibitor after immobi-
lization was demonstrated with batch extractions of MMP-12 added to buffer, resulting in extraction yields around 97%. Quantification was done indirectly using an activity assay to determine unbound MMP-12. Active MMP-12 could also be selectively extracted when added to serum (after inactivation of the endogenous inhibitor \( \alpha_2 \)-macroglobulin with trypsin) at a low level. Experiments with the endogenous inhibitors TIMP-1 and \( \alpha_2 \)-macroglobulin revealed that MMP-12 extraction is strictly activity-dependent.

Chapter 3 considers the automation of the extraction which is needed for on-line coupling to downstream analytical steps. Samples containing MMP-12 in buffer were extracted at different flow rates using cartridges packed with inhibitor affinity sorbent. Besides faster extractions and a reduced number of manual manipulations, higher extraction yields (98.9% - 99.3%) were obtained over the whole flow rate range compared to batch extractions. Application of the method to synovial fluid from a rheumatoid arthritis patient followed by gelatin-zymography revealed a strong enrichment of distinct MMPs from this biological sample that were not clearly visible in the original sample. The use of an auto-sampler and a solid-phase extraction (SPE) workstation allowed full automation of the extraction procedure. MMP-12 extractions were optimized, showing that ligand density is an important factor with a clear extraction yield optimum around 4 to 10 mmol/L. Conditioning of the Sepharose affinity sorbent for 1 week prior to use resulted in a further increase in extraction yield. Under optimal conditions, an extraction yield of 99.5% was reached with a cartridge contact time of only 13 s for MMP-12. The efficacy of the extraction method for activity-based MMP profiling was further improved by the use of a broad-spectrum MMP inhibitor with nmol/L affinity (TAPI-2), which resulted in increased extraction yields for all tested MMPs. Extraction yields ranging from 98.8% to 99.8% were obtained for MMP-1, -7, -8, -10, -12, and -13, while for MMP-9 (full length and catalytic domain) extraction yields of 96.1% and 98.4% were reached (all at a cartridge contact time of 19 s). This effective enrichment of MMPs illustrates the possibility to enrich from dilute samples with low levels of endogenous MMPs.

In Chapter 4, we investigated the chemical modification (acetylation) of immobilized trypsin (on a Sepharose and a polystyrene support) for enhanced digestion efficacy in integrated protein analysis platforms. Complete digestion of cytochrome c was obtained with modified-trypsin beads with a contact time of only 4 sec, while corresponding unmodified-trypsin beads gave incomplete digestion. The digestion rate of myoglobin, a protein known to be rather resistant to proteolysis, was not altered by acetylating trypsin and required a buffer containing 35% acetonitrile to obtain complete digestion. The use of acetylated-trypsin
beads led to fewer interfering tryptic autolysis products, indicating an increased stability of this modified enzyme. Importantly, the modification did not affect trypsin’s substrate specificity, as the peptide map of myoglobin was not altered upon acetylation of immobilized trypsin. Kinetic digestion experiments in solution with low-molecular-weight substrates and with cytochrome c confirmed the increased catalytic efficiency (lower $K_M$ and higher $k_{cat}$) and increased resistance to autolysis of trypsin upon acetylation. Because of the increased trypsin activity, the digestion rate is enhanced, which facilitates on-line digestion of low-abundance proteins with higher yields and in less time. These are favourable properties of the modified trypsin reactor and should make it a valuable tool in automated protein analysis systems.

Chapter 5 deals with the implementation of the extraction and the digestion cartridge into an integrated system for automated, activity-based profiling of MMPs. Enrichment was followed by on-line digestion in an immobilized acetylated trypsin reactor. Hyphenation of the sample pre-treatment steps to a nanoLC-MS system was achieved by loading tryptic MMP-12 peptides on a reversed-phase trap column, followed by backflush elution to a 50-μm reversed-phase silica-based monolith capillary column coupled to a nanoESI interface and an ion trap mass spectrometer. The use of non-ionic surfactants in the sample pre-treatment steps resulted in better sensitivity, probably because on-line digestion efficiency is improved and non-specific adsorption is suppressed. The completely automated method is able to analyse a sample every 75 min. Spiking of MMP-12 at 4 pmol into 500 μL urine resulted in selective detection of tryptic MMP-12 peptides with high intensities. At sub-pmol MMP-12 levels the signal dropped strongly, but some tryptic MMP-12 peptides were still detected at 0.25 pmol in 100 μL injections (from buffer) with good signal-to-noise ratios.

Though the developed system has not been extensively tested with biological samples to detect active endogenous MMPs, several aspects should likely be improved in order to achieve this. One important limiting factor in obtaining low or sub-fmol sensitivity is probably the rate of digestion in the trypsin reactor. Despite the relatively high concentration of immobilized trypsin and the chemical modification, the digestion rate is still limited by the protein substrate concentration (due to Michaelis-Menten kinetics). The use of support materials with less diffusional transport limitations such as monolithic materials in both the enrichment and the digestion step will likely result in improved digestion rates at low MMP concentrations. Captured MMPs can be eluted from a monolithic extraction cartridge in a smaller volume and thus be digested at higher concentration
in digestion reactor, resulting in improved digestion kinetics. Reduced diffusional transport limitation in the trypsin reactor may also contribute to a further improvement of the digestion kinetics at low MMP concentrations.

Digestion kinetics may also be further improved through the investigation of other types of chemical modifications of immobilized trypsin. Hydrophilic protein modifications generally result in an enhanced thermal stability and may permit the use of elevated digestion temperatures to achieve faster digestion kinetics. A decreased binding of hydrophobic peptides to immobilized trypsin, resulting in higher peptide yields and a lower carry-over is an additional advantage of hydrophilic modifications.

Chapter 5 demonstrated a positive effect of non-ionic surfactant additions to the extraction and digestion buffers on the mass spectrometric MMP-12 peptide signals. Yet, higher levels of these surfactants have an adverse effect on the effective capacity (due to overloading) of the reversed-phase trap column and will pollute the ESI interface. The option to include an ion-exchange step, either on- or off-line to wash away high levels of surfactant prior to reversed-phase trapping, should thus be a subject of further research. Care must to be taken, however, not to introduce too many system steps, which would result in high system complexity accompanied by low robustness.

Validation (LOD, LOQ, day-to-day and intra-day variation) of the integrated analytical system should be performed before application to clinical studies, once the system has been further optimized. The aspect of quantification of detected MMPs should also be addressed in future research. To correct for different yields of the different steps, ideally an internal standard with a known concentration, added to the biological sample, should be used. This can be achieved by using isotopically labelled MMPs (produced by expression in a host organism which is grown on $^{15}\text{N}$-enriched medium).

To broaden the applicability of the developed system to a more diverse group of metallo-proteases, the use of affinity sorbents with a cocktail of immobilized metallo-protease inhibitors targeting different types of enzymes can be investigated. Through the use of affinity sorbents targeting completely different protein classes (of low abundance), the developed system can be potentially applied in a wide range of studies. The requirement for different elution conditions with each type of affinity sorbent affects the on-line coupling with the digestion reactor and is the main step which needs to be evaluated before the system can be used to analyse other protein classes. Digestion reactors with immobilized proteases, other than trypsin, may be required to ensure elution-digestion compatibility.