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Parallel sample preparation of proteins, from crude samples to crystals ready for MALDI-MS, in an integrated microfluidic system

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ABSTRACT

A microfluidic structure is presented where selective capture of proteins in complex samples, followed by clean-up, enzymatic processing, and MALDI-MS sample preparation of peptides generated, can be performed. The structure uses an affinity column to capture the protein while all other components in the sample are disposed of. The protein of interest is then eluted from the affinity column and captured on a second column on which the enzymatic processing is performed. Salts and hydrophilic contaminants are then removed before the products from the enzymatic reaction are eluted together with a suitable MALDI matrix and the solvent evaporated in a designated MALDI target structure. All steps can be performed automatically in 54 parallel microstructures on a microfluidic compact disc. The process is demonstrated by the selective capture and tryptic digest of recombinant IgG molecules from samples containing other proteins: an excess of bovine serum albumin or spent cell culture media.

1. Introduction

Microfluidics, the miniaturization and integration of chemical processing units facilitating the handling of volumes in the nano-litre to micro-litre scale, has developed from a purely academic endeavour into an integrated part of many modern chemical analysis instruments and analytical procedures [1–3]. Many of the advantages inherent to microfluidic platforms, such as low sample and reagent consumption, fast reaction times, reduced dilution and significant parallel processing of samples, have been realized. The compact disc (CD) shaped unit produced by Gyros AB was one of the earliest commercialized microfluidic platforms [4]. The technology is based on disposable plastic discs where chemical processes are performed while liquids are transported towards the outer rim of the disc by centrifugal forces. Spinning the disc using spin programs controls the movement of discrete liquid volumes within the CD in a highly parallel fashion [5].

CDs have been produced that target the characterization and the quantification of proteins. Sample enrichment and clean-up prior to MALDI-MS analysis [4] and the quantitative determination of a number of different proteins in a range of samples [6,7] have been shown. Several processes involved in the characterization of proteins have been implemented in the CD-format. Enrichment, sample clean-up and crystallization of peptides from tryptic digests, selective capture and dephosphorylation of phosphopeptides [8] as well as reduction, alkylation, digestion and crystallization of proteins prior to MALDI-MS analysis have been demonstrated [9]. The interaction characteristics of immunoglobulins for synthetic peptides have also been studied using the CD technology [10].

One application where the integrated and rapid isolation and characterization of proteins is of paramount importance is the manufacturing of recombinant proteins, among which recombinant antibodies intended for therapeutic intervention are a large and important group. Cost efficient manufacturing of therapeutic antibodies is hampered by time-consuming analytical methods for the characterization of the antibodies produced. The development process, including clone selection, optimization of production parameters and quality control of the final product generates a large number of samples. Information concerning whether the selected cell clone produces an antibody with desired affinity and specificity, cell productivity and if the quality of the antibody is sufficient must therefore be generated efficiently.

This paper describes the use of a microfluidic design incorporating two columns in series applied to the selective capture and characterization of a protein from a complex sample. The protein is first selectively captured by an affinity column while all effluent components in the sample are disposed to waste. The selected protein is then eluted and recaptured on a second column where tryptic digestion is performed. A microfluidic switch or router placed between the columns enables the determination of the flow-path of the liquids in the microfluidic system. The selected

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protein is digested on the second column to generate peptides that can be studied using MALDI-MS. Characterization of the peptide pattern generated can give important information concerning, for instance, post-translational modifications of the protein [11].

2. Experimental

2.1. Chemicals and materials

Human Myeloma Immunoglobulin G1 (IgG1κ) was purchased from Nordic Biosite. Recombinant human monoclonal antibody IgG1 affinity purified by Protein A and cell culture media were kindly provided by AstraZeneca AB, Sweden. α-Lactalbumin, bovine serum albumin, D/L-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, tetrafluoric acid and alpha-cyano-4-hydroxycinnamic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). Surfactant *Rapi*Gest™ SF was purchased from Waters Corporation (Milford, MA, USA). Protein A-TSK 20 particles were prepared by coupling protein A to 10 μm tresyl-5PW activated TSK beads (Tosoh) and Source 15RPC with 15 μm particles size was from GE Healthcare. All other common chemicals were purchased from Merck (Darmstadt, Germany). IgG1 antibodies, fluorescently labelled with Alexa 647, (Invitrogen) were kindly provided by Gyros AB. The labelling was performed according to the manufacturer's recommendations.

2.2. Instrumentation

2.2.1. Gyrolab

The microfluidic CDs are processed using a Gyrolab (Gyros AB, Uppsala, Sweden) equipped with a rotating carousel for handling up to 5 sample microplates and 1 reagent microplate, a liquid transfer unit mounted on a robotic arm and a CD spinner. Gyrolab is also equipped with an arm for moving CDs from loading or storage positions to the CD spinner or to and from the fluorescence detection unit used to detect molecules captured on the different columns of the CD. The fluorescence detector moves linearly from the periphery of the CD towards the CD centre while the CD is rotated using a second CD spinner. Gyrolab is automatically controlled by a computer with a dedicated software. The prototype Gyrolab (PPT 2.1) used for this study had a stroboscope (Hella Optilux, Hella KG Hueck & Co., Lippstadt, Germany) mounted that was synchronized to the CD spinner. This enabled the visualization of the movement of the liquids in the microchannels of the CD. A digital video microscope (DVM Kappa, Gleichen, Germany) connected to a digital video recorder was used to document the experiments.

2.2.2. Prototype CD platform

A prototype microfluidic compact disc (CD) has been designed to facilitate the integration of several processes. This disc contains 54 parallel microfluidic structures. The design of the structure is presented in Fig. 1A. The first column (1), in this paper referred to as the inner column, is positioned at the outlet end of a liquid reservoir (2) in direct contact with an inlet hole. The liquid reservoir can receive up to 1 μL of sample. A microfluidic liquid router (3), placed between the first and second column, enables the determination of the flow-path of liquid through the microstructure depending on the rotational frequency used. A detailed description of the liquid router is given below. This structure facilitates, for instance, the selective capture of a certain chemical compound or group of compounds on a first column while the sample liquid is being directed to a waste channel (4). The chemical compound can then be eluted and captured on a second column (5, termed the outer column) where chemical or enzymatic reactions can be performed. Liquids can be dispensed to the outer column through an inlet connected

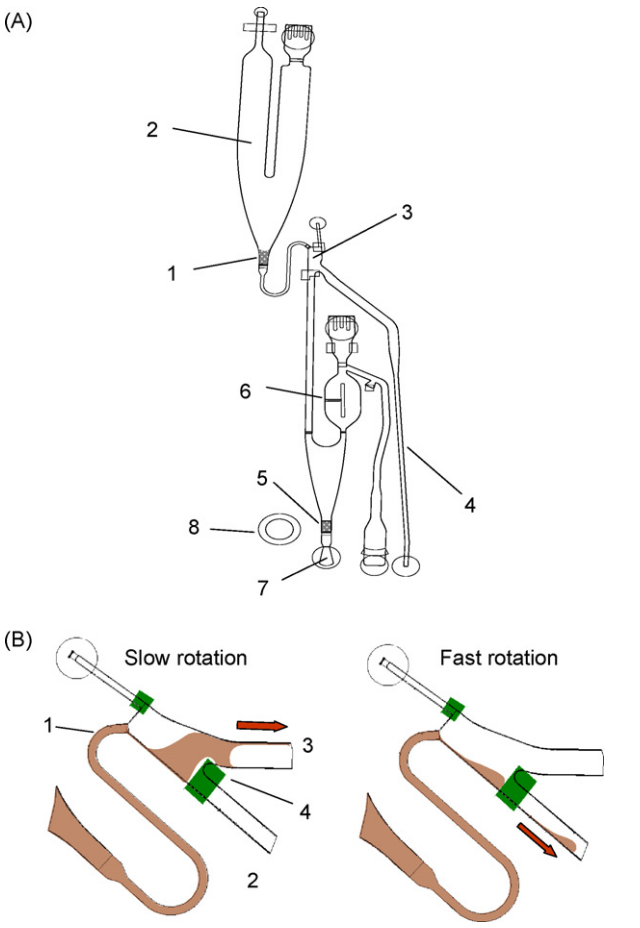

Fig. 1. The layout of the microstructure is shown in (A) and the function of the liquid router is shown in (B). A: (1) Inner column, (2) liquid reservoir, (3) liquid router, (4) waste channel, (5) outer column, (6) volume definition channel, (7) MALDI well, and (8) MALDI reference well. B: (1) Inlet channel, (2 and 3) outlet channels, and (4) hydrophobic coating. The numbers are also referenced in the text.

to a volume definition structure (6). The function of the volume definition structure has been previously described [5]. The released components or the products from, for instance, an enzymatic digest can then be eluted from the outer column and co-crystallized with a MALDI matrix in the designated MALDI target structure (7). Each structure also has a corresponding MALDI reference well (8) having the same depth as the target structure. The CD is processed in a prototype Gyrolab (PP2.1). The evaporation and crystallization part of the microfluidic structure is stamped out of the CD in groups of 9 structures and mounted, two at a time for a total of 18 structures, into a MALDI-MS target holder (for experiments performed in the ABI instrument) which is inserted into the mass spectrometer.

The prototype CD was manufactured following established procedures developed for other CDs intended for quantitative analysis of proteins in complex sample matrixes utilizing miniaturized immunoassay (Gyros AB, Uppsala, Sweden). This involves injection molding, plasma oxygen discharge treatment, incorporation of hydrophobic valves in CD by treatment with a fluorinated polymer solution, lid lamination, and surface treatment with a hydrophilic polymer solution to facilitate capillary action in microstructures. Eventually, a transparent conductive coating was sputtered on the CD-surface to enable good desorption and ionization without undesired charge accumulation. Finally, before experiments were performed, slurries of immobilized Protein A and reversed phase chromatography (RPC) material, prepared in phosphate buffered saline PBS and in 20% EtOH, respectively, were packed by manually

loading slurries to the CD channels and then spinning the CD to create columns of approximately 10 nL volume.

2.2.3. Liquid router

The liquid router and its function are described in Fig. 1B. The liquid router has one inlet channel (1) and two outlet channels (2 and 3). A reservoir separates the inlet and the outlet channels. An air inlet ensures that no backpressure from trapped gas is formed that can influence the flow-path of the liquid. Liquid will enter the reservoir and will flow along the wall in immediate contact with the inlet channel. A hydrophobic surface coating (4) is placed in the channel leading to the second column (2). This will divert liquid to the waste channel (3) unless the gravitational force generated by the rotation of the CD forces the liquid to traverse the hydrophobic barrier. If the liquid comes in contact with the opposite wall of the reservoir it can move into the waste channel (3).

The rotational frequency required for moving the liquid over the hydrophobic barrier depends on the surface tension of the liquid. A rotational frequency of 2200–2400 rpm was sufficient for liquids containing high concentrations of surface active components (such as 0.1% Tween 35, 50% ethanol or high concentrations of proteins) while liquids that do not contain surface active components require higher rotational frequencies. Liquids have an increased tendency to follow a path through the microfluidic system where liquid has passed before. This is due to the hysteresis effect of the wetting angle for the surface of the microstructure. This can result in liquid taking a route that is not intended based on the rotational frequency used. Rotational frequencies with a good margin were used to avoid the possibility that liquid takes the wrong exit channel from the liquid router: $\leq$1800 for liquids designated for the waste channel and $\geq$3500 for liquids designated for the second column.

2.2.4. MALDI TOF

Voyager-DE^{TM} STR mass spectrometer (Perceptive Biosystem, Fragmingham, MA, USA) was used for MALDI analysis of peptides. Ziptip$_{C18}$ pipette tips (Millipore Corporation, Billerica, MA, USA) were used for cleaning up of peptides digested in solution prior to analysis.

3. Methods

3.1. Comparison of CD and standard MALDI plate

A mixture of peptides generated through the solution tryptic digestion of α-lactalbumin was used to compare the mass accuracy and masspectrometric resolution of the CD substrate with a standard stainless steel plate. The solution was divided into two portions after digestion. One aliquot was desalted by C18 Zip tips and spotted on stainless steel disk with normal dried-droplet procedure. The other aliquot was transferred to the outer inlet of the CD for concentrating, salt removal, elution and crystallization. α-Cyano-4-hydroxycinnamic acid was used as matrix in both cases. The mass spectrometer was set with optimized parameters for each particular MALDI targets. MASCOT searches with NCBInr database through http://www.matrixscience.com/ were carried out for protein identification. The number of miss-cleavages allowed was set at 2 and the mass tolerance was set to 100 ppm.

3.2. Capture of antibodies and tryptic digest in the CD platform

500 nL of sample solution containing approximately 0.35 μg of IgG1 was loaded in the inner inlet and the antibody was selectively captured on the Protein A immobilized beads in the inner column. The CD was spun at 1400 rpm for 7 min to direct the effluent to waste. The column was then washed with 700 nL of phosphate-buffered saline (PBS) solution (pH ~6.8) using the same

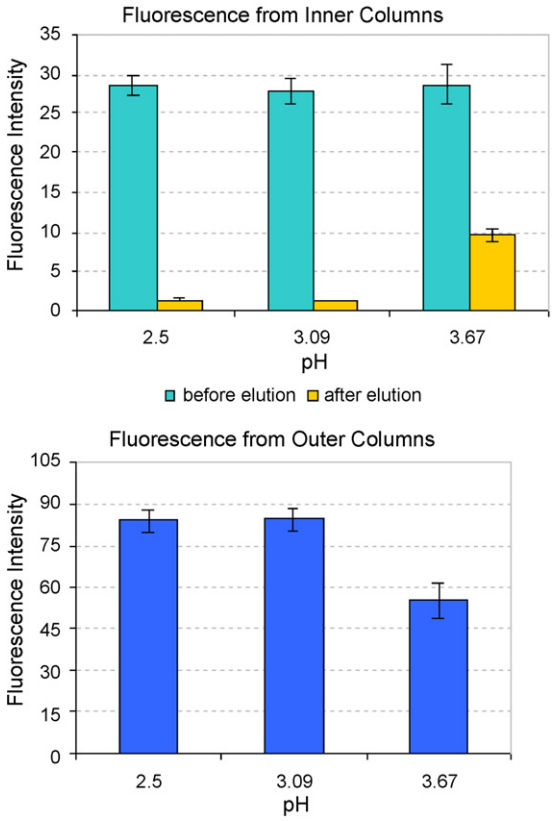

Fig. 2. Florescence signals of 0.15 μg IgG1κ samples in inner and outer columns with different elution pHs.

rotational frequency. The outer column was conditioned four times with ACN 50% and then washed twice with deionized water before the antibodies were eluted from the Protein A column. Elution was performed using a Glycine–HCl buffer (pH ~3.09) and directed to the outer column using a rotational frequency of 3500 rpm for 50 s duration. For digestion on the outer column, 400 nL of reagents was loaded in turn into the volume definition unit of the outer inlet. Reagents were then volume-defined using a spinning frequency of 600 rpm to activate the overflow channel. By increasing the spinning frequency to 1000 rpm the volume-defined liquid is forced to pass the hydrophobic barrier and out to the sample/reagent reservoir where it is kept in contact with the column for reaction using a constant spinning frequency of 500 rpm. Evaporation of the liquid at the opening to the evaporation well generates a flow of liquid through the column. This supplies the column with new reagents during the reaction. It also limits the total time that a reaction can be performed, as the liquid eventually will be expended. When the liquid volume in almost all the structures used has been reduced as to only occupy the dead volume of the column then residual solution will be pushed away by an increase in rotational speed followed by loading a new reagent. Reduction and alkylation were, due to this, performed in 15 min each while tryptic digestion was performed over 45 min by 3 consecutive additions of trypsin solution. The column was rinsed once with ammonium bicarbonate (AmBic) buffer prior to the digestion step. A halogen lamp was used to reduce fluctuation in the room temperature and maintaining a stable temperature in the vicinity of the CD spinner. Peptides adsorbed on the column were desalted by 3 additions of deionized water. The peptides were then eluted from the columns to the open MALDI target areas using an aqueous solution containing 50% ACN, 1% TFA and 2 mg/mL alpha-cyano-4-hydroxycinnamic acid. The elution droplets were held in the MALDI evaporation wells by balancing of the centrifugal force and the capillary force until the

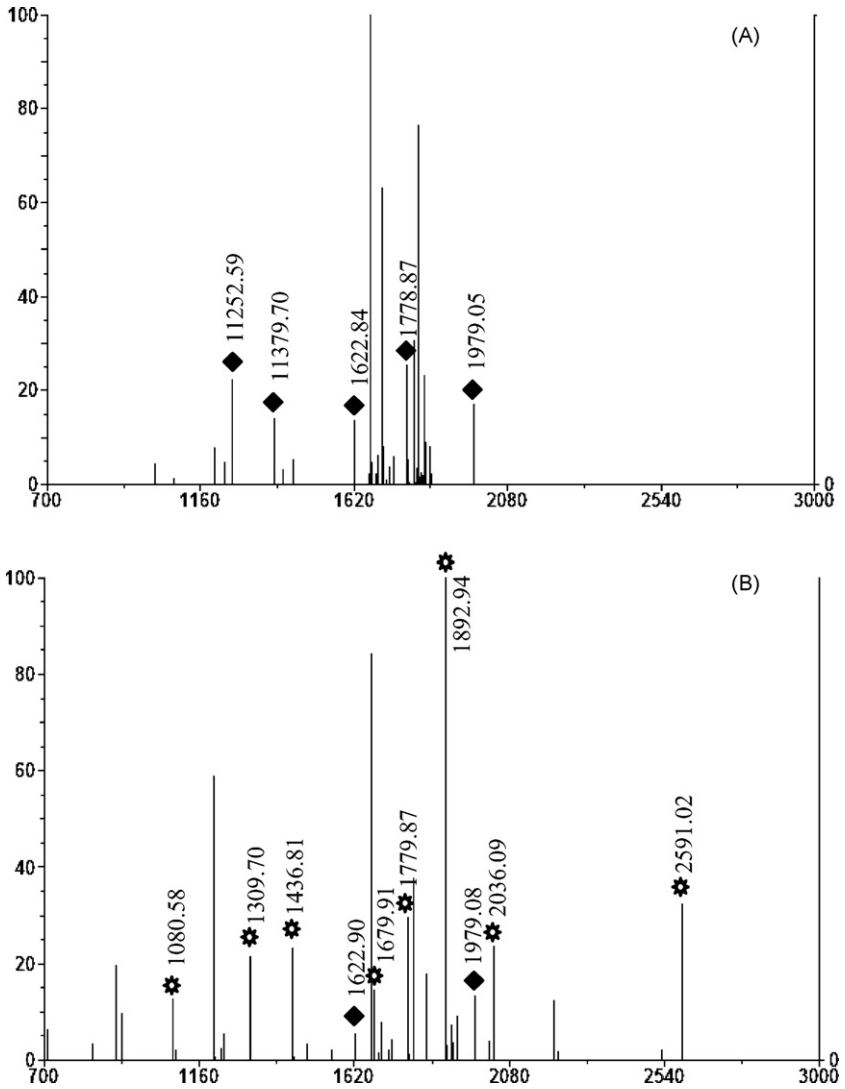

Fig. 3. MALDI spectra of a tryptic digestion of 0.14 μg α-lactalbumin performed on the CD. Protein was directly captured in the outer column by adding sample in the outer inlet (A) without alkylation. (B) With alkylation.

droplet was evaporated whereby a new droplet was pushed out by a short increase in the rotational frequency of the disc.

3.3. Fluorescence measurement

A 0.15 μg IgG1κ sample dissolved in PBS buffer containing 1% BSA was loaded on the CD in the inner inlet. This sample was prepared as a mixture of non-labelled and labelled IgG1κ, in which the amount of the latter is 0.3 ng due to detector saturation at high amount of fluorescent antibodies. This sample was captured on the inner column, eluted and then captured again on the outer column following the same method described above. Fluorescence was detected in the inner columns before the elution step. The fluorescence signal was measured both in the inner and the outer column after the elution. A blank sample not containing any IgG1κ was also measured as a reference. The images produced from recording of the emitted fluorescence from each individual column on the CD were processed using the Gyrolab Viewer (ver 1.1).

3.4. In solution digest of proteins

D/L-Dithiothreitol, iodoacetamide and trypsin were prepared in 100 mM Ambic buffer (pH 8.3). Approximately 2.5 μg of protein was mixed with 8 μL of Ambic buffer containing 1% of *Rapi*Gest surfactant in an eppendorf tube. An addition of 1 μL of 90 mM DTT was then performed, followed by incubation at 60 °C for 30 min. After that, 1 μL of 45 mM iodoacetamide was added. The sample was then incubated in the dark for 30 min. An addition of 2 μL of trypsin at 150 ng/μL was made resulting in a trypsin and protein ratio approximately of 1:8, followed by 10 μL of Ambic buffer and incubation at +37 °C for 18 h. The digested sample was then kept at −20 °C before MALDI analysis. Approximately 12 μg of α-lactalbumin was also digested with the same method using 120 ng of trypsin.

3.5. MALDI TOF MS analysis

The target adaptor containing the pieces of the CD was inserted into the mass spectrometer like a normal plate. MALDI-MS data were acquired under the delayed extraction mode with acceleration voltage of 20 kV and grid voltage of 62%. Delay times were set to 350 ns and 200 ns for MALDI analysis on CD and standard plate, respectively. The instrument was externally calibrated before acquiring the data.

Peptides generated in solution by tryptic digestion were desalted by ZipTip C18 using the protocol recommended by the manufacturer. α-Cyano-4-hydroxycinnamic acid at 2 mg/mL con-

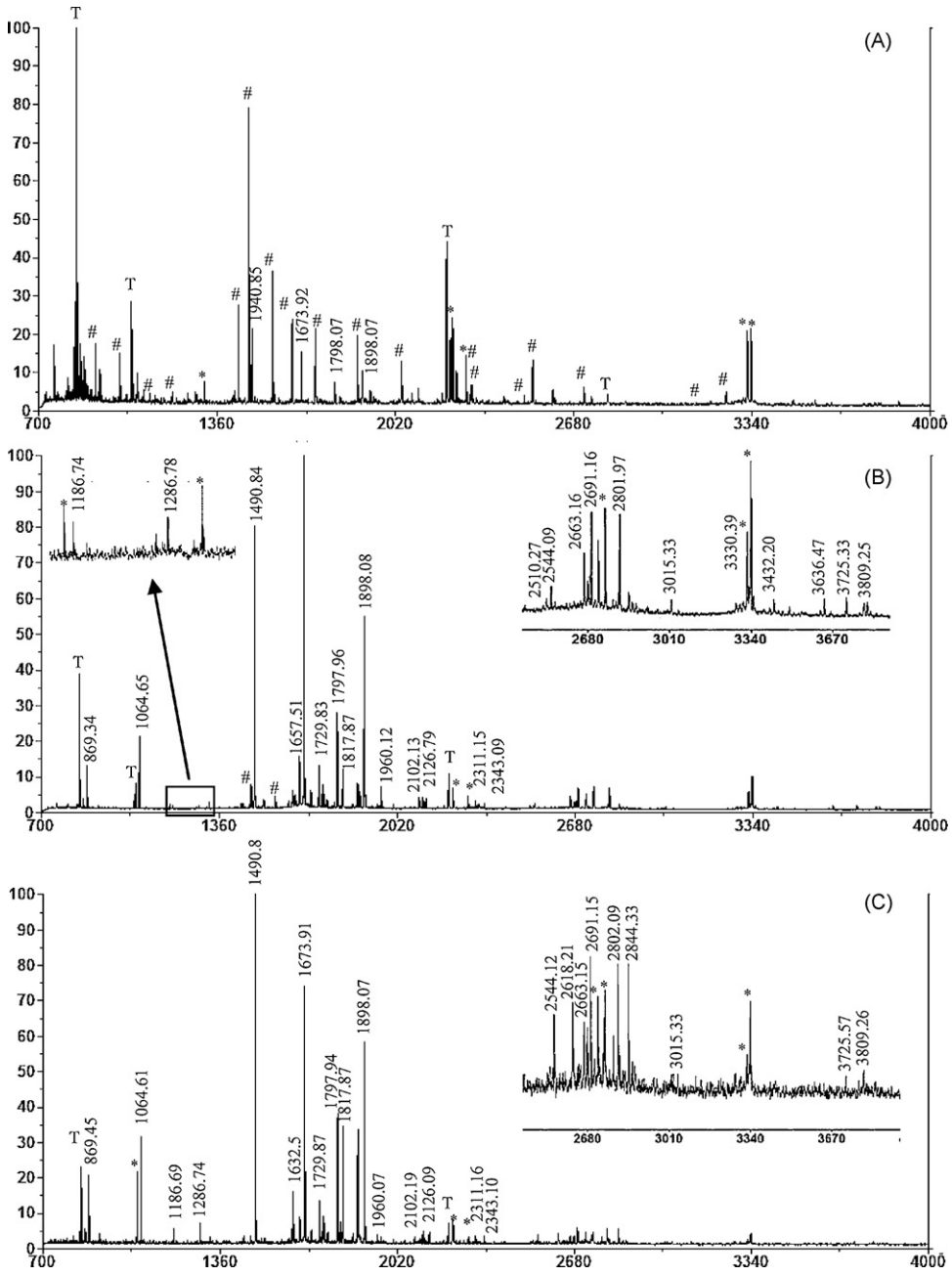

Fig. 4. MALDI spectra of tryptic peptides from a digestion of 0.35 μg IgG1κ performed in the CD using the dual column format. Digestion of IgG1 prepared in PBS containing 0.5% BSA without purification (A) and with purification (B). (C) Digestion of sample prepared in PBS. (#): BSA peptides. (T): Trypsin peptides. (*) Contamination peaks also present in the blank sample.

taining 0.1% TFA used as matrix was spotted onto the stainless steel plate and allowed to dry. Peptides eluted from C18 pipette tip were spotted onto matrix crystals and let dried under ambient conditions.

4. Results and discussion

4.1. Selective capture of antibodies

The capacity and the capture efficiency of the affinity media were studied using both columns of different size packed in the CD and packed micro-tips. Fluorescently labelled antibodies and fluorescence imaging of the columns were used in the experiments performed in the CD while UV-absorbance measurements were made of the captured and eluted antibodies from the packed micro-tips. The capacity determined using the packed micro-tips was 16 μg of antibody per μL column material, somewhat less than the capacity specified for commercially available protein A affinity media, ie MabSelect from GE Healthcare.

4.2. Release of captured antibodies

Protein A is a well studied affinity ligand for antibodies purification [12]. Antibodies typically bind to protein A at neutral pH and can be eluted using an acidic buffer. It has been reported that antibodies with different or even identical Fc-regions can have different optimal elution pH values [13]. Buffer pHs are normally chosen as low enough to give a sufficient elution and as high as possible to avoid changing the conformation and stability of the IgG molecules. Aggregation of antibodies has been found when elution with buffers

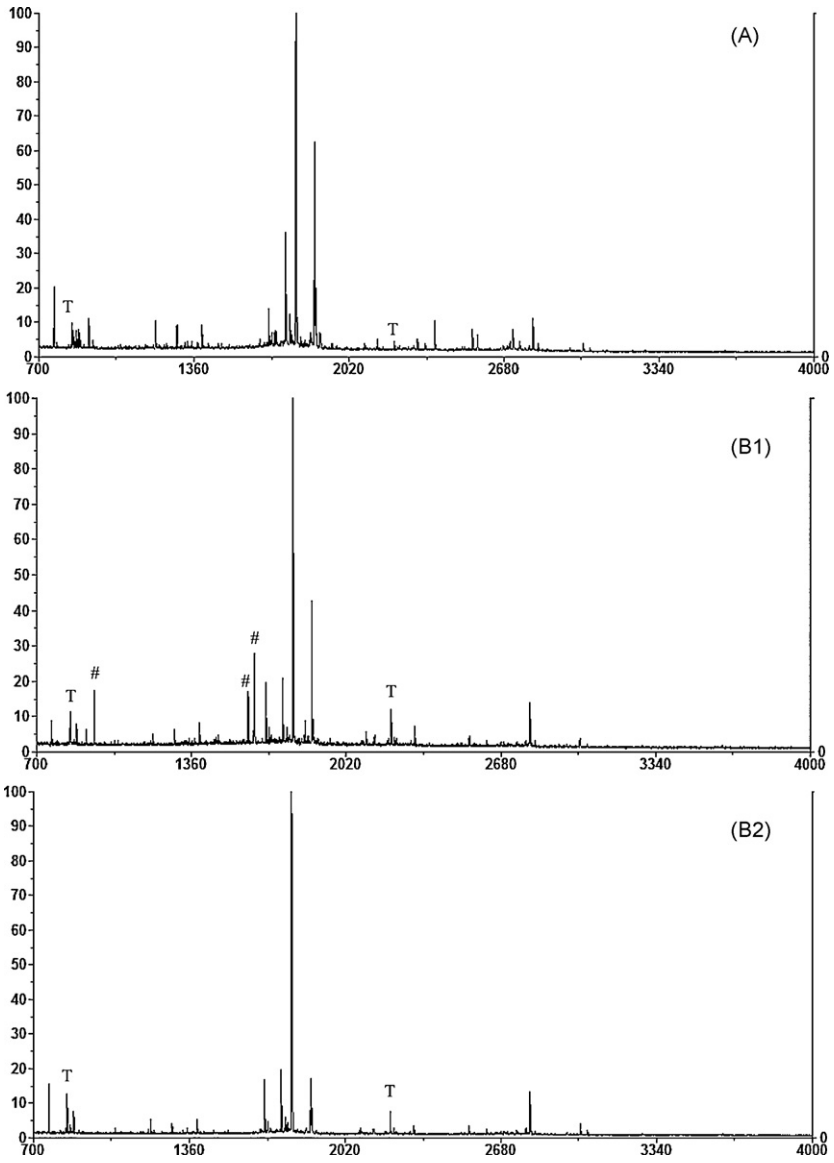

Fig. 5. MALDI spectra from 0.73 μg of recombinant monoclonal IgG1 antibodies selectively captured and digested using trypsin on the CD. (A) IgG1 sample in PBS buffer. (B) lgG1 sample prepared in cell media from untransfected CHO cells collected after POD filter. (B1) One washing step after loading samples. (B2) Three washing steps after loading samples. (T): Trypsin peptides. (#): contaminants from cell culture media.

at too low pH has been used [14–16]. Buffers with different pHs were investigated for the elution of antibodies from the protein A capture media in order to avoid undesired losses due to aggregation. The integrated fluorescence signals of a fluorescently labelled IgG1 kappa captured on the inner column and recaptured on the outer column using different elution pHs are presented in Fig. 2. There was no difference between the residual fluorescence in the inner column after the elution of the antibodies using buffers ranging from pH 2.50 to pH 3.09. The residual fluorescence after elution was very low confirming a good elution in this pH interval. When a pH of 3.67 was used then the amount of antibodies left in the inner column after elution was quite high, approximately 30% of the original amount according to the intensity of their fluorescence signals.

4.3. Tryptic digestion

The ability of performing enzymatic processing of selectively captured compounds in the CD platform is demonstrated by the tryptic digestion of immunoglobulins released from the affinity capture column. The most common steps employed for the tryptic digestion of proteins, reduction using DTT, alkylation with IAA, and digestion with trypsin were adapted from earlier work performed on a different CD design [9].

The efficiency of the alkylation step was examined using α-lactalbumin captured directly on the outer column. A digestion of 0.14 μg of α-lactalbumin was performed for 15 min with or without a preceding alkylation step. Fig. 3 shows the MALDI-MS spectra from the two different digestions of α-lactalbumin, a protein with four disulphide bonds. Fig. 3B shows the spectra obtained when α-lactalbumin was reduced and alkylated whereas Fig. 3A exhibits the digestion of α-lactalbumin without alkylation. The increased number of peaks present in the spectra demonstrated the successful implementation of reactions necessary for efficient enzymatic digestion of proteins. A total of 8 peptides with carbamidomethyl modification were found when α-lactalbumin was reduced and alkylated. Further, 5 non-alkylated peptides appeared in the spectrum without reaction with iodoacetamide whereas only 2 were found in the one with alkylation step.

Up to three subsequent additions of trypsin solution were made to evaluate the efficiency of the enzymatic digestion of an IgG1κ protein. The corresponding digestion times were 15, 30 and 45 min. The number of peptides generated increased from an average of 25 for one addition to an average of 29 for three additions. Five experiments were performed in parallel for each of the digestion experiments and the standard deviation of the number of peptides generated was 1 and 2, respectively, showing a good repeatability. Performing an ANOVA gave a significant difference at the 95%-level between the single addition and the other experiments while there was no significant difference between the digestions performed using two or three additions of trypsin. The relative intensities of the minor peaks in comparison with the more intense peaks increased, however, with the number of additions.

This demonstrates that a sequel of reactions, leading to a successful enzymatic digestion of a protein, can be performed on the microfluidic disc. The total processing time for the procedure is less than 2 h. Moreover, peptide mapping by Mascot resulted in a good protein identification of Apo-bovine α-lactalbumin with 9 matched peptides. The MOUSE score was 159 and sequence recovery was 54%.

Tryptic digestion of IgG1κ, following affinity purification on the inner column of the CD, was performed using three additions of trypsin to ensure a good digestion. The selectivity of the affinity capture was studied by dilution of the IgG1κ in a solution containing a 7-fold excess of bovine serum albumin (BSA). Fig. 4 shows the spectra of tryptic peptides of IgG1κ samples prepared in PBS solution and in PBS solution containing 0.5% BSA. BSA peptides dominated the IgG1 peptides in the spectrum without purification (Fig. 4A). Only two peptides from BSA were present in the spectrum following one column wash with PBS after affinity capture of IgG1 (Fig. 4B). The peptide profiles of the sample prepared in solution containing BSA and the one diluted in only PBS (Fig. 4C) were nearly identical. Selective capture was further tested using a recombinant monoclonal IgG1 antibody produced in CHO cells diluted in spent cell culture media. The MALDI-MS spectra from this antibody diluted in PBS solution and in the cell culture media are compared in Fig. 5. Three unknown peptides from unspecifically retained proteins were found when only one washing step was used after loading sample (Fig. 5B1). Three consecutive washes of the selectively captured antibody were then required to eliminate all contaminant peaks from other proteins present in the cell culture media (Fig. 5B2). Although there was a decrease in peak intensities of some peptides when three washes were applied, similar peptide profiles of the crude sample and the pure one was obtained. The number of observed peptides from this monoclonal antibody was at average of 26 in the range from 700 to 4000 Da, which is more or less consistent with published results from in solution tryptic digestion of same class monoclonal immunoglobulins [17,18].

4.4. Mass accuracy and resolution with CD platform

A study was performed to compare the mass accuracy and masspectrometric resolution attained when ionizing chemical compounds from CDs coated with a conductive layer and a standard stainless steel target plate. This was performed in order to see if the ionization of analytes was affected by the design of MALDI sample and calibration wells, the coating used and the crystallization process on CD. A protein digest performed in solution was desalted, eluted and cocrystallized with matrix on the CD and on the standard MALDI target. Mass spectra were collected and compared. Mascot searches in the NCBIR database with 100 ppm mass tolerance gave very good identifications for α-lactalbumin with 8 matched peptides and 54% sequence coverage for both plate and on CD platform. Table 1 shows a summary of root mean squares (RMS) of the errors presented in the Mascot search results for 8 matched peptides. These values describe the relative mass errors between experimental and calculated masses for all matched peptides. The CD target gave a good mass accuracy with the RMS lower than 100 ppm for external calibration and around 24 ppm for internal calibration. However, the average value of 79 ppm gave an evidence for a slightly poorer mass accuracy of peptides on CD compared to normal plate. Nonetheless, it is still within the mass accuracy range required for a correct identification of protein and therefore sufficient for peptide mapping. Internal calibration resulted in an equivalent mass accuracy with the standard plate. Three representative peptides were used to investigate the resolution. Results in Table 2 showed no significant difference in resolution of 1309 Da and 1669 Da peptides. The resolution of 2590 Da peptide was slightly lower on CD than on plate. More investigations with more masses included revealed that the CD generated slightly lower resolutions for peptides having masses higher than 2200 Da as compared with the plate.

Table 1
Mass errors (root mean squares) from Mascot search for peptides mapping analysis of α-lactalbumin in the NCBInr database.

	External calibration	Internal calibration
CD	79 ± 11	24 ± 3
Plate	43 ± 7	21 ± 1

The mass tolerance used was 100 ppm and the number of miss-cleavages 2.

Table 2
Resolution of three tryptic peptides of α-lactalbumin with on CD clean-up, crystallization.

	Peak 1 (1309.6 Da)	Peak 2 (1669.86 Da)	Peak 3 (2590.96 Da)
CD	10930 ± 1139	11777 ± 1396	9590 ± 1417
Plate	10061 ± 1103	12730 ± 410	11510 ± 2247

5. Conclusions

The experiments discussed above show that the centrifugal microfluidic design presented facilitates the integrated selective capture and subsequent enzymatic processing of biomolecules. This can be beneficial for performing rapid analysis of, for instance, recombinant proteins due to the speed and simplicity of the automated workflow demonstrated. An entire CD with 54 parallel structures could be processed within a total time of 2 h, not considering the mass spectrometry analysis and evaluation.

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