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Quantification of matrix metalloprotease-9 in bronchoalveolar lavage fluid by selected reaction monitoring with microfluidics nano-liquid-chromatography–mass spectrometry

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ABSTRACT

Quantitative protein analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the selected reaction monitoring (SRM) mode was used to quantify matrix metalloprotease-9 (MMP-9; ~90 kDa) in bronchoalveolar lavage fluid (BALF) from patients having undergone lung transplantation. We developed an SRM assay for microfluidics-based nanoLC–MS/MS on a triple quadrupole mass spectrometer based on two signature peptides. Samples were prepared by chloroform–methanol precipitation followed by trypsin digestion in the presence of stable-isotope-labeled internal peptide standards. The method allows accurate quantification of MMP-9 in BALF with an LLOQ of 2.9 ng/mL and an LLOD of 0.25 ng/mL without the use of extensive fractionation or antibodies.

1. Introduction

Clinical validation of potential biomarkers requires quantitative analyses. Most current quantitative analyses rely on enzyme-linked immunosorbent assays (ELISA). This technique is one of the most sensitive approaches (pg/mL range) to measure proteins in complex biological samples. To develop a specific and sensitive ELISA in the most common 'sandwich' format, two antibodies recognizing different epitopes in the same protein with high specificity and affinity are required. The need for two 'orthogonal' high-affinity antibodies may render assay development costly and time consuming. It is furthermore uncertain whether the antibodies recognize the target protein specifically, since the readout of the assay is indirect (color, fluorescence, chemiluminescence).

In recent years, there has been increasing interest in the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the selected reaction monitoring (SRM) mode for protein quantification [1–8]. The main advantage of the SRM-based protein assay is facile multiplexing due to the capacity of modern triple quadrupole mass spectrometers to monitor a range of peptides in a single chromatographic run using scheduled SRM. However, the main drawback of this technique, compared to immunoassays, is its lower sensitivity. SRM protein quantification in plasma, without immunodepletion and/or extensive fractionation steps, allows protein detection at the low μg/mL level [9,10]. To reach the low ng/mL range in serum, reducing sample complexity is mandatory and achieved by applying immunoaffinity enrichment with immobilized antibodies [11–19] and/or prefractionation [20,21] which also reduces ion suppression. Prefractionation or enrichment allows further to introduce higher amounts of the target protein into the analytical system making better use of the generally limited binding capacity of the LC column, which remains one of the sensitivity-limiting factors in protein analysis by LC–MS/MS.

SRM assays have been primarily developed for protein analysis in plasma and serum, which are easily obtained in sufficient quantity during routine medical visits. Bronchoalveolar lavage fluid (BALF) is, however, a more suitable sample when studying pulmonary disease, since BALF gives a representative picture of processes occurring in the airways. BALF is a challenging biological matrix due to its high content of phospholipids, which interfere with electrospray ionization and can lead to extensive ion suppression. This means that methodology developed for plasma or serum cannot be directly transferred to BALF.

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Transplantation remains the ultimate therapy for patients with end-stage pulmonary disease [22,23] but may be accompanied by early complications such as acute rejection of the transplanted lung. Matrix metalloproteases (MMPs), belonging to the family of zinc-dependent endopeptidases, are involved in a wide range of biological processes related to extracellular matrix (ECM) remodeling and tissue repair [24,25]. MMPs are tightly regulated *in vivo* and any deregulation may lead to broad pathological processes. Notably MMP-9 has been shown to be upregulated during chronic inflammation (e.g. during allergic asthma) [25,26]. To further assess the role of MMP-9 in pulmonary disease and notably in the rejection of lung transplants, we established a human MMP-9-specific SRM assay.

The developed method requires only generic sample preparation consisting of chloroform–methanol precipitation and trypsin digestion. The LC–MS/MS part of the assay uses a fully automated microfluidics-based nanoLC–MS/MS system coupled on-line to a triple quadrupole mass spectrometer and allows detecting MMP-9 at the sub-ng/mL level without the use of immunoaffinity enrichment and/or extensive prefractionation.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium bicarbonate, dimethyl sulfoxide, chloroform, dithiothreitol, formic acid, and iodoacetamide were purchased from Sigma Aldrich. Formic acid and methanol were ordered to Fluka. Trypsin (sequencing grade, cat. no. V5111) was from Promega, NaCl was obtained from Merck and acetonitrile from Biosolve. Ultrapure water (resistivity of 18.2 MΩ cm) was obtained from a Sartorius Stedim water purification system (model 611 VF). The following stable isotope-labeled internal standard peptides were obtained from JPT Peptide Technologies (http://www.jpt.com): AVIDDAFAXSAZ; AFALWSAVTPLTFTXSAZ (X = Arg$^{13}C_6$, 97–99%; $^{15}N_4$, 97–99%, Z = JPT-Tag, which is removed during tryptic digestion).

2.2. Human recombinant MMP-9 catalytic domain Rec. MMP-9 CD (0.1 mg/mL) in 50 mMTris

0.1 mg/mL in 50 mM Tris, 10 mM CaCl2, 20 μM ZnCl2, 150 mM NaCl, 0.02% Brij, pH 7.5, without the fibronectin type II-like repeats (residues 107–216 fused to residues 391–443 with an additional Met-Gly dipeptide at the N terminus; Table S1, supporting information for complete sequence) was a kind gift from AstraZeneca and expressed according to the protocol of Shipley et al. [27].

2.3. BALF

BALF was obtained from patients having undergone lung transplantation for COPD (8 patients), cystic fibrosis (1 patient) or idiopathic pulmonary fibrosis (1 patient) (Table S2, supporting information). BALF was collected as described elsewhere [28] and stored at −80 °C. The procedures followed were in accordance with national and local ethical guidelines.

2.4. IL-6 and IL-8 measurements

IL-6 and IL-8 were measured in BALF by a chemiluminescent immunoassay (Immulite IL-6 and Immulite IL-8, Siemens Healthcare Diagnostics Products LTD, Gwynedd, UK) performed on an Immulite 1000 system.

2.5. Determination of total protein concentration

BALF was diluted 3-fold with PBS (phosphate buffer saline; 140 mM NaCl, 9 mM Na_2HPO_4, 1.3 mM $Na_2H_2PO_4$, pH 7.4) to determine the total protein concentration with the microBCA assay according to the manufacturer's instructions (Pierce). Absorbance was measured with a Fluostar Optima plate reader (BMG, Labtech) at 580 nm.

2.6. Sample preparation

BALF (10 μg of total protein) or rec. MMP-9 CD (2.5 μg), was diluted to a final volume of 130 μL in PBS, reduced with 4 mM dithiothreitol (15 min at 56 °C) and then alkylated with 12 mM iodoacetamide (20 min at room temperature in the dark). Proteins were precipitated prior to tryptic digestion with chloroform–methanol according to Wessel and Flugge [29]. Briefly, samples were mixed with 600 μL methanol, 150 μL chloroform and 450 μL water by vortexing and centrifuged at 13,600 × *g* for 5 min. The pellet was washed with 450 μL of methanol and centrifuged as above. The final pellet was dried under the fume hood, suspended in 20 μL of 50 mM ammonium bicarbonate buffer and digested for 5 h at 37 °C (ratio trypsin/protein: 1/30, w/w).

2.7. Selection of signature peptides and SRM optimization

To assess the digestion efficiency for rec. MMP-9 CD and to select peptides for quantification, the triple quadrupole mass spectrometer was operated in full scan and product ion scan mode with the fragmentor (FR) set at 135 V, a dwell time of 150 ms and collision energies (CE) of 4, 12, 20, 30 or 50 V. The most promising transitions were further optimized with steps of 4 V for CE and 10 V for FR. This optimization was done using the Agilent MassHunter Optimizer Automated MS Method Development Software (version B.02.01) injecting 1 pmol of trypsin-digested rec. MMP-9 CD.

2.8. Optimization of MS–MS selectivity and sensitivity

To improve sensitivity, resolution of the first and third quadrupole mass analyzer were set to ±0.35 unit (u), ±0.6 u or ±1.25 u (full width at half height), respectively. To determine the optimal resolution of the third quadrupole, the first quadrupole was kept at ±0.35 u. To determine the optimal resolution of the first quadrupole, the third quadrupole was kept at ±1.25 u. Sensitivity for the selected SRM transitions was assessed by determining the peak area of the quantifier ion of the targeted peptide after injecting 14 fmol of rec. MMP-9 CD in BALF after digestion with trypsin (8 μg total protein; samples were spiked to 1.4 ng/mL of rec. MMP-9 CD corresponding to a final concentration of 12.96 ng/mL tryptic rec. MMP-9 CD digest in BALF that was injected). Selectivity of the measurements was evaluated by comparing the ratio of the transitions in the presence and absence of biological matrix at each of the resolution settings to assess whether peptides related to trypsin-digested BALF proteins interfered with the target peptides.

2.9. LC–MS/MS

Samples were analyzed by nanoLC–MS/MS using a microfluidics (chip-cube) interface (Agilent, cat. no. G4240A) on a C-18 chip (Agilent, custom-made) with a 500 nL enrichment column (Zorbax 300 SB C-18, 5 μm) and a 75 μm × 150 mm separation column packed with the same chromatographic material. The interface contained a nanoelectrospray tip (2 mm length with conical shape: 100 μm OD × 6 μm ID) that was coupled on-line to a triple quadrupole mass spectrometer (Agilent, G6410B). Tryptic digests (4 μg total protein), dissolved in 5% formic acid (FA)/5% dimethylsulfoxide (DMSO),

were loaded on the trap column in 0.1% aq. FA, 3% acetonitrile at 2.5 μL/min via the autosampler (Agilent, cat. no. G1377A) equipped with an injection loop of 40 μL (Agilent, cat. no. G1377A) and a thermostated cooler (Agilent, cat. no. G1330B) maintaining the samples in the autosampler at 4 °C. The interface was connected to an Agilent 1200 series HPLC system containing the following modules: nanopump (Agilent, cat. no. G2226A), capillary pump (Agilent, cat. no. G1376A) and solvent degasser (Agilent, cat. no. G1379B). The samples were enriched in the forward flushing mode (in 0.1% aq. FA, 3% acetonitrile, and flushed with an extra 4 μL volume using the capillary pump at 2.5 μL/min) after which the trapping column was switched in-line with the analytical column. Peptides were eluted with eluents A (0.1% FA in water) and B (0.1% FA in acetonitrile) at a flow rate of 0.25 μL/min using the following gradient program: 3%–10% eluent B at 2%/min, 10%–35% eluent B at 0.5%/min, 35–60% eluent B at 2%/min followed by 60% to 95% eluent B at 11.6%/min. Eluent B (95%) was maintained for 3 min before returning to the starting conditions over 10 min. The column was equilibrated at the starting conditions for 10 min before starting the next injection. Carry-over was avoided by washing the needle for 10 s with 0.1% FA in 20% methanol.

Calibrants and samples were analyzed with wide resolution (±0.6 u at half height) for the first quadrupole and widest resolution (±1.25 u at half height) for the third quadrupole. Each transition was monitored with a dwell time of 150 ms in segmented SRM mode as follows: 0–39 min; target peptide: 489.3 → 404.2/579.3/694.3/807.4, FR 210, CE 8/22/13/12; internal standard: 494.3 → 409.2/589.3/704.3/817.4, FR 210, CE 8/22/13/12; 39–92 min; target peptide: 841 → 219.1/290.1/1092.6, FR 220, CE 41/49/33; internal standard: 846 → 219.1/290.1/1102.6, FR 220, CE 41/49/33.

2.10. Calibration curves without and with biological matrix

A tryptic digest of rec. MMP-9 CD was diluted in 5% FA/5% DMSO to establish a calibration curve at the following concentrations (52 ng/mL, 26 ng/mL, 13 ng/mL, 8.64 ng/mL, 5.2 ng/mL, 0 ng/mL) using the optimized SRM method. Prior to analyzing BALF samples from lung transplantation patients, a calibration curve was established in a pool of 13 BALF samples (8 lung transplantation patients and 5 patients with other pulmonary pathologies such as COPD or sarcoidosis) spiked with rec. MMP-9 CD as follows: 0 ng/mL, 0.15 ng/mL, 0.5 ng/mL, 2.9 ng/mL, 9.6 ng/mL, 11.56 ng/mL, 28.91 ng/mL and 57.8 ng/mL, which corresponds to a concentration of the tryptic digests prior to SRM analysis of: 0 ng/mL, 0.28 ng/mL, 0.94 ng/mL, 5.41 ng/mL, 18.04 ng/mL, 21.73 ng/mL, 54.35 ng/mL and 108.6 ng/mL. The samples for each calibrant point were prepared separately in duplicate and analyzed once by LC–MS/MS. Stable-isotope-labeled internal peptide standards (AVIDDAFAXSAZ; AFALWSAVTPLTFTXSAZ) were added prior to tryptic digestion to a final amount of 10 fmol per sample of which 4 fmol were injected.

2.11. Repeatability of sample preparation and protein recovery

BALF samples from three different patients were spiked with 1.4 ng/mL of rec. MMP-9 CD corresponding to a final concentration of 12.96 ng/mL tryptic digest and measured by SRM as described above to assess the repeatability of sample preparation. Protein recovery was assessed in triplicate by measuring the total protein concentration after dissolving the protein pellet in 2% aq. sodium dodecyl sulfate (SDS) after chloroform–methanol precipitation.

2.12. Determination of LLOD and LLOQ

The signal to noise ratio (SNR) was determined manually by selecting a baseline region of 0.5–1 min following the peptide peak for noise estimation. The accurate LLOQ (lower limit of quantification) was based on a SNR of 10 and an accuracy of at least 75%. The LLOD (lower limit of detection) was based on a SNR of 3.

Data were processed in an automated manner with the quantitative software of Agilent (version B.03.02) and manually verified with the qualitative software (version B.01.03). The relative response uncertainty was set to ±25 for the ratio of the monitored transitions and Gaussian smoothing (15 and 5 points for function and Gaussian width respectively) was applied. Peaks were integrated automatically with the algorithm of the Agilent software.

2.13. Analysis of MMP-9 in BALF from lung transplantation patients

BALF samples from 10 patients (Table S2, supporting information, for details) were randomized (MATLAB random permutation test) and prepared in duplicate (see Fig. 1 for a schematic overview of sample preparation). Prior to and after LC–MS/MS analysis of the BALF samples, calibration curves were established to assure consistent system performance. A volume of 17.7 μL was injected for all samples and all calibration points. System performance and stability were further followed by injecting 7 control samples (trypsin-digested rec. MMP-9 CD) in regular intervals.

3. Results

3.1. Choice of signature peptides and SRM optimization

The choice of signature peptides is a crucial step to obtain a sensitive, specific, and reproducible SRM assay. In order to select the most appropriate peptides, the catalytic domain of rec. MMP-9 CD was digested with trypsin and the digest analyzed by LC–MS on a triple quadrupole mass spectrometer (Fig. S1, supporting information). Table S1 shows the detected peptides in comparison to the expected tryptic peptides. Peptides of excessive length, containing instable amino acids such as M or C or missed cleavage sites were discarded, since they may lead to poorly reproducible SRM assays. The remaining peptides WHHHNITYWIQNYSEDLPR, AVIDDAFAR, and AFALWSAVTPLTFTR were subjected to a sequence homology search using the BLASTP network service against the UniProt Knowledgebase Release 2011_07 (28-Jun-11, homo sapiens) database consisting of UniProtKB/Swiss-Prot with 530264 entries and UniProtKB/TrEMBL with 16014672 entries to assure that they do not occur in any other human protein. These three peptides were further analyzed with respect to their suitability for developing selective, high-sensitivity, quantitative SRM assays. While peptide WHHHNITYWIQNYSEDLPR showed promising results with a pure rec. MMP-9 CD digest, it was no longer detectable when rec. MMP-9 CD was added to BALF and digested. This peptide was thus not followed up. Product ion scans of the other two peptides at increasing collision energies resulted in suitable MS/MS spectra for AVIDDAFAR and somewhat less intense fragment ions for AFALWSAVTPLTFTR (Fig. 2). Optimal conditions for the doubly-charged precursor ions of AVIDDAFAR and AFALWSAVTPLTFTR are given in Table 1. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R residue were used as internal standards and subjected to collision-induced dissociation under identical conditions (Table 1).

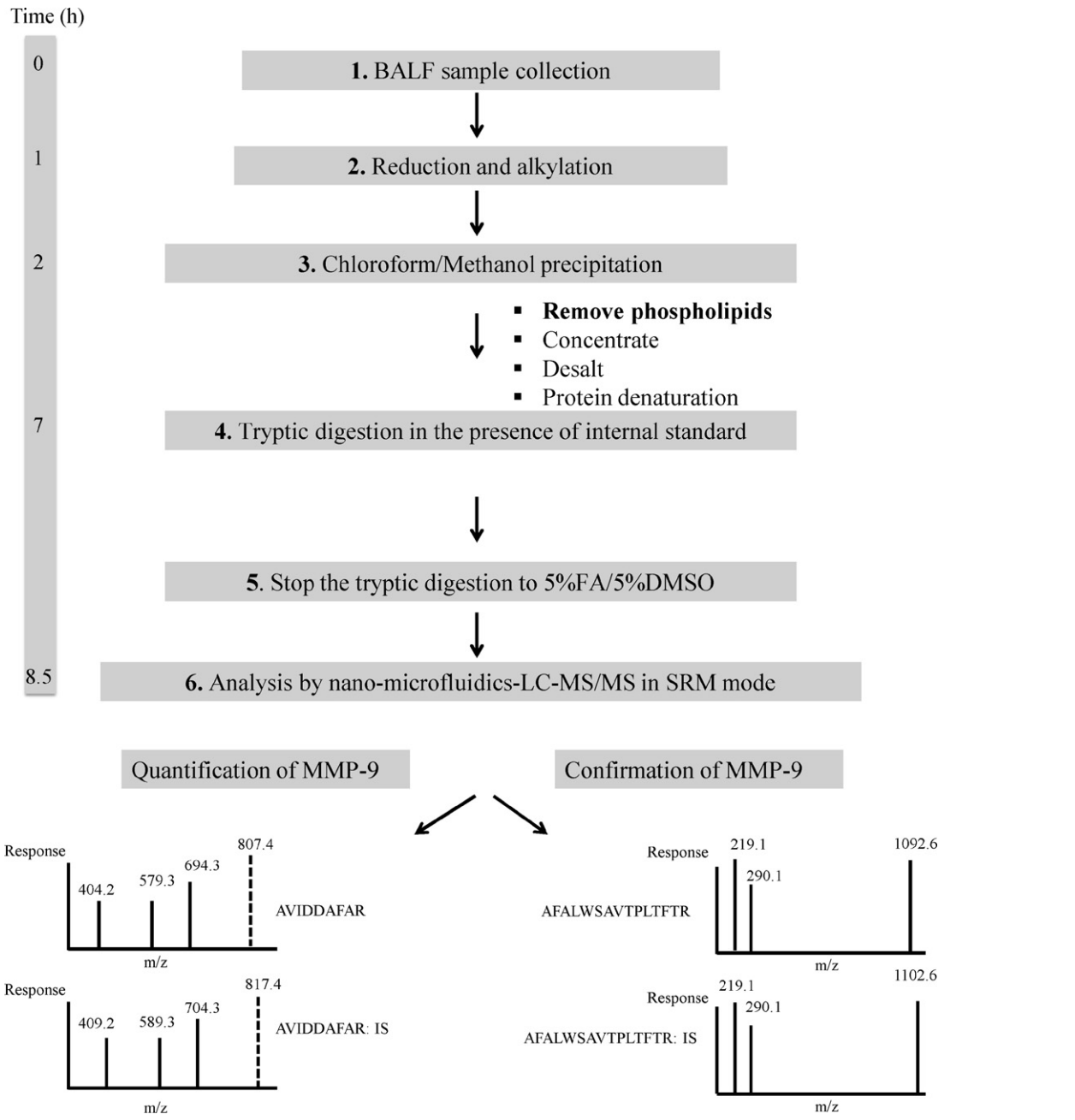

Fig. 1. Schematic overview of the overall sample preparation procedure of BALF samples for semi-quantification of human MMP-9 by SRM analysis. Aliquots of BALF are reduced and alkylated prior to chloroform–methanol precipitation. Precipitation allows to desalt and concentrate the samples, denature proteins prior to trypsin digestion, and to remove the abundant phospholipids. To develop a high-sensitivity SRM assay for human MMP-9, two peptides were targeted. AVIDDAFAR was used for quantification due to its good response and AFALWSAVTPLTFTR was followed to provide confirmatory evidence. Three or 4 transitions per peptide were followed (the transition that is represented as a dashed line is used for quantification). The time axis on the left gives an estimate of the required time, showing that the entire procedure can be completed in one day. It is possible to prepare multiple samples in parallel for SRM analysis.

3.2. Development of a semi-quantitative SRM assay for MMP-9 in BALF

In order to find the best compromise between selectivity and sensitivity, we evaluated the effect of changing the width of the selection window for parent (Q1) and fragment ions (Q3) for AVIDDAFAR between ±0.35 u, ±0.6 u, and ±1.25 u and monitoring the peak area of the quantifier ion (y7 (807.4)) in the absence and presence of BALF. To this end, a pool of BALF samples from lung transplant patients was spiked with 1.4 ng/mL rec. MMP9 CD, chloroform–methanol precipitated and trypsin digested. To determine the effect of changing resolution of Q3, we maintained Q1 at unit resolution. No interference was detected even when Q3 was opened to ±1.25 u while sensitivity increased by a factor of 2.4. Q3 was thus set to this resolution while varying the transmission window of Q1 for parent ion selection. No interferences from the biological matrix (BALF) were observed when using a width of ±0.6 u while sensitivity increased by another factor of 2.5. When widening parent ion transmission to ±1.25 u, the response of the stable isotope-labeled internal standard peptide differed significantly from the non-labeled peptide indicating interference of components from the biological matrix with the standard. For further development of the assay, we therefore used a width of ±0.6 u for Q1 and ±1.25 for Q3.

Table 1
Optimized parameters for MMP-9-specific SRM assays based on peptides AVIDDAFAR and AFALWSAVTPLTFTR monitoring 4 or 3 product ions, respectively. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R (shown in italics) were chosen as internal standards.

Peptide sequence	Parent ion (*m*/*z*)	FR (V)	Product ion (*m*/*z*)	CE (V)
AVIDDAFAR	489.3 (+2)	210	807.4 (y7; +1)	12
			694.3 (y6; +1)	13
			579.3 (y5; +1)	22
			404.2 (y7; +2)	8
AVIDDAFA*R*	494.3 (+2)		817.4 (y7; +1)	12
			704.3 (y6; +1)	13
			589.3 (y5; +1)	22
			409.2 (y7; +2)	8
AFALWSAVTPLTFTR	841 (+2)	220	1092.6 (y10; +1)	33
			219.1 (b2; +1)	41
			290.1 (b3; +1)	49
AFALWSAVTPLTFT*R*	846 (+2)		1102.6 (y10; +1)	33
			219.1 (b2; +1)	41
			290.1 (b3; +1)	49

FR, fragmentor voltage; CE, collision energy.

Based on previous observations that peptides tend to adsorb to autosampler vials [30], tryptic digests of the catalytic domain of rec. MMP-9 CD were diluted in 5% FA/5% DMSO. To assess the suitability of these peptides for quantitative work calibration curves were established in the absence of BALF. By monitoring the transitions as given in Table 1, AVIDDAFAR gave a linear response without correction based on the stable isotope-labeled internal standard peptide ($y = 480.55x + 718.87$, $r^2 = 0.9962$; Fig. 3A). Transitions for AFALWSAVTPLTFTR were also detected at all concentration levels but with poor linearity ($r^2 = 0.8164$) indicating problems with peptide adsorption (Fig. 3C). Correlation between peptide concentration and signal intensity for this peptide improved when adding trypsin-digested rec. MMP-9 CD to BALF, mostly likely since the excess of peptides derived from digested BALF proteins competed with the signature peptide for the available adsorption sites ($r^2 = 0.9112$) (Fig. 3D).

Due to the better response of AVIDDAFAR as compared to AFALWSAVTPLTFTR in BALF (Fig. 3B and D), we based our quantitative results on AVIDDAFAR, while detection of AFALWSAVTPLTFTR was used as confirmatory evidence for the presence of endogenous MMP-9.

Quantification of MMP-9 in BALF requires sample preparation notably to remove the highly abundant phospholipids [31], which interfere with electrospray ionization [32,33]. To assess repeatability of sample preparation using chloroform–methanol precipitation of proteins, three separate aliquots of BALF from lung transplant patients were spiked with rec. MMP-9 CD at 1.4 ng/mL (see Section 2 for details) and measured by LC–MS/MS in the SRM mode. The peak area of the quantifier ion of AVIDDAFAR (y7 (807.4)) showed a standard error of ±8.2%, indicating that sample preparation is highly repeatable. Protein recovery after chloroform–methanol precipitation was excellent (starting protein concentration: 36.2 μg/mL; protein concentration after sample preparation: 36.1±0.5 μg/mL).

Table 2 shows that the bias of the method ranged between 0.4 and 21% above the LLOQ of 2.9 ng/mL. Since the pooled BALF sample contained a low but detectable amount of MMP-9 of 0.6 ng/mL, this amount was subtracted from all measured rec. MMP-9 CD levels to calculate accuracy. Due to endogenous MMP-9 in the pooled of BALF used for the calibration curve, the lowest points (0.15 and 0.5 ng/mL) show poor accuracy. The lower limit of accurate quantification was thus set at 2.9 ng/mL (SNR ~ 35). To determine the

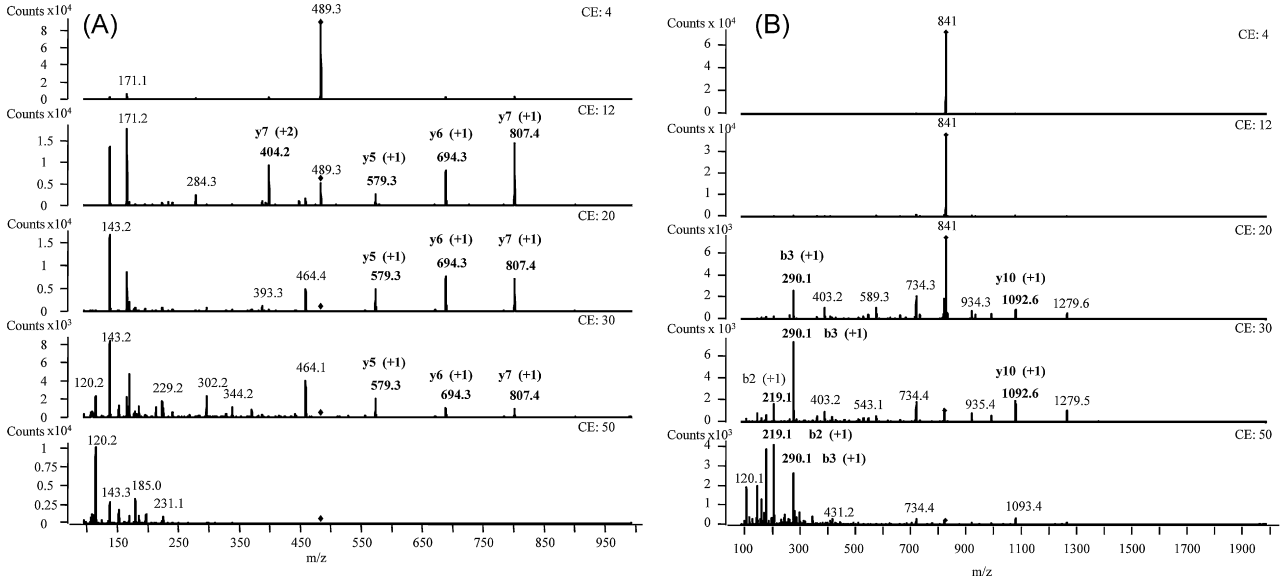

Fig. 2. Left panel: Product ion scan of peptide AVIDDAFAR (parent ion: 489.3 (+2); ♦) at different collision energies (CE of 4, 12, 20, 30, 50 V) from top to bottom. Right panel: Product ion scan of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2); ♦) at different collision energies (CE of 4, 12, 20, 30, 50 V) from top to bottom. MS/MS conditions were further optimized with the Agilent MassHunter Optimizer Automated MS Method Development Software to arrive at the final MMP-9 SRM assay (Table 1, for details).

Table 2
Determination of the bias of measuring MMP-9 in BALF by LC–MS/MS in the SRM mode based on peptide AVIDDAFAR (parent ion: 489.3 (+2)). Quantification was based on the quantifier ion (807.4 (y7 (+1)) and three qualifier ions were monitored: 694.3 (y6 (+1)), 579.3 (y5 (+1)), 404.2 (y7 (+2)). Based on these analyses an LLOQ (35-times the SNR) of 2.9 ng/mL and an LLOD (3-times the SNR) of 0.25 ng/mL were determined. Note that there was a low level of endogenous MMP-9 (0.59 ng/mL) in the pooled BALF.

Spiked MMP-9 in BALF (ng/mL)	Measured MMP-9 in BALF (ng/mL)	Mean MMP-9 in BALF (ng/mL)[a]	Calculated bias (%)[b]
0	0.6	0.6 (0)	Not applicable
	0.5		
0.15	1.1	1.0 (0.4)	180
	0.9		
0.5	1.4	1.4 (0.8)	56
	1.3		
2.9	3.6	3.3 (2.7)	7.3
	3.0		
9.6	9.4	9.3 (8.7)	9.2
	9.2		
11.6	13.7	12.2 (11.6)	0.4
	10.8		
28.9	27.1	23.4 (22.8)	21.2
	19.6		
57.8	59.2	60.5 (59.8)	3.6
	61.7		

[a] Values in brackets are corrected by subtracting the level of endogenous MMP-9 (0.6 ng/mL) from the measured values.
[b] bias was calculated based on the corrected concentrations.

lower limit of detection the SNR was set to 3. This resulted in an LLOD of ~250 pg/mL (about 3 pM for a protein of ~90 kDa). Considering that 17.7 μL were injected per calibration point, this corresponded to on-column amounts of 50 pg (~560 amol) at the LLOQ and 4.4 pg MMP-9 (49 amol) at the LLOD. Injection of 5% FA/5% DMSO between analyses showed that there was no detectable carry-over.

3.3. Analysis of BALF from lung transplantation patients

10 BALF samples (10 μg total protein per sample) from patients having undergone lung transplantation (Table S2) were prepared as shown in Fig. 1 and analyzed by LC–MS/MS. Since BALF samples showed considerable variation in total protein concentration (Table S2), we report the measured MMP-9 levels (based on the

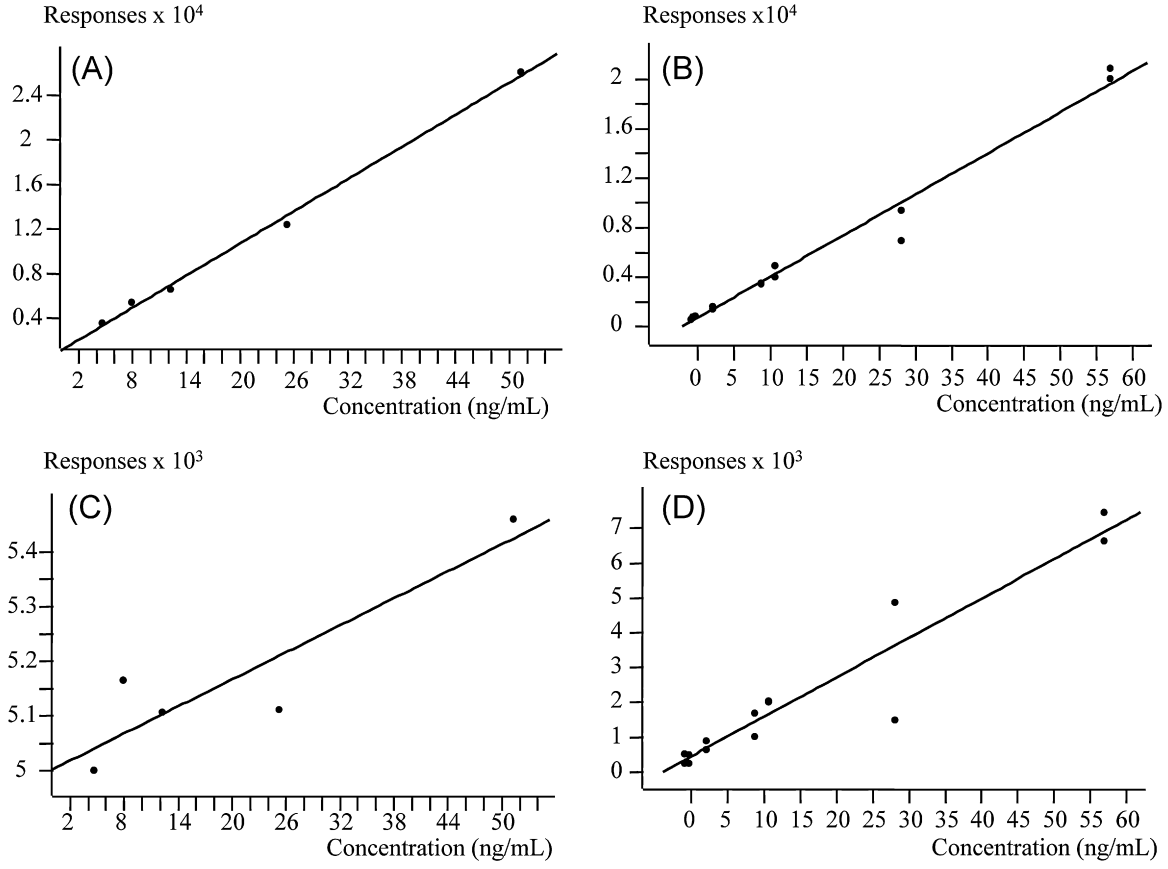

Fig. 3. (A) Calibration curve of peptide AVIDDAFAR (parent ion: 489.3 (+2)) from rec. MMP-9 CD injected in 5% FA/5% DMSO between 5.2 ng/mL and 52 ng/mL. Quantifier ion: 807.4 (y7 (+1)). Qualifier ions: 694.3 (y6 (+1)), 579.3 (y5 (+1)) and 404.2 (y7 (+2)); $y = 480.55x + 718.87$, $r^2 = 0.9962$. (B) Calibration curve of peptide AVIDDAFAR (parent ion: 489.3 (+2)) from trypsin-digested rec. MMP-9 CD in BALF at 8 levels between 0 and 57.8 ng/mL. Quantifier ion: 807.4 (y7 (+1)). Qualifier ions: 694.3 (y6 (+1)), 579.3 (y5 (+1)) and 404.2 (y7 (+2)). $y = 331.63x + 418.60$, $r^2 = 0.9801$. (C) Calibration curve of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2)) from rec. MMP-9 CD injected in 5% FA/5% DMSO between 5.2 ng/mL and 52 ng/mL. Quantifier ion: 1092.6 (y10 (+1)). Qualifier ions: 290.1 (b3 (+1)), 219.2 (b2 (+1)); $y = 8.25x + 4995.38$, $r^2 = 0.8164$. (D) Calibration curve of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2)) from trypsin-digested rec. MMP-9 CD in BALF at 8 levels between 0 and 57.8 ng/mL. Quantifier ion: 1092.6 (y10 (+1)). Qualifier ions: 290.1 (b3 (+1)), 219.1 (b2 (+1)); $y = 113.11x + 347.09$, $r^2 = 0.9112$.

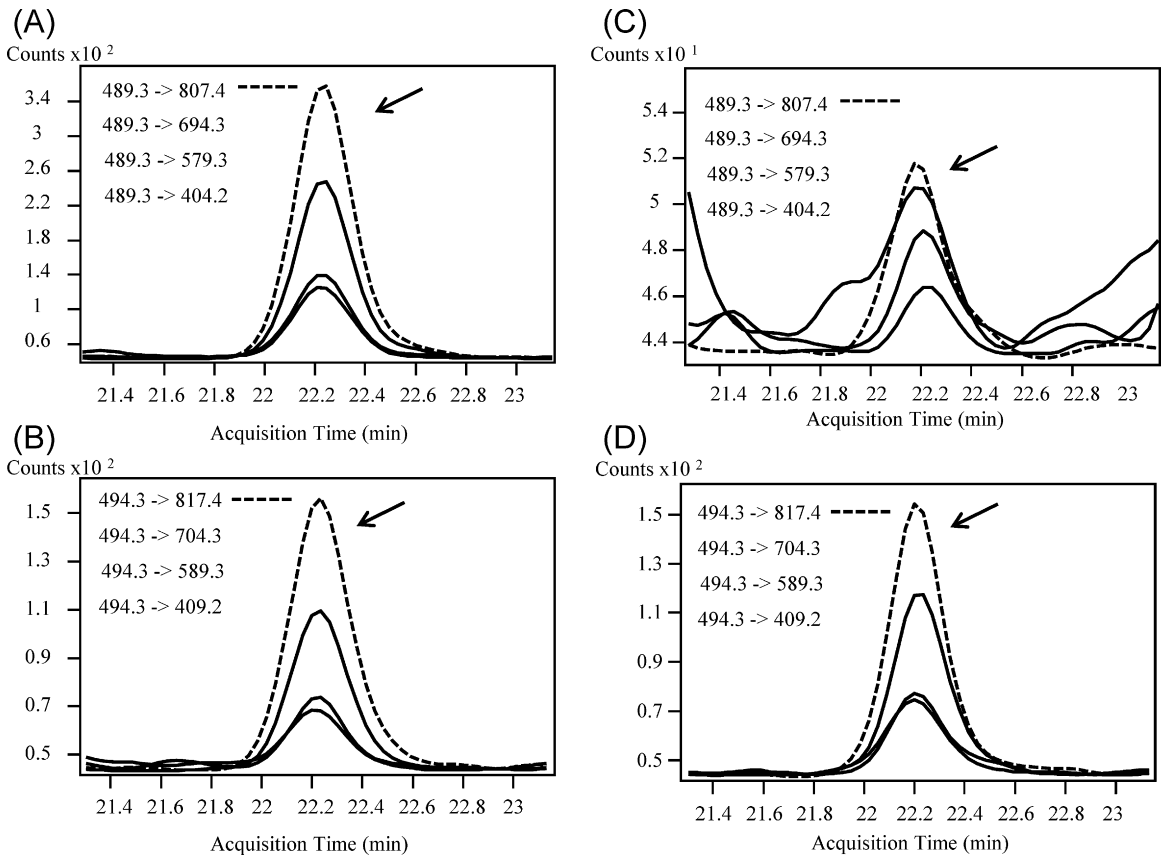

Fig. 4. LC–MS/MS analysis (SRM mode) of MMP-9 above the LLOQ (16.3 ng/mL) in BALF from a lung transplant patient with no complications due to acute rejection (sample 396; see Table S2). (A) Extracted ion chromatograms of the transitions for the target peptide AVIDDAFAR (489.3 → 404.2/579.3/694.3/807.4) and (B) for the corresponding transitions of the stable-isotope-labeled internal standard peptide (494.3 → 409.2/589.3/704.3/817.4) (lower left panel) show co-elution at the expected retention time (see arrows) (the transitions 489.3 to 807.4 and 494.3 to 817.4, represented as dashed lines, were used for quantification). LC–MS/MS analysis (SRM mode) of MMP-9 between the LLOQ and the LLOD in BALF from a lung transplant patient with no complications due to acute rejection (sample 394; see Table S2). (C) Extracted ion chromatograms of the transitions for the target peptide AVIDDAFAR (489.3 → 404.2/579.3/694.3/807.4) (C) and (D) for the corresponding transitions of the internal standard peptide (494.3 → 409.2/589.3/704.3/817.4) (D) show co-elution at the expected retention time (see arrows) (the transitions 489.3 to 807.4 and 494.3 to 817.4, represented as dashed lines, were used for quantification).

calibration curve presented in Fig. 3B) normalized to one mg of total protein and per mL of original BALF (see Table S2). Each sample was prepared in duplicate and an equivalent of 4 μg trypsin-digested total protein was subjected to microfluidics-based nanoLC–MS/MS in the SRM mode.

MMP-9 was measured at concentrations above the LLOQ in 4 out of 10 BALF samples. One sample had an MMP-9 concentration of 2.2 ng/mL, which was slightly below the LLOQ (Table S2, sample 404). Fig. 4A and B shows an example of the analysis of MMP-9 in BALF above the LLOQ. MMP-9 was detectable in 3 other BALF samples with an SNR between 3 and 10 and co-elution with the internal standard peptide but the ratio between parent ion and fragment ions did not meet the criterion of being within ±25% of the expected values (Table S2 and Fig. 4C and D). MMP-9 detection was confirmed by monitoring the transitions for AFALWSAVTPLTFTR and its stable isotope-labeled internal standard peptide.

The level of MMP-9 in BALF did not correlate with the levels of IL-8, a chemotactic cytokine for neutrophils that can activate NFκB, or IL-6, a cytokine related to the acute-phase reaction (Table S2) nor with acute rejection of the transplanted lung. Concentrations of these cytokines were previously shown to be non-predictive of acute rejection [34,35].

4. Discussion and conclusions

Current developments in the area of biomarker research emphasize that proteome-wide discovery studies must be followed by targeted quantitative validation that allow analyzing a large number of samples. While immunochemical assays (ELISA) are currently the most widely used approach, there is an increasing tendency to complement or even replace them by analytical techniques that provide direct chemical information about the analytes such as LC–MS/MS. A major challenge for LC–MS/MS is its lower concentration sensitivity when compared to ELISA, while it has clear advantages when it comes to providing more reliable qualitative and quantitative information.

To address these issues, liquid chromatography in combination with tandem mass spectrometry in the selected reaction monitoring (SRM) mode can provide high selectivity and sensitivity in complex biological samples. SRM assays have, to date, been mainly applied to serum and plasma analysis, where they are generally limited to the low μg/mL range unless immunoaffinity enrichment is used. Most potential serum biomarkers are, however, at the low ng/mL or even pg/mL level, which means that sample preparation forms a critical part of any targeted LC–MS/MS method in the SRM mode. Keshishian et al. demonstrated that multiplexed SRM assays allow detecting proteins at the 1–20 ng/mL level after removal of the 12 most abundant proteins by immunoaffinity depletion and fractionation by strong cation-exchange chromatography [20,21]. The requirement of antibodies for most high-sensitivity SRM assays in serum or plasma suffers from the same drawbacks as ELISA and makes assay development costly and time consuming. MMP-9 has been quantified in mouse serum by immunoaffinity LC–MS/MS in the SRM mode reaching a sensitivity of 30 pM [16].

While SRM assays for proteins in serum or plasma are becoming more widely used, there is, to our knowledge, currently no description of such assays for proteins in BALF. However, the study of BALF might be more meaningful than serum for the discovery and/or the validation of potential biomarkers related to pulmonary diseases.

The study of the BALF proteome by Wu et al. [36] quantified a number of proteins, including MMP-9, in the low ng/mL range. Due to the wide dynamic range of the BALF proteome, numerous prefractionaton and sample preparation steps were, however, required including concentrating BALF by ultrafiltration and depletion of the six most abundant proteins by immunoaffinity chromatography. The final steps comprised gel electrophoresis in the presence of SDS and in-gel tryptic digestion making this a very lengthy and work-intensive procedure. Another shortcoming of such a complex experimental approach is the possibility of protein loss during sample preparation. The time required for sample preparation and the number of fractions that are generated per sample are not compatible with high-throughput analysis.

The sample preparation procedure described in this study is generic and does not require antibodies or extensive prefractionation. The chloroform-methanol precipitation removes phospholipids efficiently and concentrates the proteins. This is critical, since BALF contains a high level of phospholipids but has at the same time a much lower protein concentration compared to serum or plasma. The described method is therefore suitable for the quantification of a wide range low-abundant proteins in BALF. The ultimate sensitivity of the present assay resulted further from careful optimization of the LC–MS/MS step. For example, lowering the resolution of the quadrupole mass analyzers lead to an increase in sensitivity by a factor 5 without compromising selectivity. This must, however, be checked on a case-by-case basis.

Our SRM assay reached a LLOQ of 2.9 ng/mL (SNR ~ 35) and a LLOD of 250 pg/mL (SNR ~ 3) showing that protein analysis in BALF by SRM can reach the sub-ng/mL range without the use of antibodies. Due to the use of a microfluidics-based nanoLC–MS/MS system, absolute on-column amounts were approximately ~560 amol at the LLOQ and 50 amol at the LLOD, respectively.

MMP-9 in BALF of lung transplantation patients with and without complications due to acute rejection of the transplant was measured but no correlation was found in agreement with earlier studies [23]. Indicators of an inflammatory response such as IL-8 and IL-6 or infiltration of neutrophils or lymphocytes did also not allow predicting the occurrence of acute rejection. We are currently extending our assay to combine it with the enrichment of active MMP-9 on an immobilized inhibitor material [37–41], to investigate whether active MMP-9 is a predictor of acute rejection after lung transplantation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.076.

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