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Displacement chromatography as first separating step in online two-dimensional liquid chromatography coupled to mass spectrometry analysis of a complex protein sample—The proteome of neutrophils

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ABSTRACT

Displacement chromatography provides some advantages over elution chromatography such as the opportunity to enrich trace amounts of molecules and to elute molecules in highest concentrations achievable with liquid chromatography. In a previous study we demonstrated that displacement chromatography is a well-suited alternative to gradient elution in an offline two-dimensional (2D-)LC–MS approach for the analysis of proteomes. In this study we present a method for applying displacement chromatography in an online 2D-LC–MS system including a cation exchange (CEX) column and a reversed phase column. We circumvented the problem of determining the sample capacity of the CEX column by repeated injection (pulses) of sample aliquots monitored by an LC–MS analysis of each flow-through fraction of the CEX column. Elution of tryptic peptides from the CEX column was achieved by repeated injection (pulses) of the displacer spermine. Pulsed displacer injections offer the advantage through physical separation of preventing post-column mixing of already separated compounds. As a proof of principle we analyzed the cytosolic proteome of human neutrophils.

1. Introduction

In the analysis of complex protein samples, the shotgun approach is widely used. In this approach, digestion of proteins down to the peptide level reduces complexity of the chemical heterogeneity of the molecules in a sample. The resulting peptides have more homogeneous chemical properties compared to a protein sample. At the same time, the number of different individual molecules in the sample is multiplied, resulting in well-known problems for mass spectrometric analysis such as ion suppression or identification of only high abundant peptides. Therefore, separation of the proteolytic peptides prior to their mass spectrometric identification is a crucial step in proteomic approaches. A common way of separation in shotgun proteomics is two-dimensional liquid chromatography (2D-LC) with a cation exchange chromatography as first dimension followed by reversed phase (RP) chromatography in the second dimension [1–3]. Salt step gradients are often used for peptide elution from the first dimension, especially in online-2D-LC–MS systems. Salt step gradients nevertheless possess some disadvantages such as multiple elution of peptides (also known as carry-over effect) and a decreased resolution [4–6]. Thus for 2D chromatographic approaches further improvements are desirable for getting a deeper look into proteomic samples.

Displacement chromatography (DC) was already introduced in 1942 by Tiselius [7]. Already almost 30 years ago DC was shown to work very efficiently in preparative peptide separation [8–10]. The major advantages of displacement elution mode for proteomics are the enrichment effect on trace components [11,12] and a concentrating effect yielding the highest concentrations of analytes eluting from the stationary phase compared with any other elution mode in liquid chromatography [8]. We recently demonstrated the benefits of DC using ion-exchange chromatography as a first separating step in an offline 2D-LC–MS proteomics study [13]. However, online 2D-LC–MS experiments offer several advantages over offline experiments, for example, they are more easily automatable and offer improved recovery, which in proteomics is equivalent with an increased number of identified proteins. Therefore, our aim was to develop a method for using the displacement elution mode in an online 2D-LC–MS approach integrated into a shotgun proteomics

Abbreviation: DC, displacement chromatography.

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workflow. For the successful integration of a displacement step in an online 2D-LC–MS method two problems had to be solved. 1. In DC the quality of separation critically depends on the loaded sample amount in relation to the total binding capacity of the stationary phase. If the sample amount injected onto the column is too small, the bands obtained by DC will be too narrow, resulting in a poor separation of the peptide mixture. A sample amount significantly exceeding the binding capacity of the column will result in a sample breakthrough and thus in the loss of peptides having low affinities toward the stationary phase. This problem can be solved by the determination of the binding capacity of the sample to the column. Knowledge of the binding capacity is the basis for calculating the appropriate sample amount guaranteeing a good separation. However the experimental determination of the binding capacity of the stationary phase for certain applications like proteomics can waste valuable sample. A solution is required to avoid this step. 2. In conventional DC the eluent containing the displacer is usually pumped continuously through the column as soon as the sample injection is finished. In a 2D-LC setup this is not possible without loosing those peptides that will be eluted by the displacer during the time interval needed for separating the previous peptide fraction on the second column – the RP column. In common 2D-LC approaches like "Multidimensional Protein Identification Technology" (MudPIT) [3] this problem is solved by sequential salt pulses which are pumped onto the cation exchange column for eluting a small peptide fraction, which will subsequently separated by the RP column. As soon as this separation is finished the next fraction will be eluted from the cation exchange column by injection of a salt pulse with an increased salt concentration. In this study we applied a solution for these two problems. The method was used for the analysis of the proteome of human neutrophiles.

Neutrophiles are primary effector cells of the innate immune system. These cells fight against bacterial and fungal infections and modulate inflammatory responses. Dysregulation of neutrophils can result in tissue damage which has been reported in a number of inflammatory diseases [14]. Therefore analysis of the proteome may be helpful for getting a deeper insight into the physiology and pathophysiology of neutrophiles.

2. Methods

2.1. Sample preparation

Granulocytes (10^7 cells) were isolated from human EDTA blood using LymphoPrep and PolymorphPrep (PROGEN Biotechnik, Heidelberg, Germany) according to the manufacturer's instructions. Granulocytes were resuspended in 100 μL lysis buffer (80 μL HEPES buffer (50 mM HEPES, 150 mM NaCl, 0.05% SDS) + 1 μL Protease-Inhibitor Mix M (Serva, Heidelberg, Germany) + 10 μL of 100 mM PMSF + 10 μL of 1 M NaF). Cells were disrupted using three freeze-thaw cycles and centrifuged at 660 × *g* for 10 min. The supernatant was aspirated, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Tryptic digestion

For tryptic digestion the protein sample was dried and subsequently dissolved in 50 μL 6 M urea. 1.3 μL of a 200 mM dithiothreitol in digestion buffer (100 mM $NaHCO_3$ buffer, pH 8.3) were added and the solution was incubated for 10 min at 60 °C. Alkylation was carried out at 25 °C for 40 min in the dark by adding additional 1.3 μL of 100 mM iodacetamide (in digestion buffer). 425 μL of the digestion buffer were added. 40 μL of trypsin (Promega, WI, USA) solution (0.25 μg/μL dissolved in trypsin resuspension buffer, Promega) were added. The reaction mixture was incubated for 16 h at 37 °C. The reaction was quenched by adding formic acid to a final concentration of 0.2%.

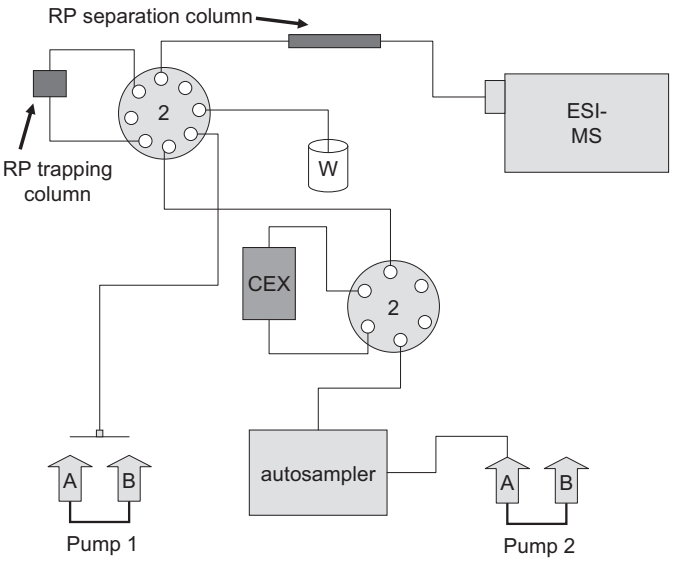

Fig. 1. Simplified scheme of the 2D-LC–MS setting in displacement mode. Note that the enrichment column and the separation column are integrated on the HPLC chip. Sample and displacer eluent are injected by an autosampler and loaded by pump 2 onto the cation exchange (CEX) column installed directly behind the injection valve (1). Non-binding and eluting components pass toward the reversed phase (RP) trapping column. Pump 1 delivers the gradient to elute peptides from the trapping onto the separation column and then into the ESI ion trap mass spectrometer. Further abbreviations: W: waste; 1 and 2: valves.

2.3. Desalting

Desalting of the sample was carried out on a μRPC C2/C8 2.1/10 (GE Healthcare, Little Chalfont, UK) using a Smart system (Pharmacia Biotech, Uppsala, Sweden, now GE Healthcare) for chromatography. Peptide elution was monitored online photometrically at 220 and 280 nm. Loading of the sample was carried out in sample loading buffer (solvent A: 0.2% formic acid (FA) in HPLC-grade water) at a flow rate of 100 μL/min. Peptides were eluted with a gradient of 0–60% solvent B (100% acetonitrile, ACN) in 5 min. The peptide-containing fraction was collected and the solvent was evaporated to complete dryness.

2.4. Two-dimensional liquid chromatography–tandem mass spectrometry

Two-dimensional liquid chromatography–tandem mass spectrometry analysis (2D-LC–MS/MS) was performed using a cation exchange chromatography as first and reversed phase chromatography as second separating dimension. The experiment was performed on an ion trap (IT) mass spectrometer (XCT Ultra, Agilent Technologies, Waldbronn), equipped with an Agilent HPLC-Chip Cube interface integrated into an 1100 series HPLC system (Agilent Technologies). The HPLC-chip (Large capacity chip, Agilent Technologies) integrates two on-chip columns, an enrichment column (internal volume 160 nL), a separation column (150 mm, both 5 μm Zorbax 300 SB-C18 material) and a nanospray emitter. As first separating dimension, a strong cation exchange column (Zorbax Bio-SCX Series II, 0.3 mm × 35 mm, particle size 3.5 mm, Agilent Technologies) was installed directly behind the autosampler of the HPLC system (Fig. 1).

A capillary pump, working at 1 μL/min, was used to load the sample onto the CEX column in 29 sample loading steps (loading volume 0.2–1 μL). The solvent was 98% solvent A (0.2% FA in

HPLC-grade water) and 2% solvent B (ACN). Non-binding components were passed directly to the enrichment column of the HPLC-chip. After each sample loading step, which included a washing step of the enrichment column, an LC–MS run was performed. Therefore, a nano pump delivering a flow of 400 nL/min was employed for gradient separation. Tryptic peptides trapped on the enrichment column were separated on the separation column using a linear gradient composed of solvent A and B. The gradient consisted of 2–40% eluent B within 40 min.

Elution of the tryptic peptides bound to the CEX column was performed by injecting pulses of 1 mM spermine dissolved in solvent A (displacer eluent). The following injection volumes were used for displacement elution: first elution pulse: 2 μL, elution pulses 2–49: 1 μL, elution pulses 50–59: 5 μL.

The following parameters were used for IT-MS identification: scanning range from 300 to 2000 m/z, electrospray voltage was set to −1750 V, nitrogen was used as drying gas for desolvation at a flow rate of 4 L/min and 325 °C. Precursor ion mass spectra were acquired in positive ion mode with automated data-dependent MS/MS of the three most intense ions of each precursor MS scan. Doubly charged ions were isolated preferentially. Isolation width was set to 4 Da, MS/MS fragmentation amplitude to 1.25 V. Peptides were actively excluded after three spectra and released after 1 min.

2.5. Bioinformatic analysis

Peaklists for MS/MS database search have been generated using Data Analysis Software for 6300 Series Ion Trap LC/MS version 3.4. The protein identification was carried out with TOPP (The OpenMS Proteomic Pipeline) [15,16] version 1.7 and its graphical user interface TOPPAS using three different search engines (OMSSA [17] version 2.1.1, X!Tandem [18] version 2007.07.01.1, Mascot [19] version 2.1.03) in a consensus identification pipeline [20]. The search parameters included potential residue mass modification for carbamidomethylation on cysteine residues and oxidation on methionine residues, one missed trypsin cleavage, precursor ion mass tolerance was 1.2 Da, fragment ion mass tolerance was ±0.6 Da. The spectra were searched against a decoy database (SwissProt Human database version 55.4) to estimate false positive rates of the peptides. The identified peptides were validated using a tool (IDDecoyProbability) similar to PeptideProphet [29]. This tool used the peptide identifications of the different search engines to learn the distribution of scores and peptide probabilities of correct and incorrect identifications. Afterward the distribution was used to calculate for each peptide identification the probability that it was correctly identified. The identified peptides were filtered using a q-value threshold of $q < 0.05$. For protein validation ProteinProphet [21] was used to estimate protein probabilities and to adjust the probabilities for each peptide. Finally, only proteins and peptides with a reported probability of higher/equal 99% and at least two identified peptides per protein including one unique peptide were accepted.

To interpret and compare the elution behavior of both elution modes, the charge of each individual peptide was calculated based on the amino acid sequences determined by the database searches as described above and on the assumption, that each peptide was fully protonated in eluent A in the presence of 0.2% FA.

3. Results and discussion

In this study we describe an online 2D-LC–MS approach in which the first LC dimension – a cation exchange column (CEX) – is operated in the displacement mode followed by a RP trapping column coupled to a RP separating column as second dimension. Both RP columns are integrated on a HPLC-chip system. Via an emitter, which is part of the HPLC-chip, the eluent of the separation column is directly passed into the ionization chamber of an electrospray ionization ion trap mass spectrometer (ESI-IT-MS). HPLC-chip–ESI-MS guarantees a robust LC–MS performance which was mandatory in this study as the total operating time of our analysis of a tryptic digest of neutrophil proteins took about 90 h. A critical point in online 2D-LC–MS approaches is the stability of the system in particular with respect to stability of the chromatographic separation and stability of the spray in the ESI source. Commonly used nanoESI-MS is often impeded by instability of the spray, a problem that is circumvented by using an HPLC-chip system. We demonstrated chromatographic performance of the HPLC-chips regarding their stability and reproducibility previously [22]. In the system used in this study we additionally installed a CEX column directly behind the autosampler (Fig. 1). The analysis of the proteome of neutrophils was started by directly injecting sample aliquots onto the CEX column. In our system non-binding or eluting peptides are directly trapped on the RP trapping column. Substances with no affinity toward the RP material in the trapping column were eluted into the waste. Each injection of a sample aliquot was followed by a short washing period thereby removing salts and other substances with no affinity to the RP material from the trapping column. Sample injection and desalting was followed by a switch of valve 2 (Fig. 1). As a result the nanopump (pump 1 in Fig. 1) delivered a water-acetonitrile gradient via the trapping column to the separation column by which the peptides were separated and eluted toward the mass spectrometer.

In this study DC was chosen for the analysis of a proteome because this elution mode promises properties, which are especially useful in 2D-LC proteomic approaches. In contrast to gradient elution the substances eluting from the column are not diluted but rather concentrated. Already during sample loading the individual molecules compete with each other for binding sites on the stationary phase. As a result of this competition the individual peptides form bands in which the band of the peptide with the highest affinity toward the stationary phase is located on the column inlet. A band containing the peptide with a lower affinity follows this band. The band of the peptide with the lowest affinity but which will still bind to the CEX column is located nearest to the outlet of the column. Once the sample application is finished the peptides will not move significantly down the column if pumping of the sample application buffer will be continued. In gradient chromatography elution of the peptides from the CEX stationary phase is induced by increasing the suppression of electrostatic interactions between the negatively charged functional groups of the CEX and the positively charged peptides by an increase in salt concentration in the eluent from 0 M to 0.5 M or even 1 M. Mass action [23] requires an excess amount of salt, because sodium ions have a very low affinity toward the charged functional groups of the CEX.

In contrast, in DC the analytes are eluted from the column by pumping a displacer molecule onto the column that is dissolved in the sample application buffer. Hence, the displacer molecule should have a higher affinity toward the stationary phase than any analyte.

By continuously pumping the displacer onto the column the stationary phase will become increasingly saturated by the displacer molecule thus forcing the analytes to move down the column. During movement of the bands – termed displacement train – down the column a self-sharpening effect increases the purity within the bands.

The velocity ν of a component running through a chromatography column is proportional to the amount of the analyte C in the mobile phase divided by the amount of the analyte bound to the stationary phase Q at equilibrium (ν is proportional to C/Q) [32]. A compound possessing a low affinity toward the stationary phase, such as NaCl, requires a large amount C to displace all of the analytes from the stationary phase. As a result the NaCl front propagates

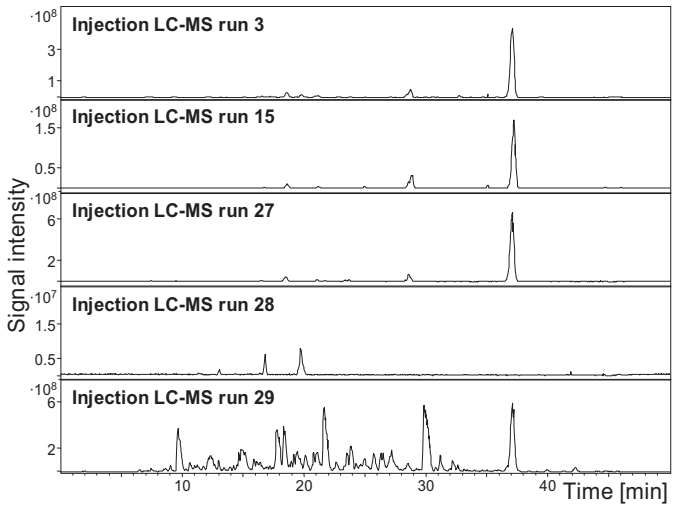

Fig. 2. Exemplified base peak chromatograms (BPC) of sample pulses for online DC. Each chromatogram represents one LC–MS run of peptides that did not bind to the CEX column after one sample pulse. The separation took place on the on-Chip RP column.

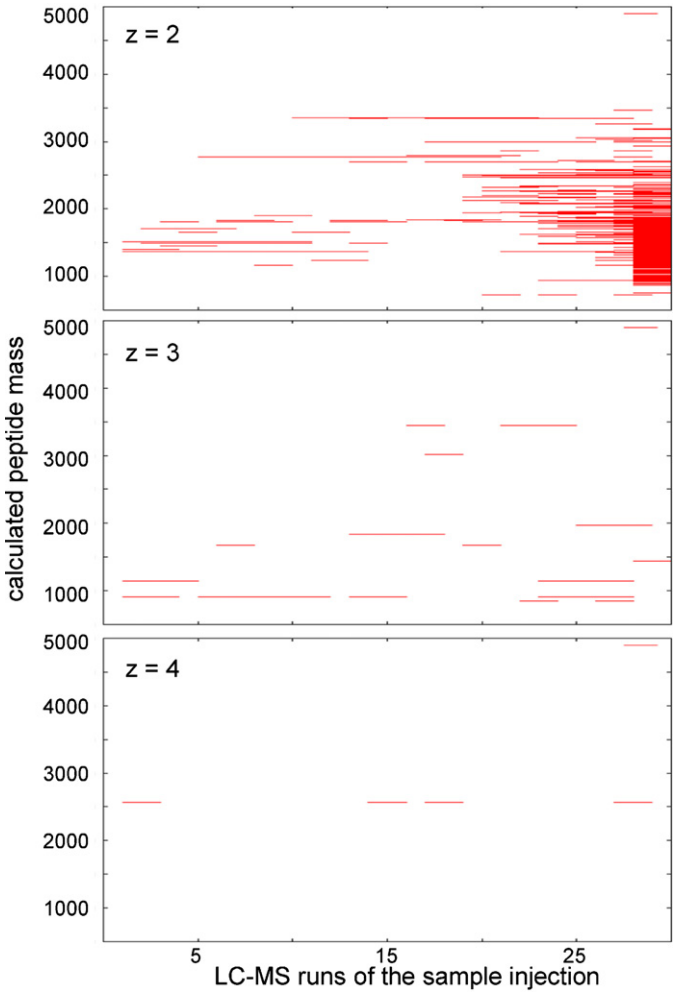

Fig. 3. Chromatogram of the sample pulse for online DC displaying individual peptides as lines. Each line represents an individual peptide, characterized by its molecular mass (ordinate), and its affinity toward the cation exchange material (number of LC–MS-run, in which the peptide was detected, abscissa). The molecular masses and the charges of the individual peptides were calculated from their amino acid sequences identified by the LC–MS/MS experiments and data base searches.

through the column too quickly to allow a displacement train to form, and in general does not allow enough "space" for the train to form between the NaCl front and the mobile phase frame of reference. In contrast a displacer with a very high affinity can displace the analytes at a much lower concentration which provides for a very low "C/Q" value, which means the displacer front propagates much slower and allows time and "space" for the displacement train to form.

The DC is finished as soon as the stationary phase is saturated by the displacer molecule. The concentration of the eluting analytes in DC are the highest concentrations achievable with liquid chromatography and can be governed by the concentration of the displacer. For example, an increase of the concentrations of eluting analytes will be obtained by increasing the concentration of the displacer molecule [24]. Typical concentrations of displacers are in the low mM range and therefore are one to two orders of magnitude lower than the common concentrations of salts in ion-exchange gradient elution [25].

According to Zhu et al. [30], displacer impurities negatively influence a displacement separation. However in the method which we present here, impurities of spermine should not have a significant effect on the peptide separation because spermine is not continuously pumped onto the CEX column like in conventional displacement chromatography but by repeated injections of small amounts. A contamination which will bind to CEX will displace spermine or some peptides or both but this has no effect on the subsequent separation of the peptides on the reversed phase column. If the contamination will not bind to CEX it may either pass the reversed phase column without binding or it will bind to the trapping column. In the latter case it may occupy a small part of the trapping column thereby slightly reducing the binding capacity of the trapping column. This type of contamination will elute as a peak from the reversed phase column which should not have an effect on peptide identification.

A problem that had to be solved in this study was the estimation of the proper sample feed as explained in the introduction. According to DC theory optimal sample loading for DC applications occurs between 50% and 75% column binding of the stationary phase [31]. However, in our experiment for practical reasons we overloaded the column by a small excess of sample until the first low affinity peptides eluted from the column (see Figs. 2 and 3). Using this procedure we circumvented to determine the binding capacity of our sample to the stationary phase thus avoiding loss of our small sample amounts. We solved this problem by repeated injection of sample aliquots. Since every sample injection was accompanied by a LC–MS analysis of the substances that passed the CEX, a full control of the sample application process was given (Figs. 2 and 3). In Fig. 2 representative base peak chromatograms of the LC–MS analysis recorded after different numbers of injections are presented. There is no significant difference in the chromatograms following the sample injections 3, 15 and 27. A change of the profile is obvious in the chromatogram taken after the 28th sample injection. This change is even more dramatic in the chromatogram following the 29th injection. The eluting peptides represent low affinity components that are pushed down from the column by a sample displacement effect starting as soon as the column is fully loaded and the binding places are completely saturated. This effect is even more obvious in the chromatogram in Fig. 3 ($z = 2$) summarizing the results of LC–MS analysis of all sample injections within a single chromatogram. For a better overview the chromatogram was split into three parts representing peptide ions with a calculated net charge of +2, +3 or +4 in the sample application buffer in the presence of 0.2% formic acid. As expected doubly charged ions are the most abundant species during sample application.

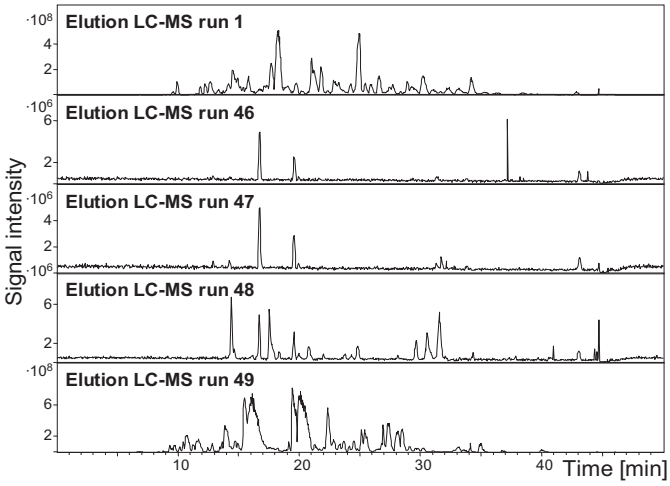

Fig. 4. Exemplified base peak chromatograms (BPC) of peptide elution in displacement mode. Each chromatogram represents one LC–MS run of peptides that were eluted by a single displacer pulse.

Elution of the peptides adsorbed to the CEX column was started directly after injection run of the 29th sample aliquot. For peptide elution from CEX the second problem had to be solved as in an online 2D-LC setting a continuous elution of analytes from the first column is difficult. A common solution for this problem in online-2D-LC is the application of salt pulses for eluting analytes from ion exchange columns. The application of a displacer pulse is even better suited than application of a salt pulse in gradient elution as the displacer molecules present in the pulse will completely bind to the stationary phase thereby pushing those analytes from the column that are located nearest to the column outlet. In contrast, in gradient elution a salt pulse will enter, traverse and leave the column thereby mobilizing in the pulse those analytes from which the electrostatic interactions with the stationary phase were interrupted. The problem of this process is that the establishment of the equilibrium of the analytes according their distribution between the stationary phase and the mobile phase is consistently repeated in every segment of the column that the salt pulse, comprising already desorbed analytes, passes. As a result in every segment of the column an aliquot of the analytes will remain at the stationary phase (according to the law of mass action) and thus the recovery decreases by distribution of the analytes over multiple fractions as documented in Le Bihan et al. [4]. In this study, the elution of peptide fractions by displacer pulses was performed by repeated injections of the displacer, here spermine, onto the CEX column. Each displacer injection is followed by a sequence comprising trapping of the eluted peptides on the RP trapping column, washing of the trapped peptides to remove the displacer and by a water-acetonitrile gradient resulting in the separation of the peptides on the RP separation column and subsequent MS analysis. Fig. 4 shows some of the representative base peak chromatograms obtained after elution of peptides from the CEX column induced by the injection of the displacer. The chromatogram in run 1 (first injection of the displacer) already shows a profile, which is completely different compared to the chromatogram in Fig. 2, run 29. The chromatograms obtained after the displacer injection No. 46, 47 and 48 (Fig. 4) contain two abundant peptides (17 min and 19 min), which obviously form a broader band on the CEX column. The comparison of the chromatograms of runs 48 and 49 shows significant differences, thus demonstrating the separation power of the displacement approach. Fig. 5 represents a chromatogram in which all individual chromatograms of the displacement elution are summarized. Surprisingly sharp zones between doubly charged peptides ($z=2$), triply charged peptides ($z=3$) and quadruply charged peptides ($z=4$) are recognizable, again demonstrating the separation power of the displacement elution.

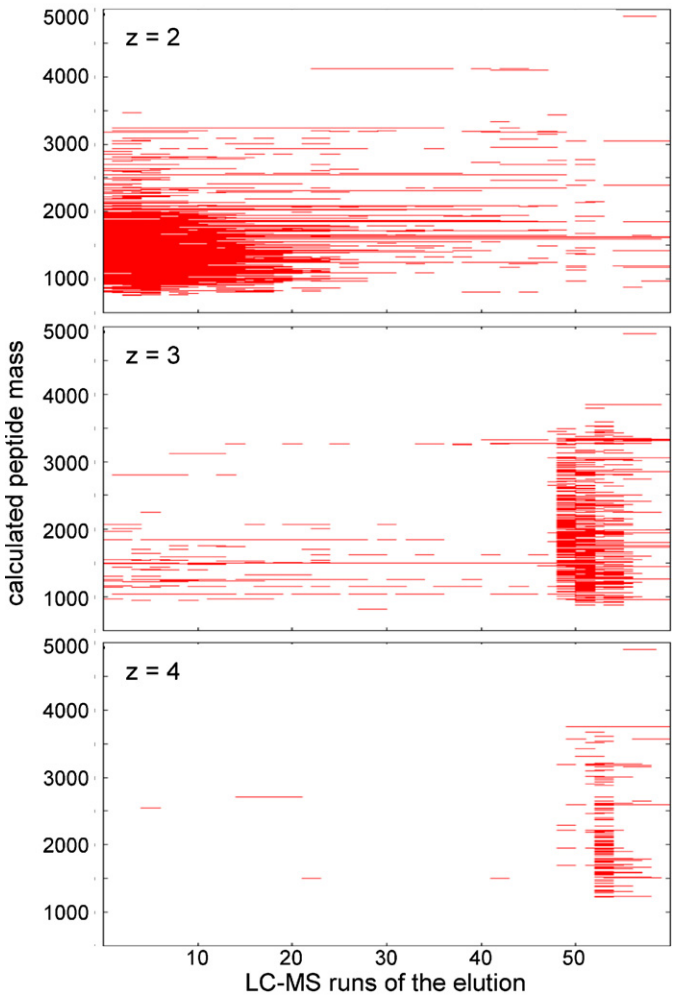

Fig. 5. Chromatogram of the peptide elution in displacement mode displaying individual peptides as lines. See Fig. 3 for further explanations.

The method described in this study has further advantages. By changing the amount of the injected displacer the results obtained by the reversed phase separation can be optimized "on the fly". If the signal intensities of the peptides eluted from the CEX in the reversed phase chromatogram are too low, a larger amount of displacer can be injected. The ability to control the amount of the peptides eluting from the CEX by changing the amount of the injected displacer is also useful for responding to the increasing concentrations of analytes eluting from the column in displacement chromatography.

Another advantage in displacement 2D-LC–MS is that fractions eluting from the CEX do not contain salt like fractions in gradient elution. Higher salt concentrations can result in a loss of hydrophilic peptides on the reversed phase chromatography as the presence of salt can disrupt the effect of ion pairing [26]. Potential residual spermine entering the chromatographic system by repeated injections via the autosampler, should not have any effects on the MS results for the following reasons. Spermine will bind more or less quantitatively to the CEX column until the binding capacity of the CEX column is nearly saturated by spermine. Those minute amounts of spermine eluting from CEX will pass the trapping reversed phase column. Because of its hydrophilicity spermine will not bind in significant amounts to the latter column and will be removed by the washing step. Thus the trapping

column is preventing that significant amounts of spermine will enter the separation column. Additionally in 0.2% formic acid, the solvent that has been used in the experiment, the low pH of about 2 ensures all peptides to be fully protonated and therefore no binding of spermine to the peptides via electrostatic interaction is expected.

Pulsed application of the displacer solution instead of continuously pumping the displacer onto the column provides the advantage of a physical separation between the eluted fractions and therefore prevents zones of the displacement train to mix up in after-column dead volumes as it happens in all chromatographic systems that are not free of after column dead volumes. An improvement of the separation of the zones should be achievable by further reducing the displacer amount per pulse. Besides the possibility to apply very low displacer amounts, one has to consider that a zone of the displacement train can be "cut" into two fractions of displacement elution which might result in the loss of low abundant compounds.

A limitation of the online-2D-LC–MS system applying displacement as first dimension is given for minute sample amounts if these amounts are significantly smaller than the binding capacity of the column of the first dimension. In this case, separation of the peptides occurs only over a small range of the column resulting in a bad separation of eluting peptides after the application of the displacer. However, this problem can be solved by a reduction of the column dimension. It is important to mention that a reduction in column dimension does not mean to simply reduce the length of a column as this would dramatically decrease the resolution. Instead, a reduction of the diameter is beneficial or, if this is not applicable, one should try to maintain a high aspect ratio of column length to diameter, e.g. an aspect ratio of at least 120 as used in this study.

Nevertheless, compared to gradient elution the approach described here is more time consuming because the sample is sequentially loaded in many aliquots.

By the 2D-LC–MS approach using displacement CEX chromatography in the first dimension we were able to identify more than 2000 peptides belonging to 253 proteins (Supplement Table 1). The list of identified proteins reveals many proteins known to be present in neutrophils. E.g. lactotransferrin was identified with 65% sequence coverage and 57 tryptic peptides, neutrophil elastase with 44% sequence coverage and 10 tryptic peptides, neutrophil gelatinase-associated lipocalin with 58% sequence coverage and 12 tryptic peptides, neutrophil defensin 1 with 52% sequence coverage and 3 tryptic peptides, neutrophil cytosol factor 1 with 17% sequence coverage and 5 tryptic peptides, and neutrophil collagenase. Lactoferrin was identified with sequence coverage of 11% and via identification of 11 unique peptides. The presence of very few peptides of albumin (7 unique peptides, 7% sequence coverage) or serotransferrin (2 unique peptides, 1.6% sequence coverage) as well as the absence of other typical plasma proteins like immunoglobulins demonstrates the high quality of the sample preparation of neutrophils. We compared the list of proteins identified in this study (Supplement Table 1) with the findings of Kotz et al. [27], who investigated the time-dependent gene expression and abundance of proteins of stimulated neutrophils. 140 proteins presented in this study (55%) match with the lists of Kotz et al. However the percentage of consent is even higher, since the Kotz et al. only included those proteins which showed a time-dependent change in their abundance. Therefore proteins like the ubiquitinously synthesized intracellular protein GAPDH were not present in the lists of Kotz et al.

Kennedy et al. analyzed a neutrophil protein extract by 2D-gel electrophoresis [28]. The authors published 23 proteins identified from the gel. Between the results of these authors and the identified proteins of our study, we find a consensus of 73%.

4. Conclusion

In this study we demonstrated a solution for applying DC for the first dimension in an online 2D-LC–MS system. In DC, optimal results will be obtained if the binding capacity of a column is used in the range of 50–75%. Since especially in proteomics studies determination of binding capacity will "cost" valuable sample an alternative procedure was required. We solved the problem by slightly overloading the column by repeated injection of sample aliquots until low affinity peptides eluted by a sample displacement effect and by monitoring the flow-through of each injection with an LC–MS system coupled to the CEX. Maximum loading of the CEX is achieved if the base peak chromatogram of the LC–MS analysis shows a significant change compared to those of prior injections, indicating the sample breakthrough. Elution of peptides from the CEX is accomplished by repeated injections of the displacer. Since after every displacer injection a chromatogram is obtained by RP-LC–MS analysis of the eluted peptides, full control over the appropriate amount of the injected displacer is possible. By changing the amount of the displacer in the subsequent injection an optimization during the separation is feasible. In contrast to gradient salt step elution (salt pulse elution), carry-over effects do not occur with displacement elution. As demonstrated by the analysis of the proteome of neutrophils studies of complex protein mixtures are possible with displacement 2D-LC–MS.

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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.2012.02.029.

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