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LENA ERIKSSON

Integrated matrix metalloprotease assays in CD-microlaboratories

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Author

Lena Eriksson

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Abstract

Matrix metalloproteases (MMPs) are subject to diagnostic assessment due to their up-regulation in cancer tissue. In this study, miniaturised CD-based methods for analysing activity and quantity of MMP-2 were developed. Enzyme activity was measured by a homogeneous assay based on the FRET technique. Enzyme quantification was performed with a sandwich immunoassay, using antibodies to capture and enable detection of MMP-2 on an affinity column. Sub-nM detection limits were demonstrated for both methods. Further, the feasibility to serially integrate enzyme activity and quantification assays in a single CD-microstructure was investigated.

Keywords

MMP, Homogeneous enzyme activity assay, FRET, Sandwich immunoassay

Supervisors

Mats Holmquist
Gyros AB

Scientific reviewer

Mats Inganäs
Gyros AB

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Integrated matrix metalloprotease assays in CD-microlaboratories

Lena Eriksson

Sammanfattning

Matrix metalloproteaser (MMPs) är en familj proteinnedbrytande enzymer med viktiga funktioner vid normal och sjukdomsrelaterad omformning av cellvävnad. Mängden MMPs och deras enzymaktivitet har visat sig vara förhöjd vid vissa cancertillstånd. Därför utförs analyser av MMPs i diagnostiska sammanhang. Enzymerna bildas i en inaktiv pro-form som aktiveras genom att en del klyvs bort. I vävnader finns pro- och aktiv form både fritt och bundet till särskilda molekyler (inhibitorer) som kan förhindra aktiviteten. Funktionerna och samspelet mellan olika MMPs är komplext. För att kartlägga detta finns behov av att bestämma både mängd och enzymaktivitet. Det här examensarbetet syftade till att göra detta i miniatyriserade laboratorier baserade på CD-teknik.

För bestämning av enzymaktiviteten blandades MMP-2, en av medlemmarna i enzym-familjen, med ett syntetiskt substrat vilket genererade ljussignaler proportionella mot MMP-2 aktiviteten. Totala mängden av pro- och aktiv form erhölls genom utnyttjandet av antikroppar för specifik infångning och detektion av MMP-2. Möjligheten att utföra bägge analyserna i serie i en CD-struktur, och därmed få information om både aktivitet och mängd från samma prov, utvärderades också. Då metoderna utfördes var för sig, i separata CD-strukturer, möjliggjorde de för analys av biologiska prover. Då de gjordes i serie blev det stora störningar vid mängdbestämingen.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet februari 2004

CONTENTS

ABBREVIATIONS

APMA	- p-Aminophenylmercuric Acetate
BSA	- Bovine Serum Albumin
CV	- Coefficient of Variation
DMSO	- Dimethyl Sulfoxide
ECM	- Extracellular Matrix
FRET	- Fluorescence Resonance Energy Transfer
LIF	- Laser Induced Fluorescence
MMP	- Matrix Metalloprotease
MT-MMP	- Membrane Type Matrix Metalloprotease
MTP	- Microtiter Plate
PBS	- Phosphate Buffered Saline
PMT	-Photo Multiplier Tube
TIMP	-Tissue Inhibitor of Matrix Metalloproteases

1 INTRODUCTION

1.1 Matrix metalloproteases in diagnostics

Matrix metalloproteases (MMPs) are a family of Zn-dependent endoproteases involved in normal tissue remodelling processes such as embryonic development, wound healing and cell migration (Johnson et al., 1998). They also take part in enzyme cascades, by processing cytokines and growth factors into products of altered biological activity (Coussens et al., 2002). The family consist of at least 17 members and can, based on their preferred extra cellular matrix (ECM) substrates, be divided into four subclasses: gelatinases, collagenases, stromelysins and membrane type MMPs. Matrix metalloproteases have been revealed to play key roles in cancer and autoimmune diseases like rheumatoid arthritis (Johnson et al., 1998). Due to its massive up-regulation in malignant tissue and their ability to degrade components of the extracellular matrix, cancer research has been focused on MMPs and identified them as promising drug targets for cancer therapies (Coussens et al., 2002). In the cell MMPs are synthesised as a latent pro-form that is activated upon protolytic cleavage. In tissue, the pro-form exists together with the active form. Besides, the enzyme activity is *in vivo* regulated through binding to tissue inhibitors of matrix metalloproteases (TIMPs) (Coussens et al., 2002). Normally, there is a delicate balance between MMP, proMMP and MMP/TIMP concentrations and disturbances in their ratio (Bode et al., 1999 and Johnson et al., 1998), as well as an increased expression of proMMP mRNA (Coussens et al., 2002), can result in pathological conditions.

To investigate the function of various matrix metalloproteases in different disorders, methods have been developed that enable levels of MMPs in biological samples to be measured. Enzyme activity assays are used to analyse MMP activity, thus only giving information about the level of active protease. Sandwich immunoassays allow quantitative measurements of the different MMP forms (Fujimoto et al., 1993). The specificity of such an immunoassay depends on the antibodies used. There are antibodies directed against proMMP, pro/active MMP, and against TIMPs. Through combined use of such antibodies various forms of MMPs can be detected. However, when performing a single immunoassay it is not possible to determine the ratio of quantities of active enzyme and pro-forms. Conventional enzyme activity and sandwich immunoassays are performed in microtiter plates, requiring sample volumes of 60-100 µl. The assay times for such methods are at least a few hours (Zucker et al., 1992 and George et al., 2003). Also, a gel-based electrophoretic method (zymography) has been extensively used to analyse gelatinolytic MMPs. The advantage of zymography is that both proMMP and active MMP and can be quantified simultaneously. However, it is a slow method taking approximately 24 h. Only a few samples can be analysed at a time and it requires sample volumes of at least 20 µl (Kleiner and Stetler-Stevenson, 1994). In conclusion, the existing methods for analysing active and pro-forms of matrix metalloproteases are time consuming, require large sample volumes and involve several manual steps.

1.2 Characteristics of MMP-2

Matrix metalloprotease 2 (MMP-2), also called Gelatinase A due to its ability to degrade gelatine, was first discovered as a result of cloning cDNA from malignant mouse tumour cells (Coussens et al., 2002 and Morgunova et al., 1999). It is secreted as an inactive 72 kDa pro-

enzyme, mainly by fibroblasts, and upon activation the 66 kDa active enzyme is formed. On the contrary to most other matrix metalloproteases, MMP-2 is constitutively expressed (Johnson et al., 1998) with normal proMMP-2 serum levels of approximately 8 nM (570 ± 118 ng/ml) (Fujimoto et al., 1993). MMP-2 influence many processes in the human body and altered activity is correlated to several types of cancer. Its involvement has been implicated at various stages of cancer progression. During early tumourigenesis MMP-2 is required for the formation of new blood vessels (angiogenesis), thereby supplying tumour cells with nutrients and enhancing their growth (Fang et al., 2000). In metastasising tumour cells the gelatinolytic activity of MMP-2 enable cells to traverse basement membranes at tissue boundaries and in blood vessels. Hence, the protease is highly expressed in metastasising tumour cells (Morgunova et al., 1999). In order to find biological cancer markers, measurements of the amount of proMMP-2 present in serum have been performed. The proMMP-2 levels in cancerous states have been shown to differ from normal concentrations. For example, in patients with hepatocellular carcinoma there is a 1.2 fold increased proMMP-2 concentration compared to normal serum levels, whereas a similarly large reduction is observed in patients with stomach and pancreatic cancer. Activation of the pro-enzyme might account for the reduction in the latter cases (Fujimoto et al., 1993). The ratio of active to total MMP-2 levels have been correlated to tumour aggressiveness (Foda and Zucker, 2001). Due to its key role in cancer, MMP-2 has been the target for the development of antitumour drugs inhibiting angiogenesis as well as metastasis. Since the structural properties of MMP-2 have been revealed, further insights into the desired characteristics of the inhibitors have been gained (Morgunova et al., 1999).

Matrix metalloproteases share structural properties. They are all synthesised with a signal peptide, a pro-peptide and a catalytic domain. In addition, most MMPs have a hemopexin-like domain C-terminal of the catalytic domain and a linker region in between (Bode et al., 1999). The different domains of proMMP-2 are illustrated in figure 1.

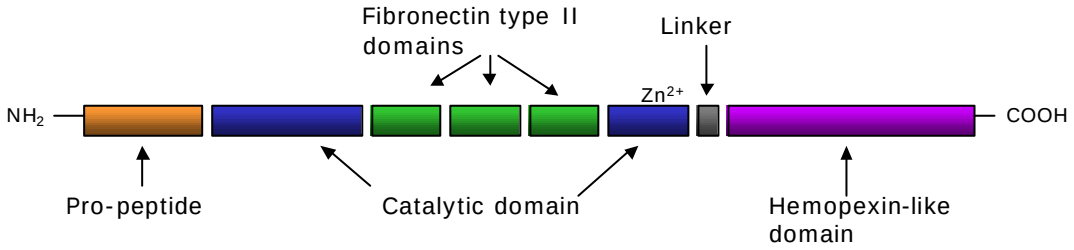

Figure 1. Schematic drawing of the different domains of proMMP-2. The illustration was adapted from (Nagase and Woessner, 1999)

The catalytic domain consists of two modules separated by a hydrophobic active site cleft with a catalytic zinc ion at the bottom. When the zinc ion is coordinated to three histidine residues in a highly conserved motif in the domain, it can activate a water molecule and thus promote hydrolysis of a peptide bond within the substrate. In proMMPs a cysteine residue in the pro-peptide is coordinated to the catalytic zinc and thereby the enzyme is maintained in a latent state. Activation occurs in a two step process, with disruption of the Cys - zinc interaction followed by autoproteolytical removal of the pro-peptide by the target MMP. *In vivo,* proMMP-2 is primarily activated on the cell surface by a complex between membrane

type MMP-1 (MT-MMP-1) and TIMP-2 (Johnson et al., 1998). *In vitro*, the process is accomplished with proteases or more commonly by SH-reactive compounds, such as organomercurials (*e.g* p-aminophenylmercuric acetate APMA), breaking the cysteine to zinc coordination. The hemopexin-like domain is involved in the interaction with the activation complex MT-MMP-1/TIMP-2 and is contributing to gelatine binding. The latter is also the function of the fibronectin type II domains, which are unique for the gelatinolytic MMPs (Morgunova et al., 1999 and Ngase and Woessner, 1999). However, there seems to be no need for these domains upon cleavage of small peptide substrates. An excess of TIMP-2 has been shown to inhibit MMP-2 activity *in vivo*, but also synthetic compounds have been evaluated for that purpose (Johnson et al., 1998). Near all synthetic inhibitors have a zinc-chelating group and a peptidomimetic moiety, mimicking substrate binding (Bode et al., 1999). In summary, there is an intricate interplay of regulation of MMP-2 activity, which is not easily controlled or understood.

2 AIM OF PROJECT

The aim of the project presented in this report was to develop miniaturised methods for analysing both enzyme activity and quantity of MMP, at concentrations expected to be found in serum and plasma. The project was based on Gyros technology platform, which miniaturise and integrate common laboratory processes into application-specific CD-microlaboratories. Within the CDs natural forces control movement of liquids and one disc can automatically process several sample in parallel. The feasibility of serially integrating enzyme activity and quantification assays within a single CD-microstructure was to be investigated. On a small sample volume such analyse would generate two pieces of information, which is valuable when studying MMP biology in diagnostic situations. Also, integration of assays would enable specific enzyme activity (that is the enzyme activity per enzyme molecule) determinations. Commercially available CD microlaboratories as well as explorative microstructures were investigated. Prevention of evaporation, unspecific losses of sample on surfaces and mixing of laminarly flowing liquids (that is liquids moving as discrete plugs) were addressed. All these are challenges coupled to miniaturisation of assays. In the project matrix metalloprotease 2 (MMP-2) was used as a model system. Experiments were performed with three preparations of the protease: proMMP-2, active MMP-2 and the catalytic domain of MMP-2.

3 METHODS

3.1 Gyros Technology Platform

3.1.1 The Gyrolab™ workstation LIF

The Gyrolab™ workstation LIF is an integrated system for running biological assays within a CD microlaboratory. The reactions occur at nanoliter scale and are monitored by an on-line laser induced fluorescence (LIF) detector, moving from the periphery to the centre of the CD during detection. Included in the instrument is also a robotic arm with capillaries transferring samples and reagents from microtiter plates to inlets of the CD and a spinner for spinning the CD. Robotic arms load the CDs into the spinner and move them between the spinner and the detection unit. Software controls the sequential loading of samples into specific inlets of the CD. Spinning rates and intervals are also automatically controlled. At most it is possible to load five discs in the workstation. The kind of reaction being executed in the workstation is depending on the microstructures in the CDs.

3.1.2 The Gyrolab CD

The CD microlaboratories consist of individual application-specific microstructures, each structure having individual inlets, chambers for volume definition, common distribution channels, overflow channels and hydrophobic brakes, as shown in figure 2. Common distribution channels connect multiple microstructures and enable loading of sample, wash buffer and reagents into several microstructures, when desired in a parallel manner. Capillary forces draw liquids into the channels of the structure, hydrophobic brakes localise it to different compartments and centrifugal force, created when spinning the CD, moves sample between the different parts of the microstructure. The flow rates through the structures are controlled through spinning programs. The channels of the microlaboratories are produced through injection moulding into a plastic CD. Hydrophobic brakes and surface modifications are made at desired spots before a lid is laminated on to the CD. Since there are up to 100 structures in one CD, several samples can automatically be processed in parallel, which is advantageous in the fields of proteomics, drug discovery and diagnostics.

Miniaturisation of assays present technical challenges. In miniaturised systems there is a large surface-to-volume ratio, which results in increased evaporation compared to larger systems. The CDs are made of a hydrophobic plastic material, which in combination with the large surface-to-volume ratio contributes to a risk of losses of proteins on the surfaces. To overcome this, the surfaces of the structures have been modified with hydrophilic agents. Within the narrow channels of the microstructures liquid moves as discrete plugs (laminarly flowing liquids) so when two liquids are to be merged into one flow they end up next to each other and diffusive mixing occurs only at the solution boundaries. Proper mixing has to be promoted through certain mixing structures. Since only nanoliter volumes are loaded into the structures, implying quite few molecules, the CD microlaboratories require a sensitive detection system. In brief, the development and application of the microlaboratories bring together the disciplines of microfluidics, surface chemistry and biochemistry.

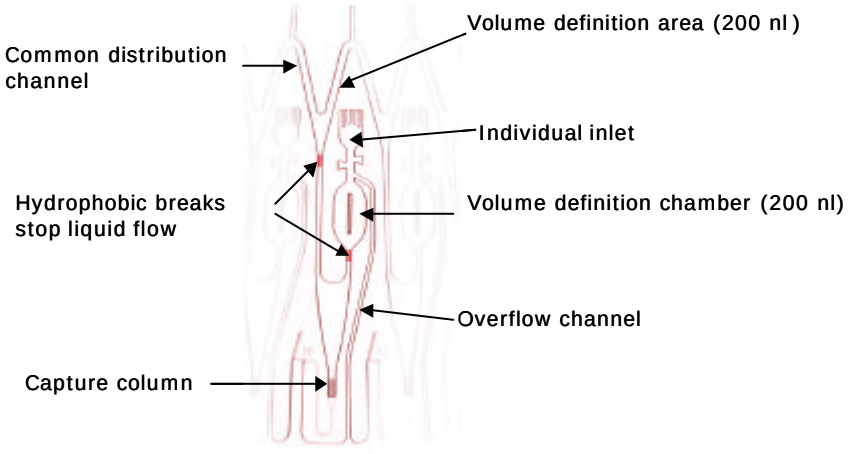

Figure 2. Illustration of the common features found in the microstructures on the CDs. This particular structure has an affinity column at the lower end and is used for sandwich immunoassays. The illustration was used with permission from Mats Inganäs at Gyros AB.

3.1.3 Laser induced fluorescence detection

Fluorescence is a sensitive detection method that can be used to quantify molecules present in concentration too low for absorption spectroscopy (Wilson and Walker, 2000). Some molecules, called fluorophores, emit light when falling back from an excited electronic state (S_1) into the ground level (S_0). In order for fluorescence to occur an earlier excitation event,

caused by illumination, must have taken place. The principle of fluorescence is illustrated in figure 3. When fluorophores, which often are polyaromatic hydrocarbons, absorb photons of energy ($h\nu_{EX}$) they are excited to a higher electronic level. At the same time they gain vibrational energy, thus entering an unrelaxed excited electronic state (S_1'). The molecule stays in the excited state for a few nanoseconds and during this time some energy is dissipated to the surroundings as heat, causing the molecules to enter the lowest vibrational level within the excited state. When returning to the ground state, photons with energy ($h\nu_{EM}$), corresponding to the difference in energy between the two electronic levels, are emitted. Since some energy is lost as heat, the emitted light will have longer wavelength (lower energy) than the light causing the excitation. This difference in wavelength is called Stoke's shift. The greater the Stoke shift is, the easier it is to distinguish emitted from light used for excitation. The principle of fluorescence is further described in Wilson and Walker, 2000.

In the LIF detector of the Gyrolab™ workstation LIF, excitation is originating from illumination with a laser, *i.e.* a monochromatic light source containing only one wavelength (633 nm (red) or 532 nm (green)), focused into the structures of the CD. The emitted light is filtered through a bandpass filter, before entering a photo multiplier tube (PMT) where the photons are converted to electrons. When wavelength and intensity of the incoming light is held constant, the intensity of the emitted light is directly proportional to the number of fluorophores present.

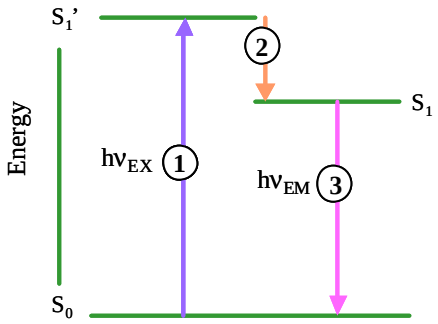

Figure 3. Principle of the molecular event of fluorescence (Jablonski diagram). Fluorescence occur in a three step process with 1) excitation, 2) loss of energy through vibration and 3) emission. The illustration was adapted from (Amersham Biosciences, 2002)

3.2 Homogeneous enzyme activity assay

3.2.1 Assay principle

Homogeneous assays, where all substances are in a homogeneous solution, can reveal the enzyme activity in a sample. The principle is that upon mixing of an enzyme with a substrate, to which it has specificity, a detectable change occurs. The more active enzyme present, the greater the signal will be. An activity assay is a suitable tool to screen for inhibitors. This is achieved by adding increasing amounts of inhibitor to constant concentrations of enzyme and substrate. The potency of the inhibitory compound is described by the IC_{50} value, which is the concentration where the activity in the sample is reduced by 50 % compared to a sample without inhibitor. The lower the IC_{50} is, the stronger does the inhibitor bind to the enzyme.

3.2.2 Microstructure design

The microstructure (CDE6 Q) used for homogeneous assays are closed in one end, forming a basin large enough for the substrate and enzyme mixture (figure 4). Besides having common distribution channels, each structure has one individual inlet, both defined to 75 nl. In between the inlets and the reaction chamber (basin) are structures enabling adequate mixing of the sample and substrate. This microstructure is located on an explorative disc.

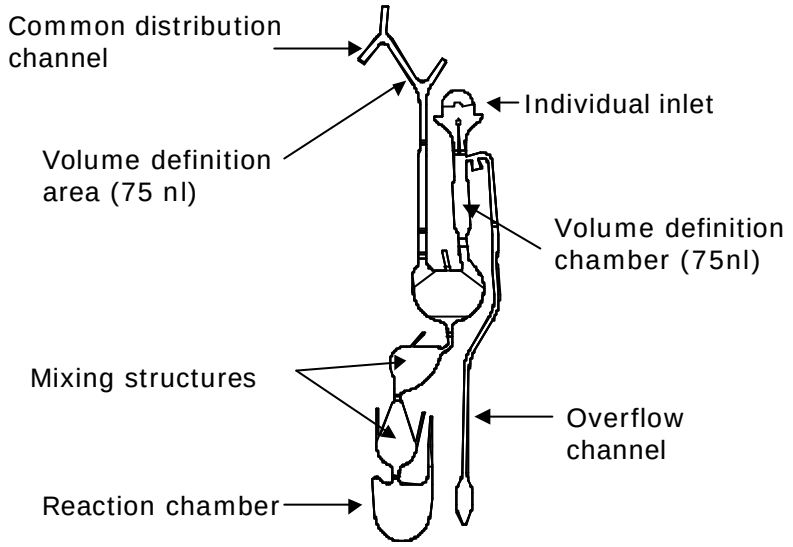

Figure 4. Design of microstructure CDE6 Q for enzyme activity assay. The illustration was used with permission from Mats Inganäs at Gyros AB.

3.2.3 FRET substrate

In some cases, the energy expected to be released as fluorescence (see 3.1.3) is transferred to other molecules, and no light is emitted. This is seen in fluorescence resonance electron transfer (FRET), where the light emitted from one donor fluorophore has appropriate energy to excite another molecule, the acceptor (Stryer, 1978). FRET is a highly distance dependent phenomenon and can successfully be utilised in homogenous activity assays by covalently coupling a donor and an acceptor at opposite ends of a short peptide substrate. When the substrate is intact the signal is quenched and no light is emitted from the donor. When the enzyme cleaves the substrate, the product results in fluorescence. Figure 5 shows the principle of FRET for the synthetic MMP-2 peptide substrate (Cy3B-PLG?LAARK(Cy5Q)-NH_2) used in this study. The donor, Cy3B, can be excited in a green LIF detector and the fluorescence emitted from Cy3B serves as a signal in the enzyme activity assay. The acceptor Cy 5Q, which is another CyDye™ molecule, is designed to loose the energy of the excited state through routes other than fluorescence (Osborn, 2002 and Hardwicke et al.).

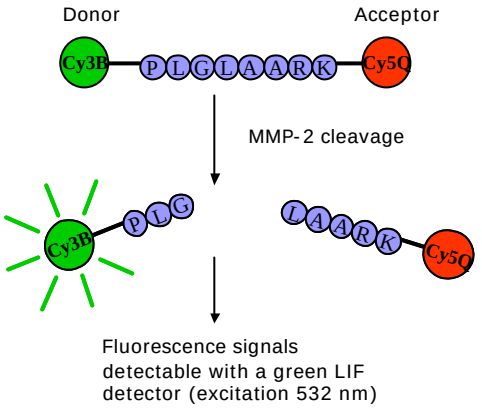

Figure 5. The principle of fluorescence energy transfer (FRET). The illustration was adapted from (George et al.)

3.3 Heterogeneous sandwich immunoassay

3.3.1 Assay principle

The immunoassay performed in the CD-microlaboratory is a sandwich immunoassay, where an antibody attached to the solid phase of the column captures the target protein. By subsequently in excess adding a detectable fluorophore conjugated antibody, the analyte can be quantified. Since all unbound constituents are washed away, the fluorescence signal from the column is directly proportional to the amount of target protein present. A sandwich assay requires antibodies directed against different binding sites (epitopes) of the analyte. Preferentially monoclonal antibodies (antibodies with affinity for a single epitope of the analyte) are used. Combinations of monoclonal and polyclonal antibodies (a population of antibodies specific for different epitopes of the same analyte) can also be used in capturing and detecting steps. Since antibodies can be produced against many analytes, this system can be used for analysing various types of proteins, such as cytokines, growth factors and proteases (Diamandis and Christopoulos, 1996).

3.3.2 Microstructure design

The microstructure shown in figure 2 was used for running heterogeneous sandwich immunoassays. Specific for this structure is a 10-15 nl column pre-packed with polystyrene particles coated with a phenyldextran layer to which streptavidin is attached. Below the column there is a restriction channel through which unbound samples leave the microstructure. All inlets are defined to 200 nl.

3.3.3 Streptavidin- Biotin system

Streptavidin is a 60 kDa tetrameric protein from the bacterium *Streptomyces avidinii* having four biotin-binding sites and the ability to bind biotin (also known as vitamin H) with an affinity that is 10^3-10^6 times higher than that between an antibody and antigen. The very strong interaction (affinity constant $>10^{15}$ M^{-1}) is commonly utilised in immunoassays, to immobilise antibodies to surfaces. In the CD, the streptavidin-biotin system is responsible for attaching capturing antibodies to the columns. Methods and commercially available reagents have been developed for covalently linking biotin to proteins. Since biotin is a relatively small molecule (M_w = 244 g/mol), it has been shown to have little effect on the functionality of the protein upon attachment. Another great advantage of the system in immunoassays is the

strong interaction, persisting extreme pH values as well as washing with detergents and organic solvents (Diamandis and Christopoulos, 1996).

3.4 Integrated enzyme activity and quantification assay

3.4.1 Assay principle

The principle of the assay is to perform a homogeneous activity assay and downstream of that apply the reaction mixture onto a column specific for the enzyme.

3.4.2 Microstructure design

A specifically designed microstructure (CDE6 D), containing a reaction chamber emerging into a space with a column, was used to integrate the activity and immunoassays, see figure 6. Between the reaction chamber and the column is a tight hydrophobic brake and through controlling spinning, liquid can be maintained in the reaction chamber. On one disc there are 10 of these structures connected through a common distribution channel with a volume defined to 75 nl. Each structure has two individual inlets, one inner at the top (75 nl) and one outer emerging into the column space (100 nl).

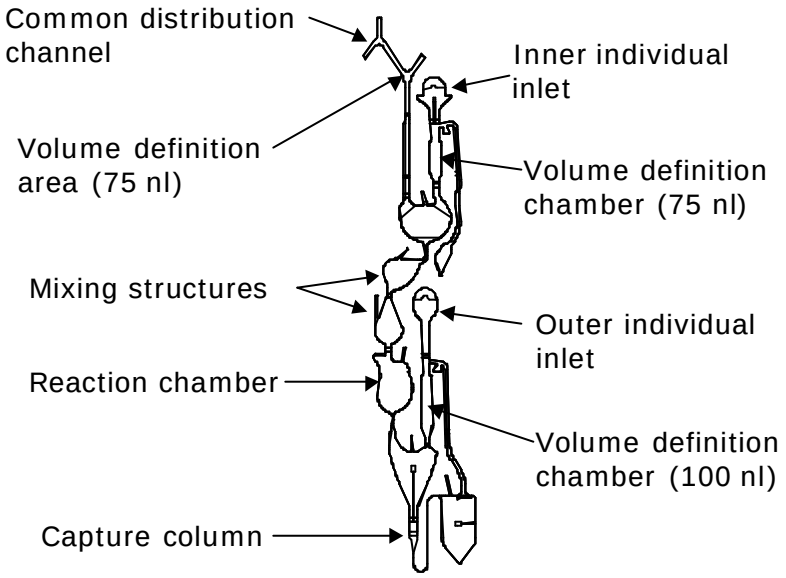

Figure 6. Design of the microstructure CDE6 D. The microstructure was used for performing integrated enzyme activity and quantification assays. The illustration was used with permission from Mats Inganäs at Gyros AB.

4 EXPERIMENTAL

4.1 Enzyme preparations

In the assays, three enzyme preparations were used: the catalytic domain of human MMP-2 (BIOMOL Research Laboratories Inc, PA, USA), human proMMP-2 (R&D Systems; Novakemi, Stockholm, Sweden) and MMP-2 (proMMP-2 activated with p-aminophenyl-mercuric acetate, see 4.2.2).

4.2 Homogeneous enzyme activity assay

4.2.1 Enzyme activity assay protocol

The Q-structure of CDE6 was utilised for performing homogeneous activity assays. MMP-2 (75 nl) in concentrations of 1-20 nM in buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 0.025% (w/v) Brij-35; pH 7.5) was added through the individual inlet of each structure. The MMP-2 peptide substrate of sequence Cy3B-PLG?LAARK(Cy5Q)-NH_2 (Mw = 2159 g/mol; Amersham Biosciences, Uppsala, Sweden) was diluted in buffer and loaded in the common channel. Thus, 75 nl of substrate was distributed to each microstructure. Mixing of enzyme and substrate occurred through spinning the CD, followed by LIF detection in a detector (532 nm excitation, 600 nm emission; green) not integrated in the workstation. Different substrate concentrations were evaluated for optimising the assay. The results were analysed using the LIF Compare 2.00 Software (Gyros AB), which visualise the distribution of fluorescence in the reaction chamber. Algorithms find the highest intensity on each radius in ten radiuses from the centre of the basin and the lowest of the collected fluorescence intensities is the response value. Samples were run in triplicates.

4.2.2 Activation of proMMP-2

Human recombinant proMMP-2 was activated using p-aminophenylmercuric acetate (APMA). APMA was dissolved in DMSO to a concentration of 10 mM and then transferred to vials with enzyme in 50 mM Tris pH 7.5, containing 150 mM NaCl, 10 mM $CaCl_2$, 0.025% (w/v) Brij-35, giving a proMMP-2 concentration of 100 nM and APMA concentration of 60 µM in 70µl samples. In negative controls DMSO was added instead of APMA. For comparison, the catalytic domain of MMP-2 was simultaneously incubated with APMA or DMSO at 37 °C. Enzyme activity of the catalytic domain dissolved in buffer was also measured.

4.2.3 Product quantification

A product calibration curve was generated relating fluorescence intensity to quantity of product. To generate product, a non-specific protease (subtilisin), was used to fully digest substrate. Subtilisin was mixed with equal volumes of MMP-2 substrate (0.125 - 8 µM; eight standards) and incubated in eppendorf tubes at + 4 °C for 48 hours. After incubation, samples were loaded in the Q structures of CDE6 and fluorescence intensity was recorded in a LIF-detector (532 nm excitation, 600 nm emission; green).

4.2.4 Inhibition of MMP-2 by galardin

The broad range MMP inhibitor galardin(10 mM in DMSO), GM 6001 (BIOMOL Research Laboratories, PA, USA) was diluted in buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 0.025% Brij-35; pH 7.5). Each galardin concentration was applied to a microtiter plate well (7.2 µl) and MMP-2 substrate (1.8 µl) was added to each well, resulting in 10 µM substrate and galardin ranging from 0 to 100 nM (0, 0.1, 0.4, 1.56, 6.25, 25, 50, 100

nM). In CDE6 Q, substrate and inhibitor mixtures (75 nl) were added through the individual inlets and 10 nM MMP-2 in buffer (75 nl) was introduced into the common distribution channel. Mixing was followed by LIF detection. Data was collected up to 16 minutes.

4.3 Heterogeneous sandwich immunoassay

4.3.1 Biotinylation of antibodies

Monoclonal antibodies with specificity for pro/active forms of human MMP-2 (MAB 902 and MAB 903 from R&D Systems; Novakemi, Stockholm, Sweden) were labelled with biotin, using a 24-fold molar excess of biotin reagent (EZ-Link Sulfo-NHS-LC-Biotin from PIERCE Biotechnology; Boule Nordic AB, Huddinge, Sweden). Lyophilised antibodies were dissolved in phosphate buffer saline (PBS) (0.015 M $NaPO_4$, 0.15 M NaCl; pH 7.4) to a concentration of 1.11 mg/ml and further diluted in 1 M $NaHCO_3$ to 1 mg/ml. Antibodies (100 µl) was mixed with biotin reagent solution (9 µl; 1 mg/ml in MilliQ) and incubated at room temperature for 1h with occasional mixing using a pipette. After incubation free biotin was removed with a Nanosep® Device 30K(PALL® Life Sciences; VWR International, Stockholm, Sweden), centrifuged at 10 000 x g for 4 x 1 min. The biotinylated antibodies were recovered in PBS (100 µl) and protein concentration was determined by spectrophotometric measurements of absorbance at 280 nm. The concentration was calculated with an extinction coefficient of 1.38 $cm^{-1}*l*g^{-1}$.

4.3.2 Fluorophore conjugation of antibodies

Antibodies were conjugated with fluorophore using the Alexa Fluor® 647 Monoclonal Antibody Labelling Kit from Molecular probes (Termometerfabriken, Gothenburg, Sweden). Monoclonal mouse antibodies MAB 902 and MAB 903 (90 µl; 1.11 mg/ml) were mixed with 10 µl 1M $NaHCO_3$ (from the kit). Lyophilised goat polyclonal antibody AF902 (100 µg) against pro/active human MMP-2 (R&D Systems; Novakemi, Stockholm, Sweden) was dissolved in 0.1 M $NaHCO_3$ (100 µl). Further, the conjugation procedure was performed on 100 µl of antibody solution according to the manufacturers instructions. Absorbance was measured at 280 and 650 nm. The antibody concentration and the degree of fluorophore labelling was determined with the following equations:

Antibody concentration (M) = $(A_{280} - (A_{650} * 0.03)*\text{dilution factor})/ 203\,000$

Degree of labelling (moles Alexa dye/moles antibody) = $(A_{650}*\text{dilution factor}) / (239\,000 *$ antibody concentration (M))

4.3.3 Sandwich immunoassay protocol

Heterogeneous sandwich immunoassays were run in CDBA2 (CD Bioaffy 2, Gyros AB) having columns (10-15 nl) pre-packed polystyrene particles (Dynal Biotech, Oslo, Norway) coated with a phenyldextran layer (Amersham Biosciences, Uppsala, Sweden) to which streptavidin was attached. Initially the columns were reconditioned, by washing twice with PBS-Tween (0.015 M $NaPO_4$, 0.15 M NaCl, 0.02 % (w/v) Tween-20; pH 7.4). Biotinylated monoclonal anti-MMP-2, diluted in PBS–Tween were immobilised onto the columns, making them specific for MMP-2. Samples of MMP-2 were passed through the columns, followed by fluorescence conjugated detection antibodies. MMP-2 was diluted in Tris buffer containing 1% (w/v) BSA (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 1% BSA; pH 7.5) and antibodies were diluted in PBS with 1% (w/v) BSA (0.015 M $NaPO_4$, 0.15 M NaCl, 1 % BSA; pH 7.4). Each solution (200 nl) was loaded per microstructure, antibodies through

the common distribution channel and MMP-2 through the individual inlets. Wash steps were included in the assay to reduce interference from species not bound to the columns. Two washes with PBS-Tween followed after addition of biotinylated antibody and 2-4 with PBS-Tween after MMP-2 addition. When fluorescence labelled antibodies had flown through the columns they were rinsed twice with PBS - Tween and four times with PBS –Tween containing 20 % (w/v) isopropanol. Fluorescence intensity was measured over the entire column, with an on-line LIF detector (633nm excitation, 650-700 nm emission; red) with photo multiplier (PMT) sensitivity of 1, 5 and 25%, before and after addition of detection reagent. The fluorescence distribution over the columns was visualised as collected image files (TIFF images) by the LIF Protein Array Analyser v 3.6 software (Gyros AB), which also calculated the integrated fluorescence signal in an area at the top, where the analytes are enriched. Integration occurred over an equally large area in all columns. Standard curves were generated using the Gyrolab Evaluator Software (Gyros AB).

4.3.4 Evaluation of capturing and detecting antibody pairs

Four different combinations of capturing and detecting antibodies were tested in order to obtain the pair giving best sensitivity in the MMP-2 quantification assay. In two cases 667nM (0.1 mg/ml) monoclonal antibody MAB 902 was used as capturing agent with either monoclonal MAB 903 or polyclonal AF 903 as detecting reagent. When 533 nM (0.08 mg/ml) monoclonal MAB 903 was tested as capturing antibody, monoclonal MAB 902 or polyclonal AF 903 served as detection reagent. The concentrations of all detecting fluorescence labelled antibodies were 100 nM. ProMMP-2 was in run in triplicates (0.05 – 50 nM; 6 standards). Two wash steps with PBS-Tween followed after protease addition.

4.3.5 Quantification of MMP-2

Standard curves were generated having 1000 nM (0.15 mg/ml) of biotinylated monoclonal antibody MAB 903 as capturing agent and 300 nM of the detecting polyclonal antibody AF 902. ProMMP-2, MMP-2 (activated proMMP-2) and the catalytic domain of MMP-2, and was run in triplicates in concentrations ranging from 0.51 to 50 nM (6 standards).

4.4 Integrated enzyme activity and quantification assay

4.4.1 Packing of miniaturised columns in a CD microlaboratory

The structures were initially wetted with PBS-Tween (0.015 M $NaPO_4$, 0.15 M NaCl, 0.02 % Tween-20; pH 7.4). Slurry (8 % (w/v) in PBS pH 7.2, containing 1 % BSA and 0.02 % NaN_3) of polystyrene particles (Dynal Biotech, Oslo, Norway) coated with a phenyldextran layer (Amersham Biosciences, Uppsala, Sweden) and coupled with streptavidin, was introduced into the outer (lower) individual inlets using a pipette. One structure was loaded at a time and spinning followed each loading.

4.4.2 Integrated enzyme activity and quantification assay protocol

The integrated enzyme activity and sandwich immunoassays were run in the D structure of CDE6 and comprised two distinct software methods for the Gyrolab Workstation LIF. In between the execution of the methods, enzyme and substrate mixture was incubated in the reaction chamber and fluorescence was recorded off-line (532 nm excitation, 600 nM emission; green). After an initial needle wash, biotinylated monoclonal antibody MAB 903 (2000 nM (0.3 mg/ml) in PBS-Tween) was applied through the outer individual inlet (100 nl),

followed by a wash step with PBS-Tween introduced through both the outer individual inlet and the common distribution channel. Further, substrate (2 µM in reaction buffer) was added through the common distribution channel (75 nl), MMP-2 through the inner individual inlets (75 nl) and simultaneously PBS-Tween was applied in the outer inlets. Spinning was performed in such a way that substrate and enzyme mixed and stayed in the reaction chamber and PBS-Tween was spun down to wet the column. The CD was rotated for 4 min before unloading and off-line LIF detection (532 nm excitation, 600 nm emission, 3.8 mW power). Incubation times were between 8-12 min. Subsequently, PBS-Tween was spun out through the column and the enzyme and substrate mixture was allowed to interact with the capturing antibodies. To enable quantification of bound MMP-2, fluorescence conjugated detection antibodies were added at a concentration of 300 nM. Analytes were diluted in Tris buffer containing BSA (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$, 1% (w/v) BSA; pH 7.5) and detection antibodies were likewise in PBS containing 1% (w/v) BSA. Wash steps were included in the assay to reduce interference from species not bound to the columns. Five washes with PBS-Tween applied through both common distribution channel and outer individual inlet followed after MMP-2 addition. After fluorescence labelled antibodies had been passed through the columns they were rinsed twice with PBS-Tween and four times with PBS –Tween containing 20 % (w/v) isopropanol. The latter six washes were performed only through the common distribution channel. Fluorescence intensity over the entire column was measured on-line (633 nm excitation, 650-700 nm emission; red) before and after addition of detection reagent. The LIF Protein Array Analyser v 3.6 Software (Gyros AB) was used to monitor the distribution of fluorescence over the columns. The enzyme activity part was analysed by the LIF Compare 2.00 Software (Gyros AB).

5 RESULTS

5.1 Homogeneous enzyme activity assay

5.1.1 Enzyme activity of MMP-2 catalytic domain

The standard curve in figure 7 shows a linear increase in fluorescence intensity with concentration of MMP-2 catalytic domain (1 - 20 nM). Data was generated with 10 µM of MMP-2 CyDye™ substrate at a reaction time of 16 minutes. The detection limit of the assay was 0.16 nM (calculated with three standard deviations added to zero sample average) and the coefficients of variation (CV; n = 3) were 1 - 7 %. For the catalytic domain, the specific enzyme activity was 22 pmoles/min/µg. This was based on the finding that the fluorescence intensity increased with 7.39 units when 1 µM product was formed. With substrate concentrations below 10 µM there were large variations in fluorescence intensity, which was reflected in high CV values. Besides at low substrate levels the fluorescence was not evenly distributed in the reaction chamber. When performing assays with 20 µM substrate high detection limit were obtained due to large background fluorescence from the FRET substrate.

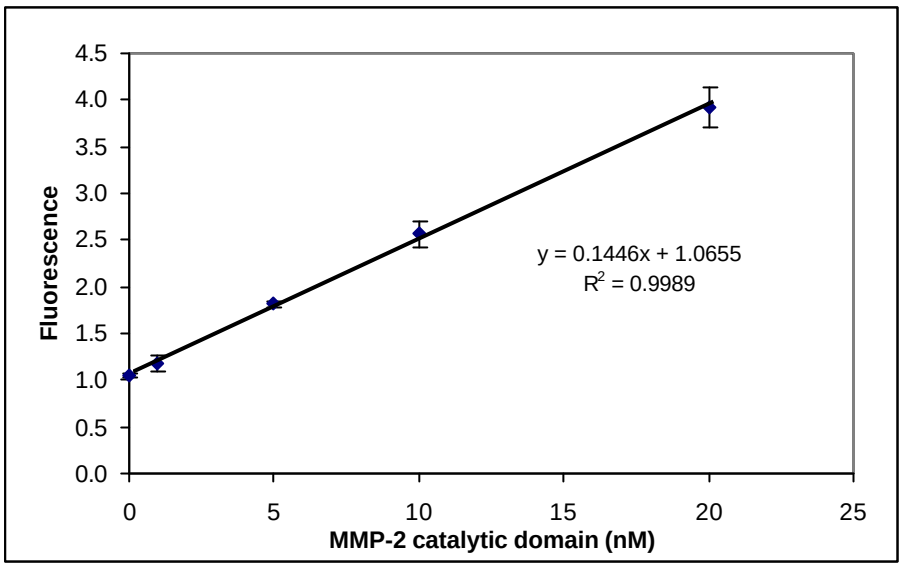

Figure 7. Enzyme activity of MMP-2 catalytic domain in a CD-microlaboratory. The protease (75 nl, 1-20 nM) was mixed with FRET peptide substrate (75 nl, 10 µM) in CDE6Q and the catalytic activity was assayed after 16 minutes through off-line fluorescence detection with a photo multiplier tube (PMT) setting of 0.35.

5.1.2 Reaction time

Enzyme activity measurements of the catalytic domain were done after different reaction times. Profiles showing the fluorescence distribution in the reaction chambers revealed that initially there was no adequate mixing of MMP and substrate. After approximately 16 minutes of reaction time the solutions became homogeneous and the fluorescence profiles were flattened out in all structures. However, the higher the activity was in the sample, the sooner the flattening occurred. Figure 8 shows fluorescence profiles in a microstructure containing 5 nM MMP-2 catalytic domain. It clearly illustrates the increase in fluorescence with time as well as the uneven fluorescence distribution at early measurements. The fluorescence increased linearly for 30 minutes even in samples containing 20 nM of the catalytic domain, see figure 9. The CV (n = 3) values were found to be below 10 % (0.6 – 9.6 %) at all time measurements and there was a reduction in detection limit concentration with time, for the first 23 minutes. The minimum detectable levels are listed in table 1.

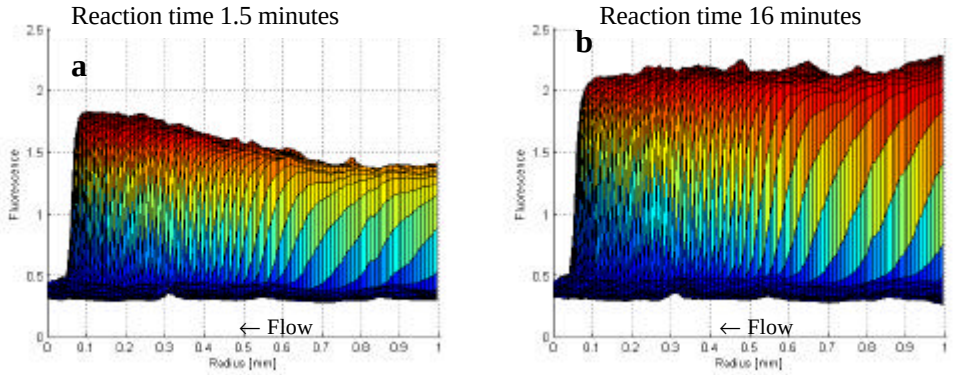

Figure 8. Enzyme activity assay with MMP-2 catalytic domain in a CD microlaboratory. Fluorescence distribution in reaction chambers containing 5 nM MMP-2 catalytic domain. Both pictures show the same microstructure; a) after 1.5 minutes of reaction and b) after 16 minutes. Initially substrate is enriched at the bottom of the basin, but after 16 minutes of incubation the solutions are homogeneous with flat fluorescence profiles.

Table 1. Detection limits of matrix metalloprotease 2 (MMP-2) in enzyme activity assays. For the catalytic domain the detection limit was reduced with reaction time up to 23 minutes.

Incubation Time (min)	Detection limit for MMP-2 catalytic domain (nM)	Detection limit for MMP-2 (nM)
1.5	4.32	1.71
5	0.73	0.66
8.5	0.50	0.42
12	0.28	0.17
16	0.16	0.17
19.5	0.13	-
23.5	0.09	-
27	0.12	-
30	0.12	-

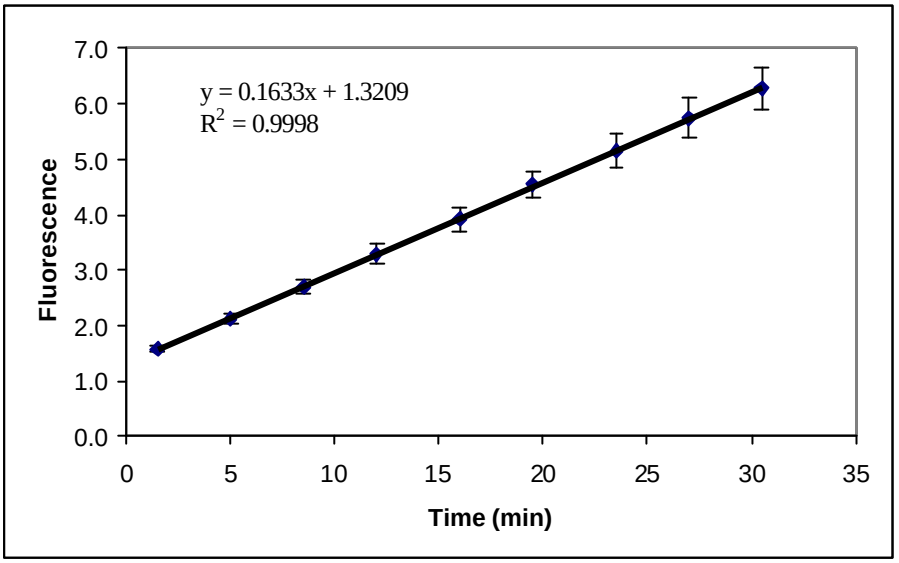

Figure 9. Progress curve of MMP-2 catalytic domain activity in a CD-microlaboratory. The reaction proceeds for at least 30 minutes in the CD (CDE6Q). The figure shows the increase in fluorescence with reaction time in the highest tested concentration of the catalytic domain (20 nM). Fluorescence was measured off-line with a PMT setting of 0.35. CV(n=3) was 4-7 %. The assay was performed with 10 μM substrate.

5.1.3 Activation of proMMP-2

The enzyme activity was determined in samples of proMMP-2 incubated at 37 °C with a 600 fold molar excess of p-aminomercuric acetate (APMA). One and two hours of incubation resulted in the same fluorescence signals, indicating that APMA converts proMMP-2 present in the sample to active MMP-2 within one hour (figure 10). The negative controls of proMMP-2 showed activity, which was approximately 30 % compared that occurring in APMA-treated samples. Apparently the preparation of proMMP-2 contained active MMP-2. This was further confirmed by performing an enzyme activity assay with proMMP-2 directly diluted in reaction buffer. The activity of the catalytic domain did not change with incubation with APMA at 37 °C for two hours, see figure 11.

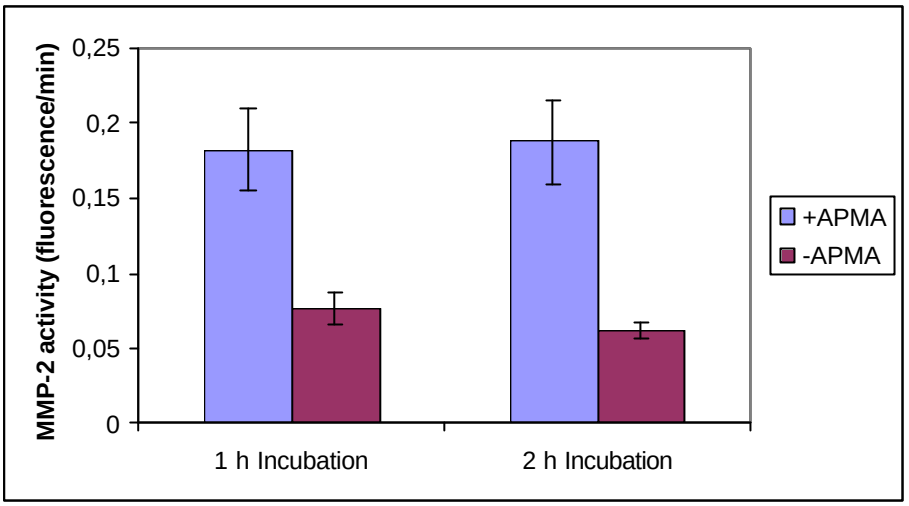

Figure 10. Activation of proMMP-2. The pro-enzyme was incubated with p-aminophenylmercuric acetate (APMA). After one and two hours of incubation, MMP-2 activity was assayed in triplicates with 10 µM substrate in CDE6 Q at a protease concentration of 10 nM.

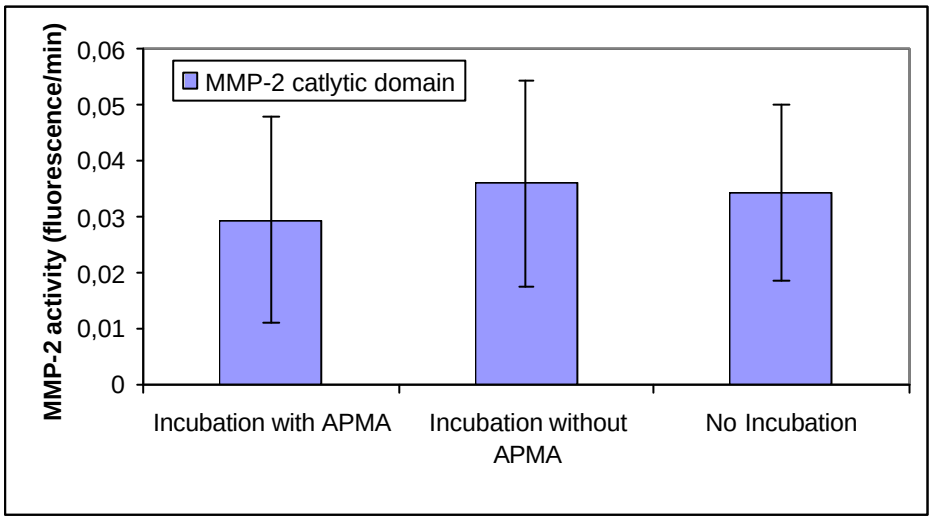

Figure 11. Enzyme activity of the MMP-2 catalytic domain after incubation at 37 °C. The enzyme activity in samples of the catalytic domain incubated for 2 hours at 37 °C with or without p-aminophenylmercuric acetate (6 µM) was within experimental error the same as in non-incubated samples of the catalytic domain. The catalytic domain (10 nM) was assayed in triplicates with 10 µM substrate in CDE6 Q.

5.1.4 Enzyme activity of MMP-2

Matrix metalloprotease 2 (activated proMMP-2) showed six times higher activity compared to the catalytic domain of MMP-2 (figure 12). The specific enzyme activity was 119 pmoles/min/µg for active MMP-2. In spite of the higher activity of MMP-2 compared to the catalytic domain, the detection limit concentration was basically the same (0.17 nM) for both forms (table 1). Due to the higher activity of MMP-2, this preparation of the enzyme could be assayed already after 8 minutes. The variation in triplicate samples ranged from CV 2-14 %.

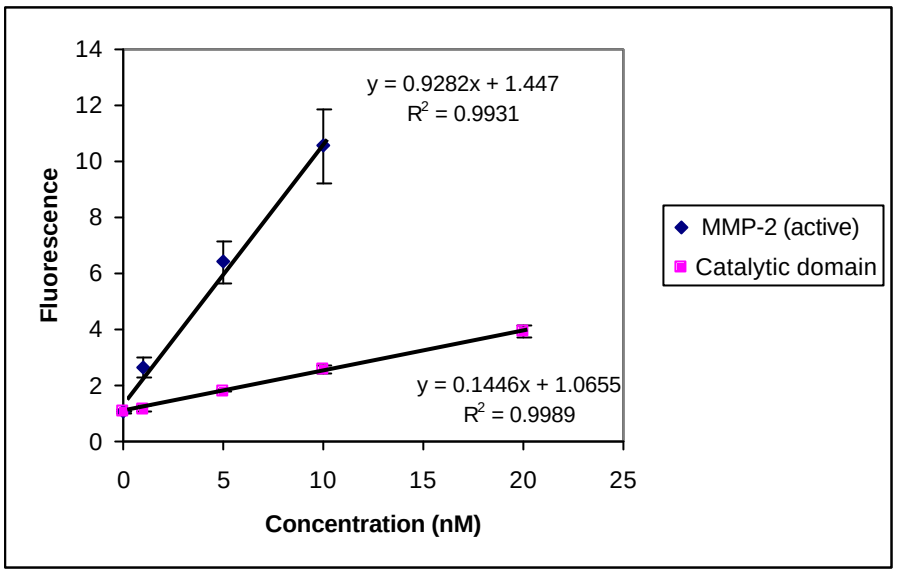

Figure 12. Enzyme activity of MMP-2 and the catalytic domain of MMP-2 in CD-microlaboratories. The various forms of the protease were assayed in triplicates with 10 µM FRET substrate in CDE6Q, at different occasions. The activity of MMP-2 was approximately 6 times higher than that of the catalytic domain. The assay time was 16 minutes.

5.1.5 Inhibition of MMP-2 by galardin

The activity of matrix metalloprotease 2 can be inhibited with galardin, a broad range MMP inhibitor (figure 13). At low inhibitor concentrations there were only an infinitesimal reduction in protease activity, but at higher galardin levels the effect was considerably higher and the activity of MMP-2 approached zero. The $IC_{50,}$ *i.e.* the concentration where the enzyme activity is reduced by 50 %, was determined to 2.7 nM at 5 nM MMP-2 and 5 µM MMP-2 CyDye™ peptide substrate.

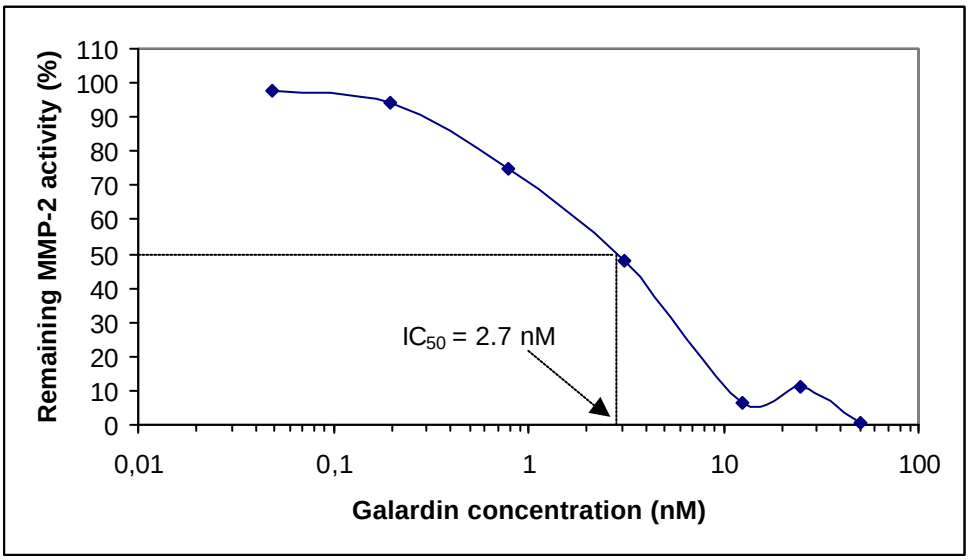

Figure 13. Inhibition of MMP-2 by galardin. Galardin was mixed with substrate (10 µM) and activity was determined through a homogeneous assay in CDE6 Q with MMP-2 (10 nM) added through the common channel.

5.1.6 MMP-2 activity in presence of protein or detergent

To investigate if the enzyme activity assay could be performed in a protein environment, tests were made with BSA replacing Brij-35 detergent in the reaction buffer. After 19 minutes of reaction time, MMP-2 activities in triplicate samples diluted in buffer containing 1% (w/v) BSA were only slightly lower compared to the activities seen in samples diluted in buffer with 0.025% (w/v) Brij-35 (figure 14). The differences in fluorescence signals were however within experimental error for each dilution media. The hydrophobic brakes of the microlaboratory were able to withstand both buffers, meaning that the volume definition of the chambers in the CD structures worked.

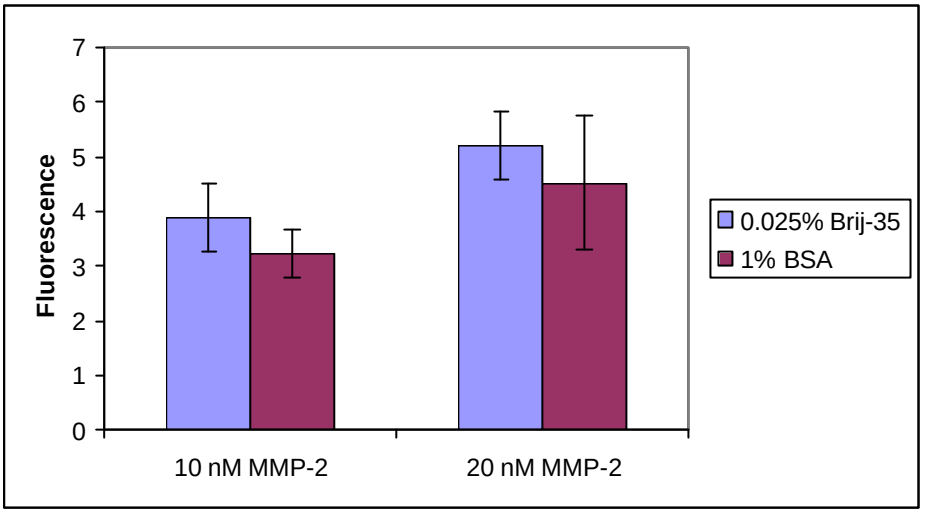

Figure 14. MMP-2 activity in the presence of protein. The fluorescence intensities obtained when performing an enzyme activity assay in buffer containing 1% (w/v) BSA were slightly lower, but within experimental error the same as in runs with Brij-35 buffer. All samples were run in triplicates in Q structures on the same CDE6. MMP-2 activity was analysed with 10 µM substrate.

In other protocols for MMP-2 enzyme activity assays the reaction buffer contains 0.05% (w/v) Brij-35 detergent. When tested in the CD-microlaboratory, a detergent concentration of 0.05% resulted in unacceptable wicking. Also, in some cases the hydrophobic brakes did not work satisfactory and reaction chambers were overfilled. By lowering the Brij-35 concentration to 0.025% the problems were overcome and the microfluidic processes functioned as desired.

5.2 Heterogeneous sandwich immunoassay

5.2.1 Identification of capturing and detecting antibody pairs

The standard curves generated when testing different capturing and detecting antibodies are shown in figure 15. The minimum concentration of proMMP-2 that could be detected, calculated with three standard deviations added to the mean of zero standard, was 0.40 nM with MAB 903/ AF 902, while it was higher in both cases when having the monoclonal antibody MAB 902 as capturing agent (2.97 nM with MAB 902/ AF 902 and 7.51 nM with MAB 902/MAB 903). The capturing monoclonal antibody MAB 903 in combination with MAB 902 did not work at all. As seen in figure 15, the response did not increase with

increasing proMMP-2 concentrations for MAB 902/MAB 903. Since the combination of MAB 903/ AF 902 had the lowest detection limit and the broadest dynamic range, the result indicates that it was best suited for further experiments. Also, the profiles showing the distribution of fluorescence in the affinity columns revealed that the fluorescence signals were higher and more enriched in an area at the top of the column when proMMP-2 was captured with the monoclonal antibody MAB 903 and detected with the polyclonal antibody AF 902, compared to having other antibody pairs (figure 16). The antibody concentrations used during the evaluation (533 nM MAB 903 and 100 nM AF902) resulted in CV values between 2 and 78 % and at low proMMP-2 concentrations (0.78 nM) the fluorescence distribution showed no smooth gradients.

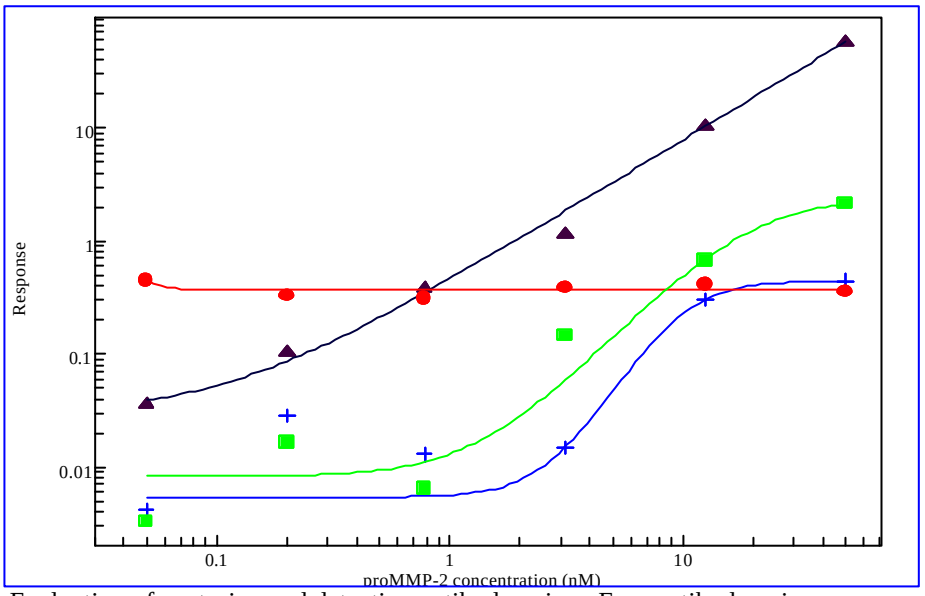

Figure 15. Evaluation of capturing and detecting antibody pairs. Four antibody pairs were assayed in parallel in CDBA2; ▲ MAB 903(monoclonal antibody)/AF 902 (polyclonal antibody), detection limit 0.4 nM; •MAB 903 /MAB 902 (monoclonal antibody); +MAB 902/ MAB 903 and ■ MAB 902 /AF 902. The curves were generated from on-line fluorescence recordings with PMT settings of 5%.

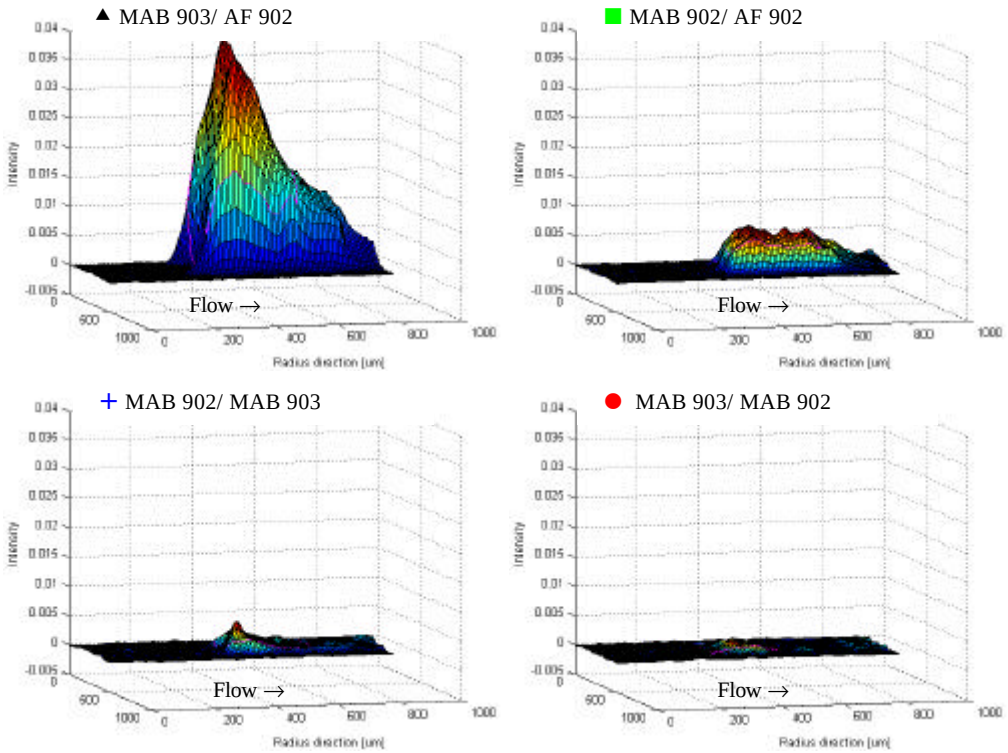

Figure 16. Evaluation of capturing and detecting antibody pairs. The fluorescence distribution in columns of CDBA2 loaded with 12.5 nM proMMP-2 and different antibody pairs. ▲MAB 903 capturing / AF 902 detecting. ■ MAB 902 capturing /AF 902 detecting, + MAB 902 capturing / MAB 903 detecting, ● MAB 903 capturing / MAB 902 detecting.

5.1.2 Quantification of MMP-2

Running sandwich immunoassays with the antibody concentrations used during the evaluation of antibody pairs resulted in different responses with proMMP-2 and active MMP-2 (activated proMMP-2), as seen in figure 17. The curves in figure 17 were generated from LIF detection with PMT settings of 1% and with no background subtracted values. Increasing the concentration of the capturing antibody MAB 903 from 533 nM (0.08 mg/ml) to 1000 nM (0.15mg/ml) and the fluorophore labelled detection agent AF 902 from 100 to 145 nM yielded identical responses for both pro- and active form. The standard curves in figure 18, shows the background subtracted fluorescence signals as a function of MMP-2 concentration. The curves are similar for proMMP-2 and active MMP-2, meaning that total amount of MMP-2 (pro-form + active form) can be quantified with this assay. When assaying the 40 kDa catalytic domain of MMP-2 the responses were 2.5 to 4 times higher than for the larger proteases. However, detection limits were found to be 0.3 nM for all three forms. Each sample was run in triplicate and large variations were seen in blanks, CV 53-95%. In samples containing enzyme the CV percentage appeared to be within 2-13 % for the catalytic domain, 4-21% for active MMP-2 and 1-14 % for proMMP-2. Profiles showing the fluorescence distribution in the affinity columns were similar in shape for proMMP-2 and the active enzyme, while fluorescence was more enriched at the top of the column when analysing the catalytic domain. For all forms clearly visible fluorescence profiles were obtained at all tested concentrations (0.51-50 nM). Increasing the concentration of detecting antibody from 100 to 145 nM resulted in fluorescence profiles with smoother gradients at MMP-2 concentrations below 1 nM, as revealed in figure 19.

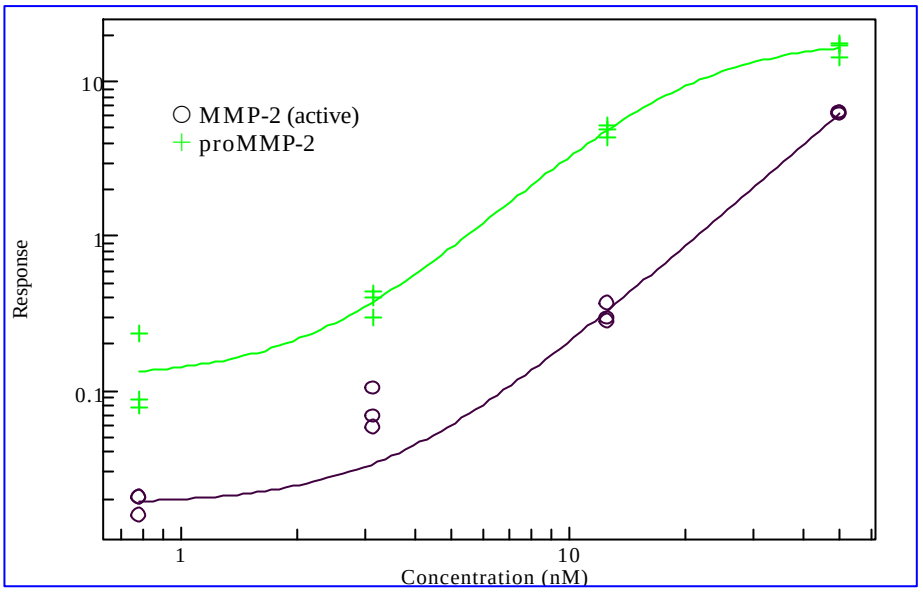

Figure 17. Sandwich immunoassay of MMP-2 in a CD-microlaboratory. ProMMP-2 (+) and active MMP-2 (○) quantified in CDBA2 with 533 nM (0.08 mg/ml) capturing antibody and 100 nM fluorophore conjugated antibody (degree of labelling: 3.09 mol Alexa™647 /mol AF 902). PMT settings were 1% and background fluorsescence was not subtracted from the responses.

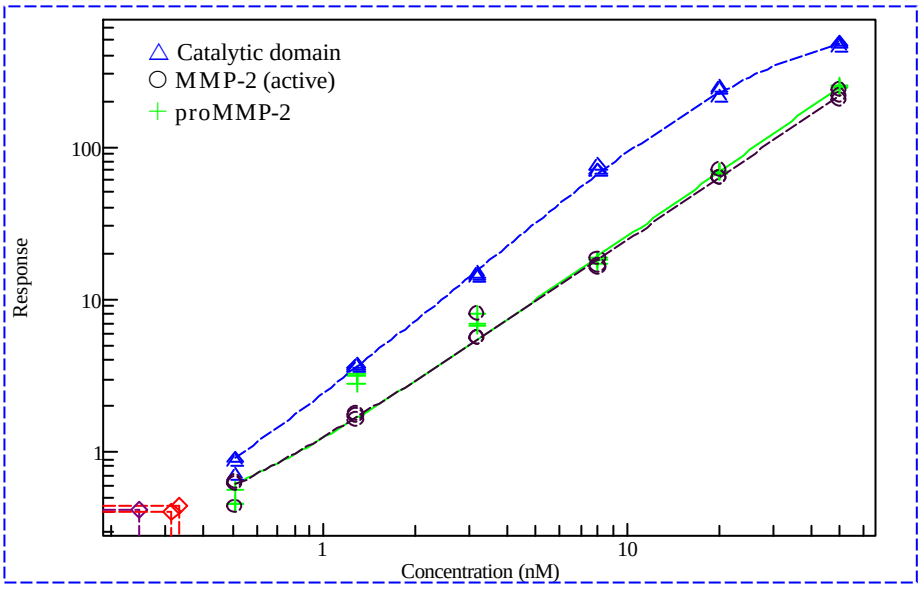

Figure 18. Sandwich immunoassay of MMP-2 in a CD-microlaboratory. ProMMP-2 (○), active MMP-2 (+) and the catalytic domain of MMP-2 (△) quantified in CDBA2 with 1000 nM (0.15 mg/ml) of capturing antibody MAB 903 and 145 nM of detecting polyclonal antibody AF 902 (degree of labelling: 3.09 mol Alexa™647 /mol AF 902). The detection limit was 0.3 nM for all MMP-2 forms. The curves were generated from detections with PMT settings of 5%.

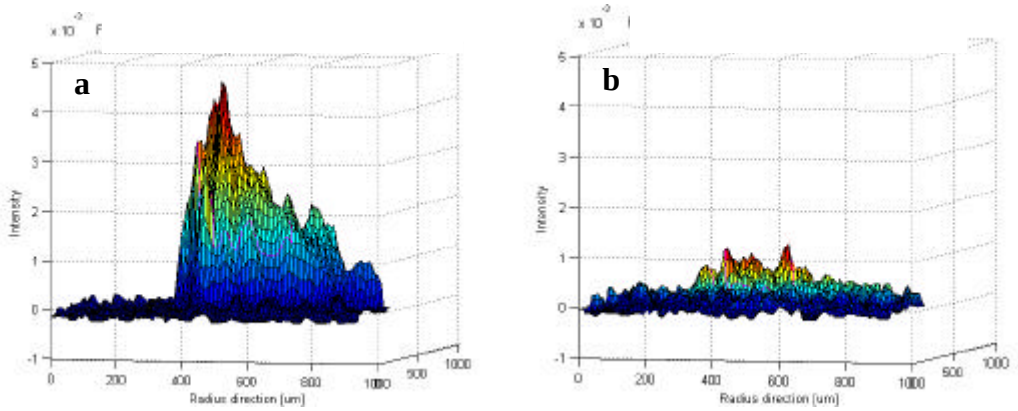

Figure 19. Sandwich immunoassay of proMMP-2 in a CD-microlaboratory. Fluorescence distribution in columns having 1000 nM (0.15 mg/ml) of capturing antibodies attached to the surface. a) 0.51 nM proMMP-2 and 145 nM of detection reagent and b) 0.78 nM proMMP-2 and 100 nM of detecting antibody.

5.1.3 Total assay time

In total it took 48 minutes to generate 72 data points with the CD-based sandwich immunoassay. Included in that time is every step in the assay, starting with attachment of capturing antibody and ending with the last LIF detection. Each step involving attachment of capturing antibody, application of protease and binding of detecting agent to the column, lasted for four minutes.

5.3 Integrated enzyme activity and quantification assay

It was possible to maintain enzyme and substrate mixtures in the reaction chambers having an outlet located at the bottom, long enough to measure enzyme activity. Figure 20 shows standard curves generated when analysing active MMP-2 with homogeneous activity assays, in the structure used only for activity assays (CDE6 Q) and in the reaction chamber of the structure for the serial integration of activity and quantification assays (CDE6 D). Though not being completely identical, they reveal the possibility of performing the assay within the D structure of CDE6. The minimum detectable concentration was found to be 0.10 nM. In approximately three out of ten structures, some wicking was observed during incubation in the reaction chamber.

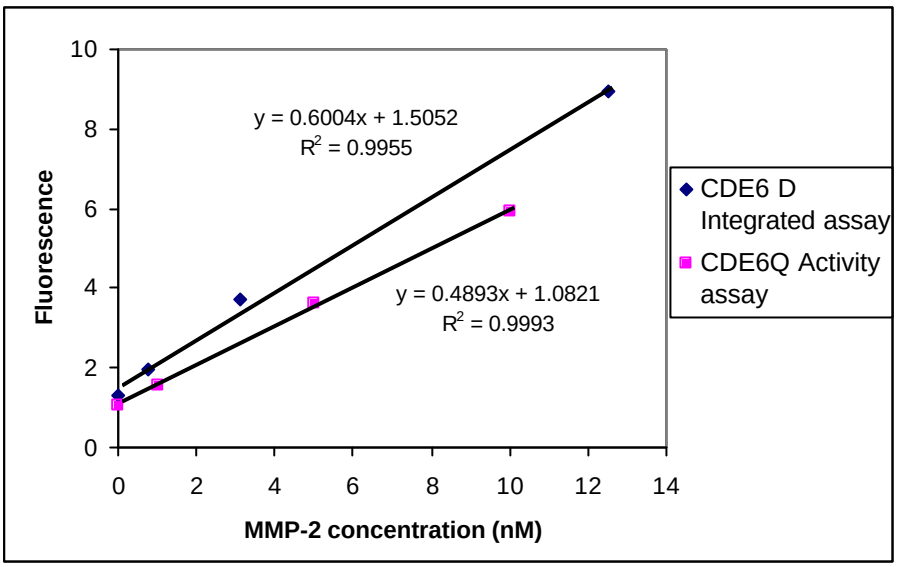

Figure 20. Enzyme activity of MMP-2 in different CD microstructures. In the enzymatic assay in CDE6 Q (■) active MMP-2 was run in triplicates in buffer containing 0.025% Brij-35. In the integrated assay in CDE6 D (◆) the enzyme was run in duplicates in buffer with 1% BSA and resulted in detection limits of 0.10 nM. The activity appeared to differ with a factor of 1.2 when analysed in the different structures. Fluorescence was recorded off-line with PMT settings of 0.35.

With 533 nM capturing antibody, 100 nM detecting antibody and 2 washes after analyte addition, as first used in the separate sandwich immunoassay, it was not possible to generate any responses in the quantification part of the integrated assay. The acceptor molecule (Cy5Q) of the MMP-2 peptide substrate yielded fluorescence upon illumination with light of wavelength 633 nm. Therefore, the substrate gave rise to background fluorescence, which was approximately 11 times higher than the fluorescence signals recorded after application of detection antibody. Besides, in the non-background subtracted fluorescence profiles from the final detection step, higher signals were seen in the lower part of the column, indicating that fluorescence arose from some unbound constituent on the way of leaving the column. By introducing five washes with PBS-Tween added through both the common distribution channel and the outer individual inlets, directly following application of substrate and enzyme mixture to the column, background fluorescence was reduced. Lowering the added substrate concentration to 2 µM decreased the background signals further. It was also revealed that substrate was contaminating the capillary used for substrate addition, leading to carry-over of substrate into the inlets during the subsequent washes. The substrate was carried over during approximately six applications following substrate addition. To overcome this, the capillary used for transferring substrate to the CD was disabled after substrate addition. Thus, wash solutions (PBS –Tween) and fluorescent-labelled antibody was applied with a clean capillary. In combination these actions resulted in a significant lowering of background fluorescence, which was approximately 3% of what was first observed. Still the signals from the substrate were to high to generate good data at low MMP concentrations.

The inlets of the structure used for the serial integration of enzyme activity and quantification assays were defined to 75 and 100 nl, which is 2 –2.7 times smaller than the volume definition in the quantification structure (in CDBA2). When having 2000 nM (0.3 mg/ml) capturing antibody and 300 nM detection antibody, which in molar amount corresponds to 1000 nM capturing and 150 nM detecting agent loaded in inlets defined to 200 nl, signals were generated down to 8.33 nM proMMP-2 in the presence of 2 µM substrate. However, trials of repeating the assay at desired MMP-2 concentrations 0 - 33.33 nM (0, 4.17, 8.33, 16.67 and

33.33 nM) in duplicate samples resulted in responses only in one of the two samples containing 8.33 and 16.67 nM. Figure 21 and 22 illustrate comparisons of responses from quantification assays, in both the presence and absence of 1 µM substrate. In the absence of substrate signals were obtained down to 2.08 nM.

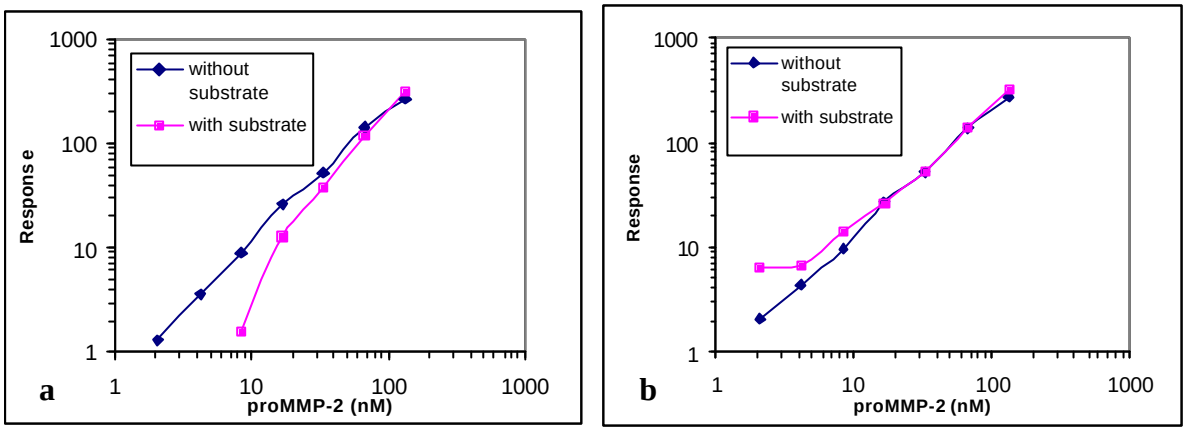

Figure 21. Sandwich immunoassay of proMMP-2 downstream of a reaction chamber in a CD-microlaboratory. ProMMP-2 quantified with 2000 nM capturing antibody MAB 903 and 300 nM detecting antibody AF902 (3.09 mol Alexa™ 647/mol AF 902) both in presence and absence of 1 µM FRET substrate. One sample was analysed at each concentration. a) background has been subtracted from the values, b) no background subtraction has been made.

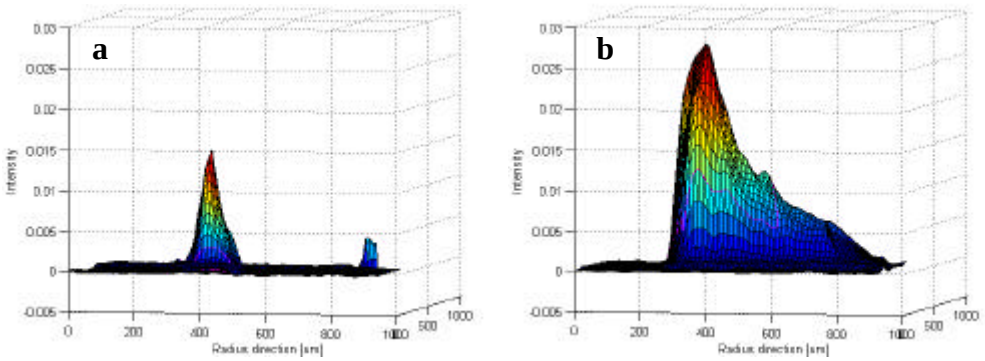

Figure 22. Sandwich immunoassay of proMMP-2 downstream of a reaction chamber in a CD-microlaboratory. Fluorescence distribution in columns loaded with 8.33 nM (75nl) proMMP-2, 2000 nM (0.3 mg/ml) of capturing antibody and 300 nM detecting antibody. a) 2 µM (75 nl) of substrate had been loaded through the common distribution channel, b) PBS-Tween was applied instead of substrate.

5.3.1 Total assay time

The time required for performing the integrated assay, in 10 microstructures in parallel was in total about 85 minutes. Included in that time was 20 minutes for making the column specific for MMP-2 and for applying enzyme and substrate (part one of the workstation method). During the following 15 minutes, incubation and fluorescence detection with a green LIF detector (excitation 532 nm) occurred. Finally, the last method starting with application of MMP and substrate mixture on to the affinity column and ending with the last red LIF detection lasted 46 minutes. The time consuming part was the 5 wash steps, with PBS-Tween added through both the common distribution channel and the outer individual inlets, after analytes had been passed through the column. That part lasted for 15 minutes.

6 DISCUSSION

6.1 Homogeneous enzyme activity assay

It was demonstrated that it is possible to perform an enzyme activity assay of MMP-2 catalytic domain and active MMP-2 in a CD microlaboratory. The assay time was only 16 minutes. Despite having different specific activities, the detection limit concentration was 0.17 nM for both MMP-2 forms. Through increasing reaction times the detection limits were even further lowered.

Different substrate concentrations were evaluated. Substrate concentrations below 2 µM resulted in fluorescence profiles with rough surfaces at low MMP-2 concentrations. Since the algorithm evaluating the data calculates the intensity over a few radii at the centre of the reaction chamber, rough surfaces will generate results with large variations. Also, such results are not representative for the intensity in the entire reaction chamber. With higher substrate concentrations the fluorescence signals were homogeneous in the entire reaction chamber in a time course of 15 minutes. Hence, the results generated were more reliable and varied less.

The incubation time was another critical parameter in the assay. During the first 5 minutes of reaction time it was found that there was inadequate mixing of substrate and enzyme. However, progressively the fluorescence profiles were flattened. In samples of higher activity the flattening occurred earlier. In order to enable earlier measurements in low-activity samples there is a need for better mixing in the structures located between the inlets and the basin. In the upper end, the small volume of the microstructure limited the incubation time with evaporation being a risk after long incubation times. However, within 30 minutes of reaction no obvious evaporation was seen in this study. During 30 minutes, it was also shown that the catalytic domain of MMP-2 remained active in the CD-microstructure.

The homogeneous activity assay developed in this project, was performed within 16 minutes of reaction time. That is to be compared with several hours, which usually is the duration of assays run in microtiter plates (MTPs). With the shorter reaction times in the CD there is a requirement for faster MMP-2 catalysis. This was achieved by performing the assay at a substrate concentration of 5 µM instead of 0.4 µM, which has been reported in a MTP based assay (George et al., 2003). Still, the consumption of substrate is below 5 ng (estimate based on 200 nl required to apply sample to inlets) per data point in the CD. Thus a commercially available vial containing 10 µg peptide substrate can generate up to 2000 and 200 data points in the CD and MTP based assays, respectively.

Enzyme activity measurements were made in the presence of a serum protein, BSA. The MMP-2 activity was only slightly reduced in the buffer containing BSA, which is a promising result considering the possibility of analysing protease activity in serum and plasma samples in a diagnostic context.

Matrix metalloprotease 2 has been a target for development of anti-tumour drugs and thus there is a need to assess the potency of inhibitor candidates. This could be done with an inhibitory assay. In this project an inhibitory study was performed by mixing the broad range MMP inhibitor galardin with substrate in microtiter plates before loading into the CD. The procedure required manual pipetting steps and resulted in higher substrate consumption

compared to the amount required when generating activity standard curves. A natural continuation of work on the inhibition assay would be to develop a method and microstructure, enabling automatic high throughput screening of inhibitor candidates. Such CD-microstructures is proposed to have an additional inlet for application of the inhibitor, which thereby would mix with enzyme and substrate in the mixing structures in the CD.

By comparing enzyme activity standard curves for MMP-2 and for the catalytic domain generated at different occasions, the specific activity of active MMP-2 appeared to be six times higher than the activity of the catalytic domain. However, the proteases were assayed in different CDs. In order to yield results that are totally comparable, multiple samples need to be processed in parallel in the same CD microlaboratory. This was accomplished when confirming the activation of proMMP-2. The activity in one concentration of APMA-treated proMMP-2 resulted in higher activities than the same concentration of proMMP-2. However, the explorative CD (CDE6) used in this project only contained 16 microstructures for homogeneous activity assays, which only allowed a few concentrations to be processed in parallel. In a diagnostic situation it would be useful to analyse unknown samples in parallel with a standard curve. During the progress of this study a new CD, which contains 96 microstructures for homogeneous assays, was developed at Gyros. That CD may allow for high throughput activity assays. Assays with 24 different samples analysed in triplicates together with a standard at six different concentrations would take less than 30 minutes.

6.2 Heterogeneous sandwich immunoassay

The sandwich immunoassay performed in a CD microlaboratory, Gyrolab™ Bioaffy is a sensitive method for protein quantification. Detection limits at low pg/ml levels have been demonstrated in the analyses of human cytokines (Gyros, 2003). In the quantitative immunoassay performed in this project the minimum detectable level of matrix metalloprotease 2 was found to be 0.30 nM, which for proMMP-2 corresponds to 23 ng /ml. With a dynamic range between 0.3 and 50 nM (23 – 3600 ng/ml) this assays should enable measurements of MMP-2 in plasma and serum and could thus be used for diagnostic purposes. Since active MMP-2 and proMMP-2 were shown to generate identical responses at given reagent concentrations this assay would quantify total amount of both MMP-2 forms. In biological samples MMP-2 may exist in complex with TIMPs. Whether such complexes could influence the response of this assay needs to be investigated. The sensitivity of the immunoassay is highly dependant on the affinity of the antibodies for the target protein (Wilson and Walker, 2000), which was clearly illustrated when identifying the best capturing and detection antibody pair. Not all combinations were applicable at MMP-2 concentrations between 0.78 and 50 nM. Also for one antibody pair (capturing monoclonal MAB 903 and detecting monoclonal MAB 902) the response was independent of proMMP-2 concentration, probably due to unspecific interactions between the detecting agent and the affinity column. Possibly, the sensitivity of the MMP-2 sandwich immunoassay could be increased with other antibodies than those tested in this study. This would further improve the assay and enable analyses of diluted samples to be made.

With 533 nM capturing antibody and 100 nM detection antibody different results were obtained for proMMP-2 and active MMP-2. Increasing the concentration of both capturing antibody (from 533 to 1000 nM) and detecting reagent (from 100 to145 nM) resulted in identical responses when assaying proMMP-2 and active MMP-2. This might be due to differences in affinity of the capturing antibody for the different forms, which becomes apparent when the capturing antibodies are more dispersed on the column surface. It could

also be due to the fact that a polyclonal antibody preparation, which might contain clones specific for neo-antigens of the pro-peptide of proMMP-2, was used as detection reagent. At low concentrations of the polyclonal antibody, the concentration of antibodies able to bind the active form might be too low to enable detection of all active MMP-2 captured to the column. Thus lower responses were yielded for active MMP-2. Unfortunately, no comparisons could be made with the catalytic domain at the lower antibody concentrations. However, with 1000 nM of capturing and 145 nM of detecting antibody, responses were higher and fluorescence signals more enriched in the top of the columns for the 40 kDa catalytic domain than for the other forms. One reason for that can be that the smaller catalytic domain diffuses more easily through the particle bed of the column. Thereby it becomes more prone for binding to the capturing antibody. Also the catalytic domain could be folded in such a way that the epitope becomes more accessible for interaction with the capturing monoclonal antibody. When producing the polyclonal antibody used for detection, goats were immunized with recombinant human MMP-2. Some of the MMP-2 might be processed in the goats and there is a chance that antibodies specific for processed parts of the intact enzyme would be produced. That could result in higher responses for the catalytic domain, a processed part of the pro-enzyme, than for the other forms of MMP-2.

The CD based immunoassay required only 46 ng of capturing and 7 ng of detecting antibody for each data point. Thus, a stock of 200 µg capturing antibody can generate up to 4000 data points. These values are based on 305 nl sample volumes being transferred to the inlets of the CD. Conventional sandwich MMP-2 immunoassays in microtiter plate format have detection limits at low ng/ml levels (Fujimoto et al., 1993 and Zucker et al. 1992). That is to be compared with 23 ng/ml in the CD. However, due to the large sample volumes loaded in the microtiter plates (100 µl) the minimal detectable amounts are between 2.4 and 200 pg. The total assay time for MTP-based methods exceeds 2 hours. Excluded in that time is the time required for attaching the capturing antibody to the solid phase, which is typically an overnight procedure. In the CD microlaboratory the minimal amount of MMP-2 being detectable was found to be 4.6 pg, which is at the lower end of the detection limit amount in the MTP-based assays. Since only nanoliter volumes are loaded into the CD structures the assay can be run with undiluted samples. Thus the assay can directly be used to analyse serum and plasma samples. All steps in the miniaturized CD method took less than an hour. Sample, reagents and wash buffers were automatically transferred; thereby several manual pipetting steps are avoided.

6.3 Integrated enzyme activity and quantification assay

One aim of the project was to investigate the feasibility of integrating enzyme activity and quantification assays within a single microstructure. Beforehand, the integration offered some challenges. For example, mixtures of enzyme and substrate were to be maintained long enough for performing an enzyme activity assay in a reaction chamber having an outlet at the bottom. During the incubation time of the catalytic reaction, capturing antibodies were attached to the affinity column, which had to be prevented from getting dry. Also, the quantitative immunoassay was to be performed in the presence of a fluorescent substrate. The enzymatic activity assay was easily transferred to the structure used for the integrated assay. The reaction mixture was maintained in the reaction chamber for at least 15 minutes and the detection limit was found to be 0.10 nM, which is somewhat lower than in the separate enzyme activity assay. In the quantification part of the serially integrated assay the FRET peptide substrate was the biggest problem. Since the CyDye™ substrate was the only commercially available MMP-2 substrate compatible with a LIF detector able to record

fluorescence signals in the CDs, it was chosen for the assay. The Cy5Q molecule of the substrate was supposed to be a dark dye, dissipating energy through routes other than fluorescence. However, in this assay Cy5Q was obviously emitting light of the same wavelength as the detection antibody. The feasibility study of the integrated assay clearly illustrates the need of PBS-Tween wash steps and sufficient amounts of capturing and detecting antibodies in the sandwich immunoassay. After application of several wash steps, reduction of substrate concentration and increased antibody concentrations the quantification part gave rise to responses with large variations in duplicates. Besides, the lowest proMMP-2 concentration that generated a characteristic signal with fluorescence enriched at the top of the column was 8.33 nM in the presence of the CyDye™ substrate. The substrate was found to influence the low-level quantification of MMP-2. In the absence of the FRET substrate responses where generate down to 2.08 nM of proMMP-2 (corresponding to 0.78 nM in a 200 nl inlet). By using a substrate that does not emit light at the same wavelength as the detection antibody, the quantification part of the integrated assay would be improved.

The enzyme activity and quantification assays were initially optimized independently. When bringing together the assays compromises, such as lowering of substrate concentration, had to be made. The idea of serially integrating the assay was to generate two pieces of information, enzyme activity and quantity, with small sample consumption. Integration of assays was shown to reduce performance of the assays compared to when running them separately. Therefore, the specific needs of the method have to be considered. Is it most important to use small sample volumes or to have high sensitivity in the assay? During the time of this project it was not possible to serially integrate the enzyme activity and quantification assays within a single microlaboratory. Instead it is suggested that the assays are performed in separate microstructures located on the same CD. Alternatively, the assays can be run in different CDs that are subsequently processed in the Gyrolab workstation. In both cases the same samples can automatically be transferred to the CD inlets and determination of specific enzymatic activity could be achieved.

7 FUTURE PERSPECTIVES

In this study all assays were performed with commercially available recombinant human MMP-2 and standard curves of activity and quantity were generated. Intra-assay variations were evaluated in triplicates. To further assess the precision of both methods, repeated trials during the same conditions are necessary. In order to test the suitability of the methods for determination of specific enzymatic activity there would be a need first to run both assays on simulated samples containing known total amounts of MMP-2 and known active to proMMP-2 ratios. This to examine if the expected responses are given by the assays in combination. The final goal would be to analyse biological samples of serum and plasma with the activity and sandwich assays. Biological samples are complex in comprising several constituents, which can affect the enzyme reaction, the interaction of antibodies with MMP-2 as well as the microfluidics within the CD-structure. Since there is a whole family of matrix metalloproteases, which share functional and structural properties, the assays have to be evaluated regarding cross reactivity with other MMP species. In cases of cross reactivity the responses would be altered in biological samples where other species are present.

A continuation on the project would be to compare the CD based assays with other methods using the same reagents. The performance of the assays is dependent of the reagents used. Besides, there can be large variations in biological material produced in different batches and thus proteins can give different responses at different occasions.

The idea of integrating both methods within a single structure is exciting and there are some possible ways worth trying in order to make the integration possible. One way would be to have a FRET peptide substrate with a donor that is excited at 633 nm and an acceptor that absorb the light emitted at a higher wavelength. The FRET pair Cy5 – Cy7™ have those characteristics, but today it does not exist in conjugation with a MMP-2 peptide substrate as an off the shelf product. By synthesizing such a substrate and simultaneously conjugate a fluorophore excitable at 532 nm to a detection antibody it would be possible to monitor the activity assay with a red LIF detector (633 nm excitation) and the immunoassay with a green LIF detector (532 nm excitation) without interference from the substrate. Another approach to the integration would be to design a microstructure in which the applied sample is split so that one part is for the activity assay and the other is for the immunoassay.

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