Surface Plasmon Resonance
on-line with
Liquid Chromatography – Mass Spectrometry
for the
Quantification and Identification
of Proteins

Surface Plasmon Resonance on-line with Liquid Chromatography – Mass Spectrometry for the Quantification and Identification of Proteins

Surface Plasmon Resonance on-line met vloeistofchromatografie - massaspectrometrie voor de bepaling van de concentratie en identiteit van eiwitten
(met een samenvatting in het Nederlands)

Proefschrift

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1.

Introduction

1.1. Surface Plasmon Resonance - Mass Spectrometry

Surface Plasmon Resonance (SPR) biosensors, have obtained a solid position in biochemical research due to their ease of use, robustness and flexibility. Various immobilisation strategies and interaction experiments can be performed without the need of chemical labelling of the ligand. The main feature of the technique is the possibility to obtain real-time data on the interaction of a ligand and its receptor allowing kinetic data to be determined. Also many analytical SPR-applications have been developed such as the analysis of interacting proteins and rapid screening methods, e.g. for illegal drugs and toxins in foods. In contrast to other affinity-based analytical methods, SPR permits sensitive, real-time determination of the amount of e.g. proteins present on the sensor surface as there exists a linear relation between the quantity of protein bound and the sensor response. However, from an analytical point of view, as the response is merely an account of the mass present at the sensor surface, there is no absolute certainty about the nature of the ligand interacting with the receptor. This is caused by both the possible occurrence of non-specific adsorption (NSA) and the fact that the immobilised receptors used in SPR recognise an epitope and not the whole ligand. Therefore, the identity of an interacting ligand has to be established using another strategy.

Mass Spectrometry (MS) is most generally employed to provide supplementary information on the nature of isolated material. The identification of proteins in MS is based on molecular weight determination or, after proteolysis, on the sequencing of the formed peptides using tandem-MS. The combination of SPR and MS allows monitoring of affinity interactions occurring at the sensor surface as well as the quantification and identification of the isolated components. Various strategies have been used for combining both techniques (see also Chapter 2, Figure 2). In short the most important approaches are:

a) *On-chip SPR-MS*, in which both the SPR monitoring of the receptor-ligand interaction and the MS identification, typically with MALDI-

ToF MS, occur on the same (sensor) surface. The most important benefits of this approach are its ease of use and its sensitivity. Drawbacks are the fact that sensors can only be used once and the necessity of manual intervention which hampers automation.

b) *Elution SPR-MS*, in which the ligand bound by the receptor present on the SPR surface is eluted and recovered enabling analysis using MS. An important feature of this strategy is the possibility of reusing the sensor. A drawback is the necessity of manual intervention as both techniques are generally physically separated. Additionally, potential damage of the ligand might occur when kept in the buffer used for ligand desorption. These shortcomings are improved or even avoided when all steps are fully automated. The development and application of such a sophisticated system combining SPR and LC-MS/MS is described in this thesis.

Apart from identification and quantification, an automated on-line SPR-LC-MS/MS system makes manual intervention, e.g. for sample transfer, unnecessary. This will improve analysis speed and reproducibility. In order to perform on-line SPR-MS several aspects need to be addressed. These concern the sensor-surface, the (on-line) digestion of the isolated proteins and the integration and control of all devices.

An important feature is the sensitivity of both the SPR and MS instruments. For SPR instruments equipped with a standard flow-cell (surface area approximately 1 mm^2) the binding of 1 fmol of a 10 kDa protein will generate a response well above SPR noise level. Unfortunately, identification using an (on-line) LC-MS approach will be difficult with such a small amount of material. This means that the surface area of the SPR flow-cell should be increased to bind a larger quantity of protein. Another aspect that will enhance on-line SPR-MS performance is the optimisation of the sensor surface to improve ligand isolation and to prevent non-specific adsorption. The latter may not only negatively influence the ligand interaction but also impede identification.

A different feature is the implementation of an on-line digestion reactor. For the elucidation of the amino acid sequence of a protein, proteolysis using e.g. trypsin is necessary. Since most accepted approaches are either time-consuming or costly, a method for on-line digestion allowing rapid digestion of the sample needs to be developed, thereby reducing the sample handling as well as the digestion time. Additionally, the digestion device should be positioned between the low-pressure SPR-system and the high-pressure LC-MS. Last but not least, for successful operation of the on-line SPR-MS setup all devices need to be integrated and operated in an automated, computer monitored way. All of these aspects are addressed and described in the following chapters.

1.2. Outline of the thesis

Chapter 2 gives an overview of the various systems used so far in the combination of SPR and MS for the quantification and identification of ligands**.** Several alternative sensors that can be used in combination with MS are also discussed. In **Chapter 3** the issue of non-specific adsorption (NSA) is discussed which is of paramount importance for all sensor applications including SPR-MS. NSA of the analyte and matrix, recombinant human-interferon gamma (IFN-γ) and bovine plasma, respectively, proved to be minimal if the sensor surface was coated with native dextran. However, surfaces coated with carboxyl-modified (CM) dextran adsorbed significant amounts of both the analyte and plasma constituents. Dextran could easily be derivatised with antibody molecules, although the immobilisation yield was higher when the CM-dextran was used. The latter is due to its open structure caused by electrostatic effects from the carboxyl groups. The different coatings are tested, improved and applied in the sensor surfaces and the enzyme reactors.

For unambiguous identification of protein ligands proteolysis of the isolated substances is necessary. **Chapter 4** describes the development and application of a trypsin IMmobilised Enzyme Reactor (IMER) capable of efficient on-line digestion of a protein. Capillaries with a multiple dextran layer and a high

immobilisation yield of trypsin allowed the rapid and complete digestion of up to 20 pmol μL^{-1} cytochrome C. Fragmentation of the peptides during tandem-MS showed excellent sequence coverages. In **Chapter 5** the development and characterisation of a pepsin micro-digestion capillary is described. Such a reactor permits the immediate digestion of proteineous ligands desorbed from SPR sensors using an acidic buffer. The reactor shows excellent stability and activity, allowing effective on-line digestion of a range of proteins and protein mixtures. The sequence recovery yields determined with nanoLC-MS/MS analysis for all tested proteins is comparable with the results obtained with the trypsin IMER.

The combination of SPR with on-line digestion followed by LC-MS/MS is the focus of **Chapter 6**. The development and application of an on-line SPR-MS approach is applied to the analysis of IFN-γ in plasma and consists of SPR quantification of the amount of protein isolated from plasma followed by ligand desorption and collection of the desorbed protein in a valve loop. Subsequently, the protein is on-line digested using the aforementioned protease micro-reactor. The peptides are collected and desalted on a trapping column and analysed using nanoLC and ion-trap MS/MS. The setup was fully automated and capable of multiple analyses. The separate use of both trypsin and pepsin micro-reactors permits reproducible unambiguous identification of the model protein. Due to the different protease specificities complete sequence recovery of the protein can be obtained when using both reactors separately. In **Chapter 7** the automated system is applied to the analysis of the protein β_2-microglobulin (B2M), a biomarker of renal failure, in urine. Using the SPR sensor, a B2M calibration curve is constructed. Desorption and digestion of the protein that is bound to the sensor surface followed by nanoLC-MS/MS analysis shows that both concentration determination and identification of the protein is feasible in a biologically relevant range.

A summary and discussion of the results is presented in **Chapter 8**. In this closing chapter further system improvements and future developments are also discussed.

2.

Hyphenating Surface Plasmon Resonance and Mass Spectrometry for quantification and identification of ligands

E.C.A. Stigter, G.J. de Jong and W.P. van Bennekom, **in preparation**

Abstract

In signal transduction and other biological processes, molecular interaction plays an important role. Real-time data on the molecular interactions can be helpful in the understanding of the relation between the occurrence of these processes and the actual binding. Surface Plasmon Resonance (SPR) sensors, which provide quantitative, real-time binding data, have evolved as useful tools in a broad range of applications. However, as the monitoring of an interaction between a ligand and a receptor molecule provides no unambiguous information on the identity of the bound ligand due to the selectivity of the receptor molecule involved, a second technique is necessary for identification. Mass Spectrometry (MS) is a method suited for this purpose and various MS techniques have been used in conjunction with affinity surfaces in recent years. In this chapter the current approaches for combining SPR interaction monitoring and ligand identification using MS, i.e. on-chip SPR-MS and sample elution prior to MS analyses, are treated. The possible setups and technological developments of these approaches are discussed. The use of SPR arrays and the application of alternative acoustic, optical and electrochemical sensor technologies in combination with MS analysis is reviewed. The future developments in the field of sensor–MS combinations are examined.

2.1. Introduction

Surface Plasmon Resonance (SPR) sensors are optical devices capable of detecting minor changes in refractive index in close proximity of a metal surface (typically gold). Since changes in refractive index and mass are related, mass changes that occur due to binding of molecules at the sensor surface are measured (Figure 1). At a specific wavelength and angle of incidence, energy originating from the incoming light is transferred to free electrons in the thin metal film of the sensor and the intensity of the reflected light decreases. This resonance angle changes with the optical density of the material (e.g. a gas or a liquid) at the sensor-surface. It has been shown for proteins that the refractive index and thus the resonance angle increases linearly with the amount present on the sensor surface [1, 2]. The difference in amount thus measured is due to binding and dissociation of interacting molecules, generally between the immobilised receptor molecule and the ligand present in the sample. A detailed description of SPR and its applications can be found in literature [3-5].

Since their commercial introduction in the early 1990s [6, 7], SPR biosensors have obtained a strong position in biochemical research due to their ease of use, robustness and flexibility. One of the features of the technique is the possibility to obtain real-time data on the interaction of a ligand and its receptor allowing kinetic data to be determined. Comparison of these data showed that the kinetic constants correspond well with those obtained from more conventional methods such as ELISA and affinity chromatography [8-10]. Moreover, compared to these technologies the amount of material necessary to perform the experiments is less, labelling is not required and variation in surface chemistries is possible, allowing various immobilisation strategies and interaction experiments. Apart from the physicochemical information [11, 12], SPR also provides an accurate determination of the amount of ligand present on the sensor surface as there exists a linear relation between the quantity of a bound protein and the sensor response [13]. SPR as analytical method allows picogram amounts of protein to be quantified [14-16]. However, from an analytical point of

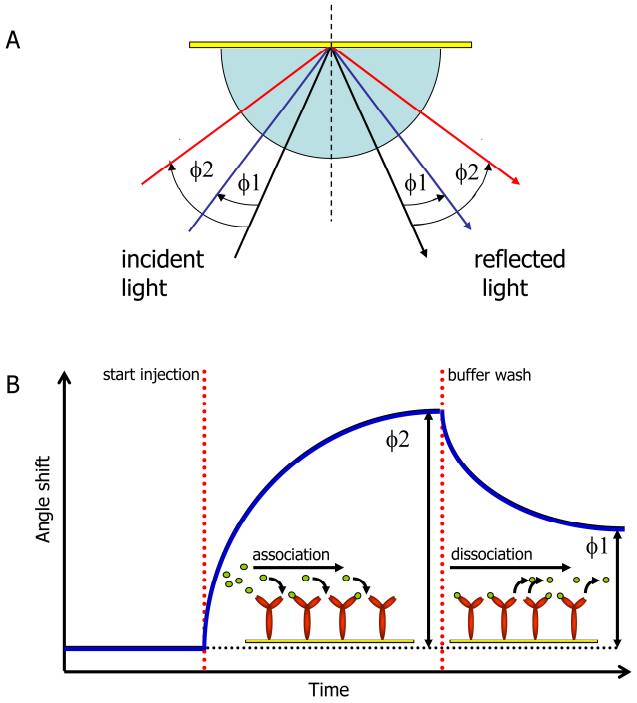

Figure 1: The principle of SPR. (A) SPR can detect association and dissociation events by monitoring the reflection of a beam of light off the interface between an aqueous solution containing a ligand and a biosensor surface coated with immobilised receptor molecules. (B) A solution containing a ligand flows past the immobilised receptor. Binding events produce a detectable change in the resonance angle which is monitored in real time and visualised in a sensorgram.

view, as the response is merely an account of the mass present at the sensor surface, there is no absolute certainty about the nature of the ligand interacting with the receptor. There might be a selectivity / validation problem.

In standard SPR experiments the selectivity is established by the character of the sensor, i.e. the surface modification and the receptor molecule used. SPR sensors with different chemistries, ranging from hydrophobic monolayers to hydrogels, are commercially available from various suppliers. These surfaces perform well with respect to non-specific adsorption in academic solutions and samples after an extensive clean-up. However, in untreated complex samples, e.g. plasma or synovial fluid, high levels of non-specific adsorption may occur. For accurate analysis the adsorption problem as well as possible cross-reactivity requires more sophisticated approaches. The use of a

surface chemistry with apparent minimal adsorption and reference channels to correct for unwanted interactions is a strategy that facilitates the study of most interactions. However, non-specific adsorption as well as receptor cross reactivity can never be ruled out. This means that the nature and composition of the interacting ligands remain partly unknown. The same applies for the determination of the identity of an unknown ligand, e.g. in the case of orphan receptor screening. In these cases an additional method is required in order to unambiguously identify the ligand interacting with the sensor.

For the identification of a protein various techniques are suited. Different approaches, such as SDS gel electrophoresis, isoelectric focussing, liquid chromatography (LC) and mass spectrometry (MS), can be used in conjunction with SPR biosensors, thus providing complementary information on the nature of the isolated material. MS is most generally employed for the identification of proteins on the basis of their molecular weight, as well as on the peptides obtained by proteolysis and the sequencing thereof using tandem-MS. The sensitivity of both SPR and MS is of the same order of magnitude and the combination allows monitoring of affinity interactions occurring at the sensor surface and the quantification and identification of the isolated components. The arrangement of both techniques can be accomplished in different ways varying from off-line approaches, using the SPR sensor as a micropurification device for quantification and isolation of a protein for further analysis, to on-line systems comprising all necessary steps including proteolysis for the identification of the isolated protein(s). Several applications in the fields of e.g. proteomics and drug screening have been developed since the first papers describing this hyphenated technology appeared. As the use of SPR-MS in these specific fields has been reviewed recently [17-20], the focus of this chapter will be on the technological advances. These developments and relevant applications are presented and discussed in the following sections and will be compared with other affinity-based methods of analysis. Finally, recent technological developments that will influence the future direction of the hyphenated systems will be evaluated and discussed.

2.2. Combinations of SPR and MS

Besides SPR many bioanalytical strategies employ affinity-based methods for cleanup and isolation of ligands of interest. Affinity chromatography and pull-down procedures are often employed for sample enrichment, drug screening or interaction proteomics in which e.g. the targets of small (signalling) molecules or enzyme inhibitors are identified. Other approaches are e.g. the Mass Spectrometry Immuno Affinity (MSIA) system and Surface Enhanced Laser Desorption Ionisation Time of Flight (SELDI-ToF) MS. The MSIA system is a 96-well robotic work station equipped with an array of affinity-modified pipette tips [21, 22]. The tips displayed minimal non-specific adsorption and the additional use of surface-functionalised target plates resulted in evenly distributed sample and hence improved Matrix-Assisted Laser Desorption and Ionisation Time-of-Flight (MALDI-ToF) MS results due to the absence of sweet spots. The analysis rate was approximately 100 samples/h which allows high-throughput analysis. Protein structural characterisation is obtained when using trypsin-modified target plates as was shown by Nedelkov *et al.* [23]. SELDI-ToF MS is an adaptation of MALDI-ToF MS using surface-modified target plates. It has been used for monitoring changes in the composition of samples collected from healthy and diseased subjects, in the search for biomarkers [24]. As these biomarkers are generally low-abundant species, extreme care should be taken in e.g. sampling and sample treatment, and thus the interpretation of the result [25, 26]. Several approaches have been reported and the use of immobilised antibodies show the potential of the technique for e.g. cancer research [27, 28]. Nevertheless, the use of SPR as an alternative affinity probe shows several obvious advantages over the methods mentioned above. Two important benefits of this technique are the facts that the interaction between binding partners can be measured in real time and that the quantitative information on the amount of material bound to the surface can be obtained.

When SPR is combined with MS a setup is created that presents both quantitative and qualitative information which can be applied in e.g. ligand

fishing and orphan receptor screening. The MS method that is most often used when SPR and MS are hyphenated is MALDI-ToF. The advantage of MALDI-ToF MS is that it is relatively tolerant towards minor contaminants present in a sample such as salt residues originating from the non-volatile buffers used in SPR [29]. Additionally MALDI-ToF is a sensitive technique with high mass accuracy. Alternatively, Electrospray Ionisation-MS (ESI-MS) has been increasingly employed, especially when nano-Liquid Chromatography (nanoLC) is used for the separation of proteolytic fragments of SPR isolated protein-ligands [30-34]. The MS-instruments that have been used in the latter case are either the IonTrap or the Quadrupole-Time-of-Flight (Q-ToF).

As mentioned above, SPR has been used in the past for kinetic studies, ligand screening and protein and hapten quantification. The strategies that can be used to combine SPR and MS for ligand quantification and identification are summarised in Figure 2. SPR-MS can be divided in two groups: one approach performing MS directly on the surface on which the protein-protein interaction takes place, i.e. the sensor surface, and approaches in which the interactants are eluted from the surface prior to further treatment and characterisation by MS. Originally, MALDI-ToF MS was performed on-chip which allows the accurate mass determination of the protein on the sensor surface. As high molecular weight proteins (> 100 kDa) are difficult to analyse with MALDI-ToF MS and, more importantly, structural information of the protein is not obtained using this method, approaches for on-chip proteolysis were developed. The peptide fragments could be analysed with either MALDI-ToF MS or, after elution of the fragments onto a trapping column, with LC-ESI-MS. Unfortunately, the on-chip analysis methods described above are destructive and allow only a single SPR-MS analysis. Therefore, elution and collection procedures were developed that enable the recovery of the protein ligand isolated with the SPR sensor. This allows multiple assays to be performed on a single sensor surface. For the unambiguous identification of the protein ligands an MS analysis of the enzymatically cleaved samples can be performed. Unfortunately, during sample handling potential material loss can not be fully excluded. Therefore, on-line

methods performing SPR analysis, digestion and LC-ESI-MS were recently reported, needing no manual intervention. In the next two sections illustrative examples of the designs and applications of the two different approaches will be described and their (dis-)advantages are discussed in more detail.

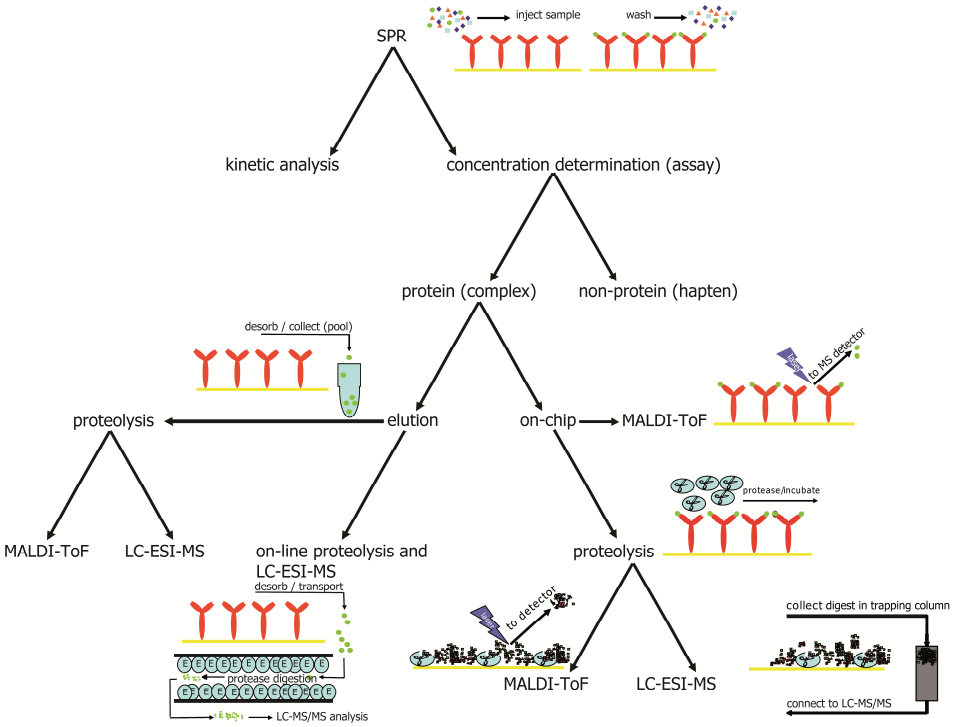

Figure 2: Schematic overview of the different approaches for coupling SPR and MS.

2.3. On-chip SPR-MS

The first combination of SPR analysis and MS was reported by Krone *et al.* [35]. The MS identification was performed "on chip" using MALDI-ToF MS to determine the molecular weight of the protein ligand bound to the receptor. Several pharmaceutical protein solutions as well as urine and plasma samples have been assayed and are summarised in Table 1. Examples are the screening for Staphylococcus enterotoxin B present in milk or mushroom samples, which could be detected at a level of 1 ng g^{-1} sample [41, 42], the simultaneous detection of biomarkers such as β_2-microglobulin and cystatin C in pooled blood serum

Table 1: On-chip SPR-MALDI MS analysis

receptor	analyte	matrix	LOD	remarks	ref
antibody	B2M, myotoxin, myoglobin	buffer	500 amol	isolated amount depending on antibody immobilisation level and sample contact-time	36-38
antibody	myoglobin	buffer	750 fmol	BIACORE probe (large surface area)	39
antibody	rat serum albumin	plasma	6.1 fmol	on-chip digestion and MS analysis of SPR-isolated proteins	40
antibody	SEB	milk / mushroom	500 amol	multiple different toxins observed upon injection of mixture	41, 42
antibody Ni^{2+}/NTA	epitope tagged GST	*E coli* lysate	800 amol 100 fmol	analysis of trypsin digested lysate containing a tagged protein same 4 peptides (ca 4 kDa) observed using Ni^{2+}/NTA surface modification	43
antibody	cystatin C, B2M, RBP, urinary protein 1	plasma / urine	fmol protein isolated	protein (biomarker) isolation single and multiple protein detection/identification on a single surface	44-48
antibody	cystatin C, B2M, RBP, urinary protein 1	buffer	ca 1nM	protein arrays of 10x10 to 100x100 antibody spots on different surfaces (MUA-SAM or traditional carboxyl-modified dextrane), SPR-imaging	49-51

and urine of several volunteers [44] and the isolation and MS analysis of tagged proteins present in cell lysates, e.g. from genetically engineered *E. coli* [43, 48]. By injecting a 50 µL diluted urine sample containing β_2-microglobulin at flow rates varying from 1 to 5 µL, capture efficiencies could be obtained of 47% and 18% respectively. As time was considered a more important factor the experiments were conducted at a flow rate of 5 µL min^{-1} even though the capture efficiency was lower. Under these experimental conditions, an injection of 50 µL β_2-microglobulin in buffer (1 µg mL^{-1}) resulted in the isolation of 9.7 pg protein per mm^2. The protein could be identified on the basis of its accurate mass thus showing that the combination of SPR and MS enabled the quantification and identification of sensor-bound material and that the sensitivity of both techniques is of the same order of magnitude which makes it an ideal combination [44]. A similar example displaying the on-chip SPR-MS analysis of retinol binding protein in serum is shown in Figure 3.

To facilitate protein identification for the on-chip SPR-MS analyses described above, a method was developed to enable MS analysis of the protein isolated during SPR analysis on the BIACORE carboxylated dextran (CM-5) cartridges [29]. The procedure consisted of several buffer washing steps to remove the running buffer and detergents after which the chip was punched out of the slide and positioned in an adapted MALDI-target. The sensor surface was connected to the target-plate in order to create an electrical field during MS analysis after which the matrix solution was applied allowing MALDI-ToF MS analysis. Although for these on-chip MS-approaches detection limits as low as 700 amol total protein originating from biological samples, such as serum or urine, have been reported [29], the practice is both rather laborious and destructive as only a single SPR-MS experiment can be conducted on a sensor surface.

In order to allow reuse of the sensor surface, a system was developed by Grote et al. [52] suited for cuvette-based SPR instruments like the IBIS-SPR. In their method a metal transfer-pin was used with the same chemistry as the SPR sensor. This pin was positioned near the SPR sensor surface and in contact with

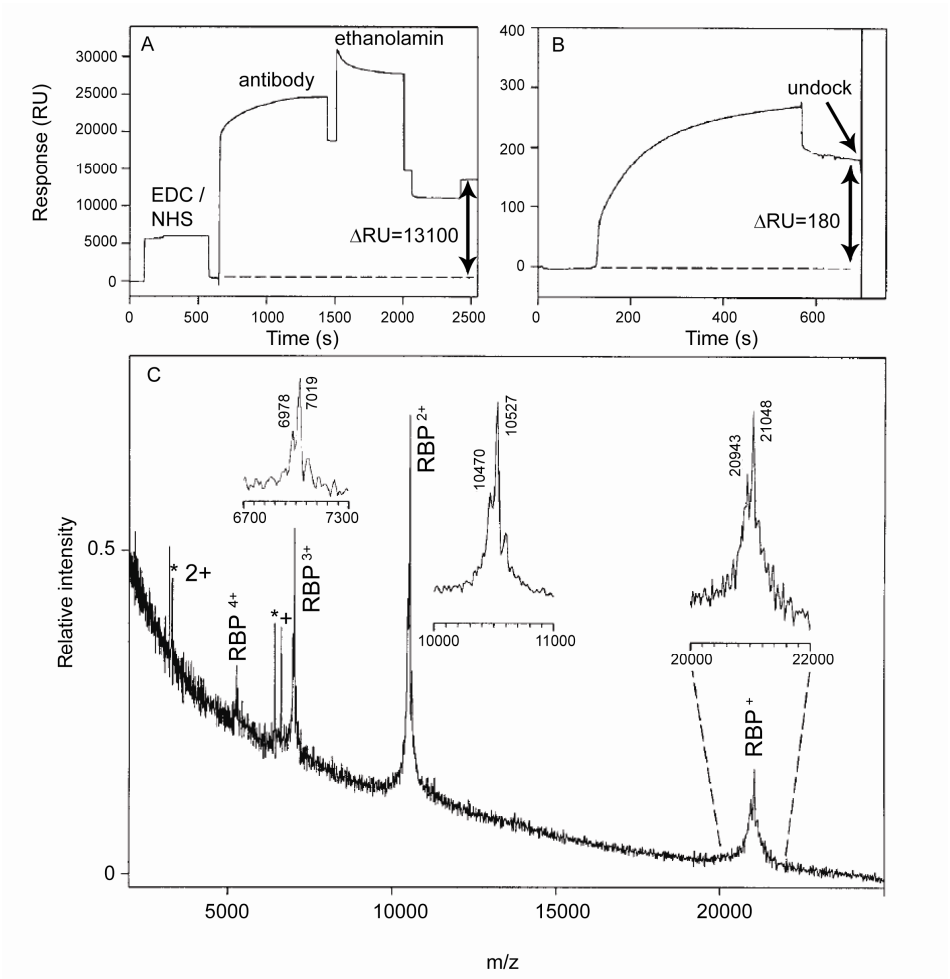

Figure 3: On-chip SPR-MS analysis. (A) immobilisation of anti-human retinol binding protein (RBP) antibody in a flow-cell of a sensor chip with a carboxymethyl-dextran coating, (B) response curve of the injection of an aliquot of a 100x diluted human plasma solution, resulting in a response of 180 RU at the time of removal of the sensor chip from the instrument and (C) MALDI-TOF mass spectrum taken from the surface of the flow-cell. The RBP signals are indicated along with the signals from two other components (labeled with *). Expanded views of the singly, doubly and triply charged RBP ions are also given to show the presence of the smaller mass RBP variant (from Nedelkov and Nelson [29]).

the sample during the experiment. After SPR analysis the pin was removed from the cuvette, washed and placed in a specially designed MALDI target-plate. Although manual intervention is necessary, the method permits several SPR-MS analyses to be conducted on a single sensor surface, thereby only replacing the transfer pin.

Although the approaches described above allow very sensitive and accurate mass determination of the isolated protein, tandem MS information on peptide fragments obtained after proteolysis is required for the unambiguous identification of an isolated protein. A strategy to accomplish this using an on-chip SPR-MS approach was reported in a recent study [40]. The isolated protein was digested on-chip and subsequent MALDI-ToF MS/MS allowed protein identification at a level of 6 fmol mm^{-2}. Unfortunately, due to performing the digestion on chip, the sample also contains fragments of the protease caused by self-digestion and partially digested receptor protein.

An alternative strategy using on-chip digestion but employing LC-MS/MS analysis was published by Natsume *et al.* [32]. In this study, His- or biotin-tagged proteins were isolated from a cell lysate. The protein interacting with the sensor surface was digested on-chip and the resulting peptide fragments were eluted onto a micro-RP trapping column. After washing the trapped peptides to remove salts and contaminants, nanoLC-ESI-MS/MS analysis was performed resulting in unambiguous identification of the isolated proteins. A similar example was recently published [53] showing the applicability of on-line SPR-MS for the analysis of protein complexes. During the on-line SPR-MS approach two different protein complexes were purified twice using the two labels present on the recombinant protein that was analysed. After on-chip digestion, the resulting protein fragments were collected onto a RP trapping column and analysed by nanoLC-ESI-MS/MS. One of the complexes employing tagged FKBP52 displayed α- and β-hsp90 fragments associated with the FKBP52 protein. In contrast to the method described by Natsume *et al.* [32], the process needed no manual intervention and was fully automated.

2.4. Sample elution prior to MS analysis

As shown in Figure 2, elution of the surface bound ligand, collecting the sample for further handling and MS analysis has been described as well. Examples of SPR-MS methods employing sample elution prior to MALDI-MS or LC-ESI-MS analysis are summarised in Table 2 and Table 3, respectively. The approach was introduced in 1998 by Sönksen *et al.* [54], who studied the interactions between two strands of dsDNA or an antibody and the proteins myoglobin or ParR using SPR. After desorption the isolated material was captured in a pipette tip and used for MS analysis. A further optimisation of the ligand recovery was described in 2002 by Gilligan *et al.* [58] who developed an isolation method based on the small volume microfluidic sample manipulation technique with oscillatory flow as previously described by Abrantes *et al.* [72]. In short, the procedure comprises a series of small volumes of buffer and sample separated by small air segments that are manipulated over the SPR surface. First the sample is allowed to interact with the immobilised receptor after which a series of washing steps is performed to remove contaminants and detergents. Finally the molecules isolated from the sample are desorbed from the surface by the regeneration buffer, which is recovered by reversing the flow direction. The method was shown to be effective by isolating lysozyme from a sample containing up to 850 fold excess of myoglobin. The protein could be identified using MALDI-ToF both by the protein mass as well as the Protein Mass Fingerprint (PMF) of the tryptic peptide fragments [58].

The sophisticated ligand-recovery strategy as described by Gilligan *et al.* [58] is integrated in newer generations of BIACORE SPR instruments, e.g. the BIACORE 3000 series, and is by now the mostly used implementation for studies in which SPR is combined with MS. A first example of this so-called microrecovery approach was presented by Lopez *et al.* [60] who isolated SHP2 tyrosine phosphatase from cytosolic extracts using SPR surfaces modified with the immunoreceptor tyrosine-based inhibitory motif sequence of the sst2 somatostatin receptor. The method allowed unambiguous identification of

Table 2: SPR-MS methods employing sample elution followed by MALDI-ToF MS

receptor	analyte	matrix	amount isolated	remarks	ref
antibody	myoglobin, ParR	buffer	4 ng myoglobin	isolation from a HSA containing solution using "sandwich"	55
antibody	A33 epithelial antigen	cell lysate	ca 14 ng per cycle	isolation of A33 epithelial antigen from crude lysates using IAsys SPR, RPLC and gel electrophoresis	56
PGIP2	endopolygalacturonase	yeast extract	40 fmol	*Fm*PG interaction, elution and further cleanup followed by study of glycosylation effects after LysC digestion and nanoLC	57
antibodies	lysozyme	buffer	50 fmol	sample manipulation using sandwich elution and oscillatory flow, identification based on protein mass and PMF	58
antibodies	B2M, cystatin C	buffer / urine	ca 10 fmol	isolation of protein from diluted urine using CMD surface for cation exchange cleanup, low efficiency	59
phosphopeptide	GST labelled SHP2 (tyrosine phosphatise)	*E coli* lysate	8.5 fmol per run (total 17 fmol)	GST-SHP2 recombinant protein isolation; non-specific adsorption observed, ca 30% sequence coverage after digestion, µZip-tip and nanoLC-MS/MS	60
(CBD) calmodulin binding domain	calmodulin	brain extract	140 fmol	95% sequence coverage after on-plate digestion	61
trypsin/CBD	protease inhibitors	plant extracts	ca 0.13 ng	enzyme inhibitor study; monitoring enzyme activity characterisation employing digestion, RP clean-up	62
C-peptide	IgG antibody	buffer	35 pmol	preparative, large surface SPR system used for antibody isolation allowing gel-electrophoresis and blotting	63
NTA/Ni2+ phopshopeptide	recombinant 14-3-3 protein phospopeptides	*E coli* lysate cauliflower homogenate	7 ng (230 fmol)	screening and isolation using recombinant 14-3-3 protein for phosphopeptide interactants. Use of isolated peptides for 14-3-3 isolation from extracts	64

Table 3: SPR-MS methods employing sample elution followed by LC-ESI-MS

receptor	analyte	matrix	amount isolated	remarks	ref
Ni2+/NTA Streptavidin and biotin-IP3	GST-labelled IP3 receptor domain	cell lysates 58 fmol IP3-binding protein	160 fmol His-xFKPB µLC-ESI-MS/MS analysis	on-chip digestion of isolated protein	32
p53	p53 binding proteins e.g. PPC1	cell lysates	up to 100 ng protein (pooled samples, 10 cycles)	screening, SDS-PAAGE, digestion and ESI/MS-MS. 4 proteins present in liver, 1 in placenta samples	65
E-cadherin β-catenin	protein interactants e.g. HSP60	cell lysate (SW480 cells)	ca 16 ng protein per cycle	screening of interactants using large sensor surface. characterisation using gel-electrophoresis and µLC-ESI-MS/MS	66
DNA-fragments	histone-like nucleoid Structuring protein	cell lysate	20 fmol protein (1.4 pmol, pooled fractions)	eluted sample trapped on RP column, ESI-MS analyzed. high background (Tween), comparison with SELDI	67
cGMP	nucleotide binding proteins	cell lysate (HEK293 cells)		selective extraction of cyclic nucleotide binding proteins, sequential elution, several proteins identified after database search	68
Cav-1 (81-100) Nav1.8 (85-103)	protein interactants e.g. caveolin-1	mouse and rat brain extracts	ca 0.5 ng protein per cycle	ligand fishing experiments using large surface unit, interaction between Cav-1 peptide and sodium channel subunit	69
anti-IFN-γ	interferon-γ	plasma (spiked)	320 fmol	on-line digestion and nanoLC-ESI-MS/MS analysis, trypsin and pepsin digestion allowing high sequence recovery	70
anti-β2-MG	B2M	urine	LOD 50 fmol	concentration range of 200 ng mL^{-1} to 10 µg mL^{-1} protein automated SPR-digestion-LC-ESI-MS/MS analysis	71

10-20 fmol isolated protein using MALDI-ToF analysis of the trypsin digests of the recovered material.

However, simpler elution modes are still in use, especially when operating non-BIACORE or older SPR instruments. The use of the cuvette-based IAsys SPR instrument for micropreparative ligand fishing experiments was described by Catimel *et al.* in 2000 [73]. The 16 mm^2 large surface area allowed microgram isolation of A33 antigen from cell lysates during a series of repetitive injection and recovery cycles. This allowed SDS-PAGE, blotting and LC analysis of the antigen to be performed. A more recent example was presented by Visser *et al.* in 2007 [68] who studied the interaction of proteins present in a human cell-lysate with immobilised cyclic-guanidine mono phosphate (cGMP) molecules. As shown in Figure 4, the selective isolation of low-abundant interactants such as cGMP-dependent protein kinase and less- and non-competitive binding proteins was enabled by sequential elution using buffered solutions containing one of the nucleotide phosphates ADP, GDP, cGMP and cAMP, respectively. The identity of the eluting proteins was further assessed by a database search of the LC-MS/MS results of the trypsin digested samples.

SPR-MS methods that are operated (semi) on-line can be distinguished into two groups: those that use on-chip protein digestion and those that elute the isolated material for further on-line sample treatment, e.g. on-line digestion. The destructive nature of the on-chip proteolysis, used in the methods described in the previous section, may contaminate the sample for MS analysis with protease autolysis or receptor fragments. Also the time associated with an off-line and an on-chip digestion process is a disadvantage which limits sample throughput. For the examples employing on-chip digestion discussed above this ranges from 3 to 11 h [48, 53]. A recent approach to limit the time needed for proteolysis uses on-line digestion of the desorbed ligands employing an enzyme-modified capillary [70]. The time needed for the fully automated analysis, i.e. from sample injection to nanoLC-MS/MS analysis, can be limited to 60 min during which a new experiment can already be started. Additionally,

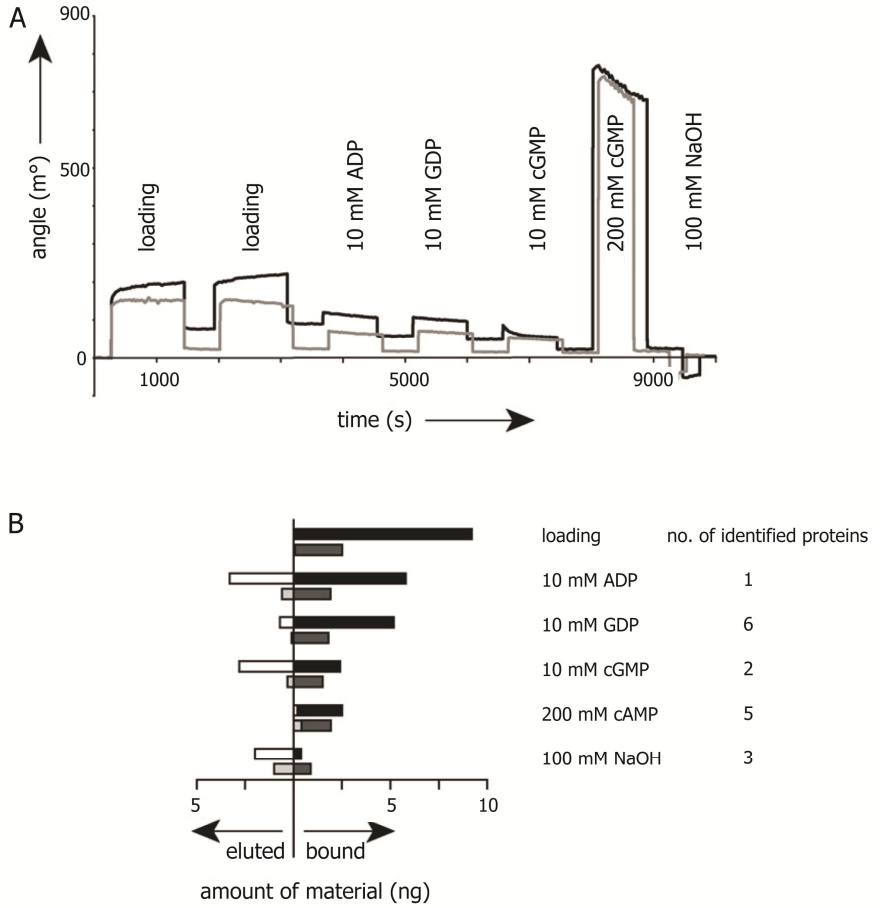

Figure 4: Sequential elution SPR-MS experiment with in (A) the loading step which represents the injection of a HEK 293 lysate. The grey line shows material bound non-specifically to a spacer-molecule derivatised sensor whereas the black line shows the signal onto a 8AETcGMP disk. The sequential elution, revealing selective dissociation, was conducted with the indicated solutions. In (B) the amount of material is depicted bound or eluted during each step for the 8AET-cGMP modified sensor (black and white) and for the non-specific binding (grey). The number of unique proteins identified in each of the eluates using MALDI-ToF MS is listed (adapted from Visser *et al.* [68]).

alternately using trypsin- and pepsin-modified capillaries almost allowed the complete sequence coverage of the model protein interferon-γ.

While different with respect to the type of material being analysed, the method reported by Marchesini *et al.* [30] is worth discussing. Although SPR sensors have been used to detect and quantify unwanted compounds such as toxins in food and feed since years [74-76], this was the first publication combining a sample

screening with a micropurification and subsequent nanoLC-MS/MS identification of the analyte. Via an SPR inhibition assay chicken meat samples were tested on the presence of ciprofloxacin, a metabolite of the antibiotic enrofloxacin. When found positive, the samples were directed towards a large-surface, off-line antibody-modified sensor surface to isolate the metabolite. This unit was interfaced via a valve with a chip-based nanoLC-ToF MS system allowing the determination of the identity of the compound. Unfortunately, the LC-ToF MS analysis clearly shows the presence of Tween 20, a surfactant residue originating from the SPR analysis. Nevertheless, analyte concentrations of 5 µg L^{-1} can readily be quantified and identified using this system.

2.5. SPR-MS arrays

The SPR-MS methods described above accommodate for the analysis of a single analyte only. For diagnostic purposes, when e.g. several protein biomarkers have to be examined, the throughput is therefore too limited. Surface Plasmon Resonance Imaging (SPRi) instruments, which recently became commercially available, open the possibility for the development of SPR-MS protein arrays and thus the simultaneous analysis of various proteins present in a biological sample [77]. Like conventional SPR equipment, in SPRi instruments imaging (see Figure 5) is performed by illuminating the functionalised area of the sensor chip surface with monochromatic polarised light from a laser diode at a specific angle close to resonance. Perturbations at the surface, such as an interaction, induce a modification of resonance conditions and can be observed as a change in reflectivity. A high resolution CCD video camera is used for monitoring the intensity of the reflected light and thus the real-time difference images across the array format of the spots of the array. The reported size of the array differs from 20 for the BIACORE A100 [78] to more than 500 for e.g. the SPRi-IBIS [79].

A first example of the use of a protein array on a SPR surface used the BIAcore X microfluidic system to transport the sample across the sensor [49]. A

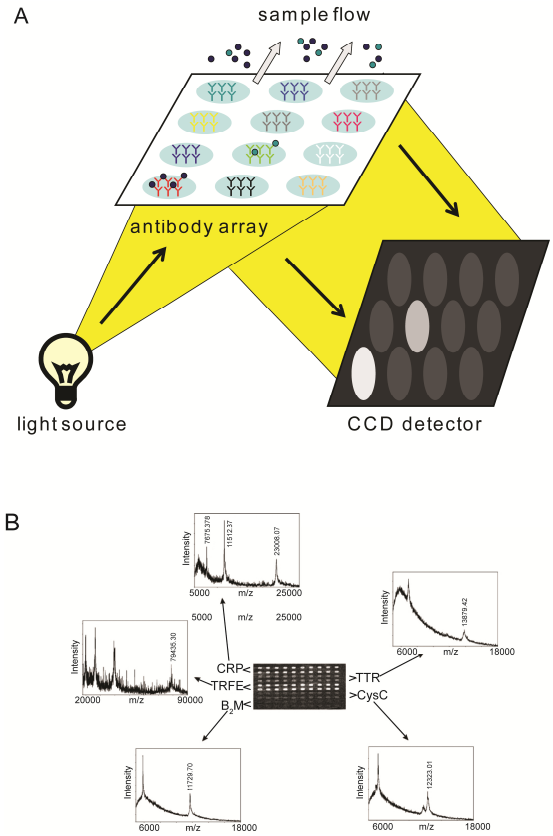

Figure 5: SPRi-MS analysis showing (A) a schematic representation of the SPRi array and (B) SPRi image and mass spectra obtained for various proteins on a CM dextran sensor surface employed in an SPRi experiment (adapted from Nedelkov [80]).

total of 50 spots of a larger array were addressed within the confines of each BIAcore X flow cell (ca 1 mm^2) with diluted human plasma sample. MALDI-MS analysis of the interaction result of five different antibodies directed against proteins present in plasma, showed that the array could effectively be used for monitoring the presence of these proteins in the sample. Extended testing of the procedure showed that the method was robust and repeatable. Parameters that were identified as critical to the success of protein arrays were, apart of the choice of MALDI matrix, the array conditions such as antibody solution pH, temperature and humidity to maintain uniform spots and effective coupling chemistry. The approach was used for SPR-MS experiments showing that simultaneous monitoring of the interaction on 100 spots was possible. Under

optimal conditions, ligand detection limits proved to be in the order of 1 nM for the tested antibody-antigen pairs [80].

A recent paper using an antibody microarray showed the potential of SPRi for high-throughput label-free bioanalysis [81]. The serum protein profiles of liver-cancer and non-liver cancer patients were compared and thirty-nine statistically significant protein changes were detected, ten of which were already known from earlier research. A similar approach was used for protein arrays spotted on self-assembled monolayers (SAM) of short polyethylene oxide (PEO) chains [82]. The PEO chains, which have shown excellent non-fouling properties in the past [83-85], carried an NHS-ester for the efficient coupling of the receptor antibodies. MALDI-MS analysis of the antigen bound after SPRi analysis showed that femtomole amounts of protein present were still detectable. Non-specific adsorption of matrix proteins was not observed due to the composition of the surface SAM. This allowed on-chip protein digestion followed by MALDI-ToF MS/MS experiments for a more definite protein identification.

An interesting development that allows high throughput protein measurements was recently presented [86]. Current SPRi instruments are limited to the analysis of a single analyte stream on multiple ligand spots. The developed, alternative system consisted of 264 element-addressable chambers with a volume of 700 pL, isolated by microvalves. Equipped with serial dilution networks allowing the simultaneous interrogation of up to six different concentrations of analyte, the presented system allows quick analysis of interaction constants. Since each chamber can be addressed independently, the array is capable of determining binding events from up to 264 different immobilised ligands against multiple analytes in a single experiment. The system has not been used in conjunction with MS analysis, but may allow high throughput SPRi-MS analysis of different samples on a single chip.

Another recent approach for real-time interaction monitoring that allows the production of protein nano-arrays is Localised Surface Plasmon Resonance (LSPR). The principle of the LSPR sensors relies on the high sensitivity of the

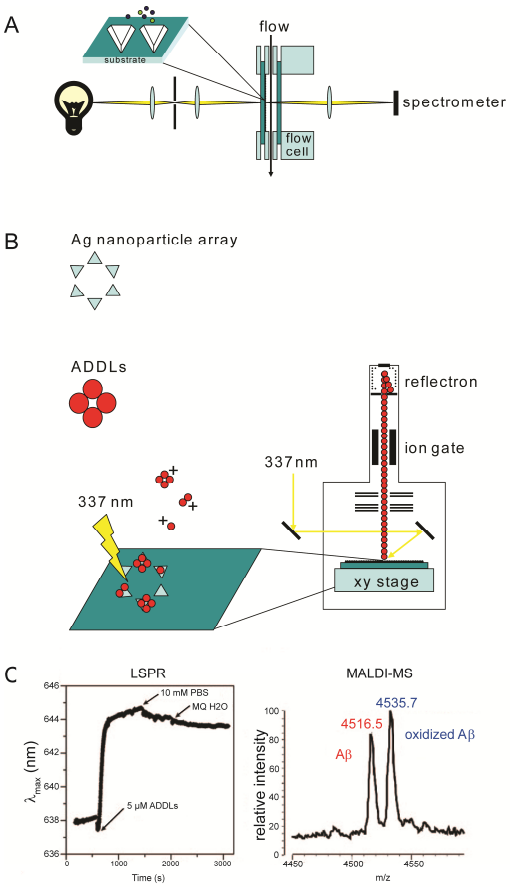

Figure 6: LSPR-MS analysis showing (A) experimental setup for LSPR extinction spectroscopy; (B) experimental setup for MALDI-MS of the LSPR substrates; (C) LSPR monitored interaction between receptor and ADDLs, and MS result showing the isolated oxidised and normal ADDLs (adapted from Anker *et al.* [97]).

LSPR spectrum of (noble) metal nanoparticles to adsorbate-induced changes in the dielectric constant of the surrounding environment. These plasmon resonances give specific metallic particles a strong and well-defined color, a property that has been used since long in e.g. stained glass. Apart from the nature of the metal and thus its dielectric properties, the LSPR is dependent on the size, shape and spacing between the particles, and the dielectric properties of the local environment that surrounds the particle [87, 88]. The extinction maximum of the nanoparticle arrays can be measured with UV-VIS spectroscopy. Since the LSPR of the nanoparticles is highly dependent on the

local environment, the extinction maximum will shift when adsorbates bind to the particles [89-92].

Although commercial instruments employing the LSPR principle are not yet available, several papers show the successful implementation of sensors for both small molecule detection such as steroids [93] as well as proteins [94-96]. Recently, a first report on the combination of LSPR and MALDI-ToF MS was published [97]. With this set-up Amyloid-beta Derived Diffusible Ligands (ADDLs) were detected and characterised (see Figure 6). These proteins play a role in the molecular pathogenesis of Alzheimer's Disease. The detection and identification of normal and oxidised ADDLs proved possible when an estimated amount of 9 pmol of protein is present. It was shown that posttranslational modifications of protein bound to the LSPR sensor can be resolved. Unfortunately, the current state of the technique is approximately 10000 times less sensitive compared to the on-chip MS described earlier [44]. This is attributed to the surface chemistry and antibody immobilisation protocols [98].

2.6. Alternative biosensors

As described above, the assembly of SPR and MS is an ideal combination as the sensitivity of both techniques is of the same order of magnitude, and complementary information on quantity and identity can be obtained. However, other sensor platforms can be used as well on basis of sensitivity as well as robustness. An example of such a sensor used for quantification and combined with MS identification is the Surface Acoustic Wave (SAW) sensor. After using the sensor for the analysis of the blood clotting cascade α-thrombin and human antithrombin III, the sensor surface was analysed with MALDI-ToF MS [99]. Alternatively, an on-line SAW-ESI-MS system was developed in which the isolated ligand was desorbed, concentrated and desalted on a RP micro-column. To identify the ligand the column was positioned in-line with the ESI and trapped proteins and peptides were desorbed and multiple charged ions could be observed. The applicability of the

system was demonstrated using a range of antibodies and (peptide) ligands [100]. With regard to sensitivity, the SAW sensors have shown to perform similar to SPR. Surprisingly, taking into account the claimed robustness of the relatively simple instrument, little experimental data are available today to show the successful integration of this type of sensor and MS.

An example of an extremely sensitive optical sensor principle is interferometry. The method claimed to be far more sensitive than traditional SPR, thus allowing the direct detection and quantification of femtomolar concentrations of protein or ppb levels of hapten molecules, such as the pesticide simazine [101-103]. Sensors based on Mach-Zehnder technology with integrated microfluidics have been applied for screening and quantification and MS-identification of vasopressin [104-107]. Unfortunately, the lack of commercial availability of these instruments has limited further investigations so far. Recently, a consortium consisting of the firm Optisense and several research institutes (www.optisense.nl) has started production and application development in the field of healthcare and food safety using these sensors.

Electrochemical sensors using non-destructive electrochemical impedance spectroscopy (EIS) are extremely sensitive and detection levels of attomolar levels of protein have been reported [108]. Sensors based on this principle have been combined with Quartz Crystal Microbalance (QCM) or SPR. Several papers describing the resulting EQCM and ESPR platforms have appeared [109-111], showing that these complementary techniques are well suited for the study of sensor construction and performance. Various applications using EIS-sensors have been reported, e.g. for the detection of neuron-specific enolase, a stroke marker, at femtomolar levels [112]. Also the development of an array for molecular immuno-diagnostics comprising 54 peptide markers and capable of detecting antibodies in diluted serum at pM levels has been described [113]. Recently a number of reviews on EIS sensors for biomedical applications has appeared [114, 115]. In spite of their high potential, EIS sensors have to our knowledge not been combined with mass spectrometry so far. This is partly due to the reported attomolar sensitivity of EIS which is far below the

detection range of current MS instruments [108]. Nevertheless, the relatively low instrumental costs and the easy miniaturisation are attractive aspects and will stimulate further investigations in this type of sensor.

2.7. Future perspectives

Although SPR-MS has been used for studying protein-protein interactions, ligand fishing, orphan receptor and drug screening has been in use for almost fifteen years, several experimental aspects remain to be further addressed. Among these are the issues of SPR surface modification in order to allow efficient protein coupling and prevent non-specific adsorption, and the increasing use of SPR arrays.

Non-specific adsorption on SPR sensors of matrix components has been studied since the introduction of the technique. Several approaches have been described, both in surface chemistry as well as in the running buffer composition. The classic surface, carboxylated dextran (CM) [116, 117] is a hydrophilic material offering a large surface area that usually enables high antibody immobilisation yields. These CM coatings have proven to perform satisfactory when employed under well-defined conditions as was shown for on-chip MALDI-ToF MS analysis reported by Nedelkov *et al.* [38] who showed that for SPR-MS experiments several hundred attomoles of protein present on the SPR surface can readily be identified. However, when analyses are performed in more complex media like plasma or serum, high non-specific adsorption has been observed [118]. Therefore SPR sensor chips providing dextran with various degrees of carboxylation were commercially introduced to accommodate for experiments conducted in complex matrices. Also native dextran was used, and compared to carboxylated dextran low non-specific adsorption was observed [15]. Other oligosaccharides with intrinsic low non-specific adsorption have been used as well [119, 120], but are not commercially available and therefore so far not often used in spite of their promising characteristics.

A different material known for its fouling-resistance is oligoethylene-glycol. This material has been extensively tested [83-85, 121]. Especially when introduced as thiol-derivative, a highly ordered structure can be composed on the gold SPR surface which is efficient against random adsorption of proteins and other matrix components. However, such a surface modification generally allows only for a single layer of receptor molecules. This means that the amount of ligand that can be isolated from the matrix is limited compared to the three-dimensional structure that the classic dextran-modified SPR surfaces offer. A recent example of the application of this kind of surface in SPR-MS research is the study conducted by Boireau *et al.* [122] showing a sensitive SPR analysis without detectable non-specific binding and good MALDI-MS/MS identification due to the absence of interfering compounds. However, in order to obtain enough material for ESI-MS analysis large surface area sensors, high receptor densities or, preferably, a combination of the two is used as was reported by several research groups [48, 53, 123]. This means that dextran-modified surfaces, which allow for high receptor immobilisation, are an obvious choice when an SPR-LC-ESI-MS experiment is conducted. Non-specific adsorption issues have to be addressed e.g. by washing/rinsing the surface or by using running buffers specially designed for the analysis of an analyte.

The composition of the SPR running buffer influences the level of (non-specific) interaction. Generally detergents such as Triton and Tween are present in SPR running buffer to avoid non-specific adsorption of hydrophobic matrix components such as membrane proteins. Unfortunately, these detergents are often not compatible with MS analysis due to the occurrence of ion suppression or adduct formation. A detergent used in SPR that has been shown to have only a limited effect on the ESI-MS result is the non-ionic surfactant N-octyl-glucoside [32]. In MALDI-ToF MS the same detergent even shows beneficial effects by reducing the high mass discriminatory effects [124]. For the removal of e.g. ethyleneglycol-based detergents from protein digests several protocols have become available that can be implemented in automated analysis

systems [125-127]. The development of cleavable detergents such as the commercially available Rapigest SF or PPS silent [128-130] also provide a possibility for creating SPR running buffers that minimise fouling and are compatible with ESI- and/or MALDI-MS. Alternatively, materials that show similarity to the SPR surface, such as dextrans, can be added to the sample [131]. Similarly, a SPR-MS study of Larsericsdotter *et al.* [132] describe the use of coated colloidal particles. These additives may competitively bind adsorbing components that would otherwise potentially disturb the experimental outcome.

The development and use of SPR arrays, i.e. SPRi instruments, improves the throughput and quantity of interaction data during a single experiment and will be the focus area for future research. Compared to other formats that allow array production such as LSPR it does not require sophisticated fabrication environments and is therefore more economical in operation. For MS analysis MALDI-ToF MS is an obvious choice, taking into account the small surface areas for every spot and thus the small quantity of isolated ligands. Although the results obtained so far, e.g. by Nedelkov *et al.* [80], are promising, the interfacing with MS has still to be optimised. Especially the potentially limited amount of material isolated during an experiment, and sample loss or carry over during processing steps such as surface rinsing and proteolysis may affect the MS result. Consequently, improving the surface chemistry to retain the sample during processing and/or the development of a sophisticated fluidic system such as described by Ouellet *et al.* [86] are issues for further research.

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3.

An improved coating for the isolation and quantification of interferon-γ in spiked plasma using surface plasmon resonance

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Abstract

A study was initiated to investigate the use of surface plasmon resonance (SPR) for the detection in plasma of a high pI model protein, recombinant human interferon-γ (IFN-γ). Initially a number of self-assembled monolayers (SAMs) and hydrogel-derivatised SAM-coatings were characterised for the adsorptive and desorptive properties of plasma components. Next a monoclonal anti-IFN-γ antibody, MD-2, was covalently attached to dextran-modified mercaptoundecanoic acid surfaces that performed best.

On coatings consisting of carboxyl-modified dextran (CMD) a difference in interaction behaviour was observed when IFN-γ was injected in either buffer or diluted plasma. During the injection of IFN-γ in buffer, an acceleration of the interaction process was observed and the signal continued to increase after the injection plug had passed. Upon injection of diluted plasma spiked with IFN-γ, the response increased without acceleration of the binding process. After the injection was finished, some of the bound material desorbed as expected, resulting in a signal decrease.

On non-charged dextrans, the interaction between the antibody-modified surface and IFN-γ in either plasma or buffer was similar. During sample injection the response increased with a binding rate depending on the concentration of IFN-γ present in solution. When the injection was finished, some of the bound material was washed away from the surface and only a minor contribution of non-specific adsorbed plasma components was noticeable.

From the coatings tested, the non-modified dextran-coated SPR sensor disks prove to be best suited for the detection of IFN-γ in complex matrices like plasma. The interaction of IFN-γ in both diluted plasma and buffer is comparable and concentrations of IFN-γ of 250 ng mL^{-1} and higher can be detected in both buffer and 100x-diluted plasma. The non-specific adsorption of plasma components is low whereas the specific IFN-γ response is relatively high.

3.1. Introduction

Due to their high selectivity, affinity-based techniques are often used for the quantification, isolation and purification of biomolecules. In the last decade many papers have appeared where receptor molecules like antibodies are integrated in electrochemical, acoustic or optical methods allowing direct, real-time monitoring of interactions of unlabelled (bio-)molecules. A robust optical method that is often used in this respect is surface plasmon resonance (SPR) [1-3]. SPR methods have been developed for various purposes including the quantification of ligands [4-9], the study of binding kinetics [10] or complex formation [11]. More recently, SPR was used as a micro-purification unit to isolate proteins, protein-complexes or enzyme inhibitors for further study as well as for the mass spectrometric identification of the isolated material [12-17].

All sensors basically consist of two elements: the (bio-)chemical recognition layer and the physico-chemical transducer. The design of the surface layer which is in direct contact with the sample is crucial. This is especially true when complex materials, like blood and other clinically relevant samples have to be analysed. In these samples the analyte (i.e. the ligand) is typically present in a low concentration compared to other molecules. Non-specific adsorption of these latter components may overwhelm or even prevent the specific interaction signal. Hence, the design and preparation of surface coatings that suppress or even prevent non-specific protein adsorption is important in biosensor design to ensure specific recognition of the analyte only.

Many papers have appeared dealing with the phenomenon of non-specific adsorption of biomolecules being present in blood and other complex media. This research was stimulated by biomedical applications such as transplant-rejection prevention [18, 19], and the demand for quick and reliable analytical methods avoiding time- and sample-consuming clean-up methods. The outcome of these investigations may be used in the development of coatings that enable direct quantification in real biological samples. Most attention has been paid to the use of poly(ethylene glycol) and derivatives [20-23], and polysaccharides [24-27]. These substances were shown to be able to minimise

or even prevent non-specific protein binding depending on the nature of the coating material and the way it was grafted to the sensor surface.

Although the ethylene glycol-based materials showed potential for the prevention of non-specific adsorption of proteins, these materials are not often used in biosensors today. In general affinity-based SPR-sensor applications make use of commercially available surfaces based on carboxyl-modified dextran like the CM5 biosensor chips from BIAcore [1]. This hydrophilic material offers a large surface area and usually enables high antibody immobilisation yields due to the preconcentration effect using the electrostatic interaction between the negatively charged carboxyl functions present in the coating and positively charged amino-acid residues in the protein. When used in well-defined standard solutions and diluted samples, these coatings have proven to perform satisfactory. However, when analyses are performed in more complex media like plasma or serum, high non-specific adsorption has been observed [28].

Therefore we have initiated a study to explore the possibilities for the application of SPR to real samples. The suitability of carboxyl-modified dextran and some other coatings for the prevention of non-specific binding from bovine plasma will be studied. Antibody-modified coatings will be used for the isolation and quantification of a model protein, recombinant human interferon-gamma (IFN-γ), from plasma. The protein is a cytokine and has a molecular weight of 15.5 kD and a high pI of 9.6. This protein was used before in various assay development studies in our group [29-31].

3.2. Experimental

3.2.1. Materials

The IBIS and IBIS II SPR equipment used was from IBIS Technologies (at present available from Eco Chemie, Utrecht, The Netherlands). The sensor disks, purchased from SSENS (Hengelo, The Netherlands), were positioned on the IBIS-prism using index-matching oil from R.P. Cargille Laboratories Inc

(Cedar Grove, USA). The flow-cell employed and the SPR sensor disks have been described before by Bart *et al.* [31]. Nuts, unions, tubing and loops were from Upchurch Inc (Santa Monica, USA). The autosampler used was a Gilson 231-401 (Villiers le Bel, France). The flow was generated by a Shimadzu 10AD*vp* HPLC pump (Kyoto, Japan). The water used for washing and to prepare buffers was produced by a MilliPore system (Amsterdam, The Netherlands, conductivity > 18.2 MΩ cm).

Acetic acid, ethanol, ethanolamine (EA), hydrochloric acid, hydrogen peroxide, sodium dihydrogen phosphate, sodium chloride, sodium hydroxide (NaOH), sulphuric acid and titriplex III (EDTA) were purchased from Merck (Darmstadt, Germany). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and acetone were purchased from Acros Organics (Geel, Belgium). Bovine serum albumin (BSA) and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma (St Louis, USA). Bromoacetic acid, carbonyl diimidazole (CDI), hydrazine, mercaptoethyl amine (MEA), polyacrylic acid (PAcA) and 11-mercaptoundecanoic acid (MUA) were from Aldrich Chemical Company (Milwaukee, USA). *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and dextran-500 were purchased from Fluka (Buchs, Switzerland). Amino-modified dextran (AMD) was from Unavera ChemLab (Mittenwald, Germany).

Piranha solution was prepared by mixing 1 part of 30% hydrogen peroxide and 6 parts of concentrated sulphuric acid. Phosphate buffered saline (PBS) consisted of 10 mM sodium dihydrogen phosphate and 150 mM NaCl in water set to pH 7.2 using 2 M NaOH. HEPES buffered saline-EDTA Tween 20 (HBS-ET) consisted of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% Tween 20 set to pH 7.4 using 2 M NaOH. Bovine plasma was prepared by centrifugation of heparinised bovine blood obtained that same day from a local slaughterhouse. The bovine plasma was diluted 4 times with PBS. Further dilutions were prepared with HBS-ET. Monoclonal mouse anti-interferon-γ (MD-2) and recombinant human interferon-γ (IFN-γ) were kindly supplied by Dr. P.H. van der Meide of U-Cytech (Utrecht, The Netherlands).

3.2.2. Preparation of SPR sensor coatings

The SPR sensor disks were extensively cleaned in a freshly prepared piranha solution. After 1 h the disks were thoroughly rinsed with water, dried in a stream of nitrogen gas and immediately incubated overnight in a 10 mM solution of either MUA or MEA in ethanol. After SAM formation, the disks were washed with ethanol and water, and dried with nitrogen gas.

The MEA SAM was modified with 100 µL of a mixture consisting of an aqueous 1% polyacrylic acid (PAcA) solution that was activated by addition of 200 mM EDC and 25 mM NHS in water in a 1:1 ratio. After 15 min, the sensor disks were washed with water and incubated overnight with a 10% solution of AMD in water. The resulting MEA-PAcA-Dex sensor coatings were modified by incubation in a 1 M solution of bromoacetic acid in 2 M NaOH in the dark at room temperature for 16 h. After incubation the resulting MEA-PAcA-CMD disks were extensively washed with water and dried in a stream of nitrogen.

MUA-Dex sensor coatings were prepared by activating the carboxyl functions on the SAM layer with a mixture containing 200 mM EDC and 50 mM NHS in water during 30 min. After removing the EDC-NHS mixture the disks were incubated overnight with a 10% solution of AMD in water. Subsequently, the dextran-modified disks were cleaned with water, ethanol and water successively and dried in a stream of nitrogen. *MUA-CMD* sensors were prepared by incubating the MUA-Dex modified sensors 40 h in a 1 M solution of bromoacetic acid in 2 M NaOH in the dark at room temperature. When less carboxyl functions were required the reaction was allowed to proceed only 16 h. After incubation the disks were extensively washed with water and dried in a stream of nitrogen. *MUA-HMD* surfaces were prepared by introducing hydrazide functions by activating the MUA-CMD sensor disks with a 1:1 mixture of 400 mM EDC and 100 mM NHS for 30 min. After removing the EDC-NHS mixture the sensor disks were incubated overnight in a 10% hydrazine solution in water. After incubation the disks were extensively washed with water and dried with nitrogen gas. All sensor disks that were not used immediately were stored dry and dust-free at room temperature.

3.2.3. Immobilisation of MD-2 on sensor disks

Dextran disks were activated with 400 mM CDI in dry acetone. After removing the acetone and rinsing the sensor surface with water, 35 µg mL^{-1} MD-2 in PBS was introduced. After coupling, the non-reacted activated groups were blocked using ethanolamine (see also Figure 1A). Hydrazide modified dextran (HMD) disks were incubated for 30 min with a solution containing 50 µg mL^{-1} MD-2, 100 mM EDC and 50 mM NHS in 10 mM HCl. After incubation the sensor disk was flushed with HBS-ET running buffer. Carboxyl-modified dextran (CMD) sensors were activated using a 1:1 mixture of 400 mM EDC and 100 mM NHS in water. The NHS-esters produced in this way reacted with amine functions present in the 50 µg mL^{-1} MD-2 solution at pH 4.7. After coupling, remaining activated groups were inactivated with a 1 M ethanolamine solution at pH 8 (see also Figure 1B).

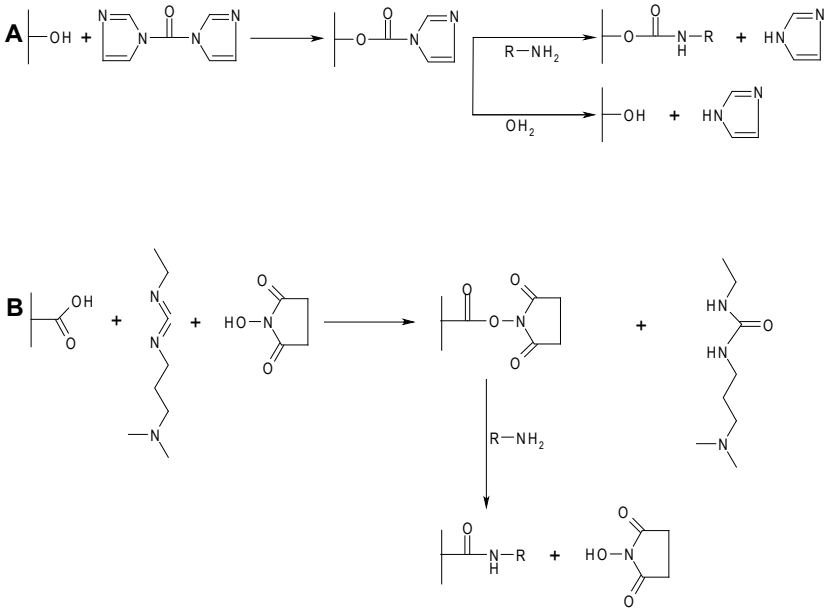

Figure 1: Immobilisation chemistry. A: carbonyl diimidazole activation on surfaces containing native dextran. B: carbodiimide activation of carboxyl groups, for example CMD coatings. R-NH_2 represents a primary-amine containing molecule, here an antibody.

3.2.4. SPR experiments

All experiments were performed with IBIS SPR immunosensors. Sensor disks were fixed on the prism using index-matching oil. After positioning of the flow cell, SPR measurements were made by recording the angle shift as a function of time. Injections of 100 µL of diluted bovine plasma, BSA and IFN-γ diluted in HBS-ET were made in a 10 µL min^{-1} flow of HBS-ET running buffer. Regeneration of material bound to the sensor surface was accomplished by injections of 20 µL of a 10 mM glycine solution, pH 1.5. Nuts, unions, tubing (ID 50 µm) and loops (ID 150 µm) were PEEK. During the experiments the system was thermostatted at 25.0 °C. The system was fully automated using a Gilson autosampler programmed to perform sample injection and regeneration cycles.

3.3. Results and discussion

3.3.1. Interaction of bovine plasma with SPR coatings

Various substances including proteins, self-assembled monolayers (SAMs) and hydrogels have been reported for modification of gold surfaces to enable the monitoring of binding processes by SPR. Currently, the carboxyl-modified dextran hydrogels are in common use because of their high binding-capacity, and their assumed low non-specific adsorption. However, compared to SAMs it is time-consuming to prepare these surfaces. This makes SAMs prepared from thiol-containing substances an interesting alternative. Therefore we have chosen to investigate coatings prepared from thiol-containing and dextran-modified materials for the development of an interferon-γ assay, as mentioned, a recombinant cytokine with a high pI.

Various coatings (see Experimental) were tested for their adsorptive properties by injecting a 4 x-diluted bovine plasma solution for 10 min. The net responses caused by the plasma components that remained after injection followed by a 5-min flow of buffer, are presented in Figure 2. All coatings give rise to non-specific adsorption, the highest amount for AcCys. The material that

adsorbed to this coating could not be removed completely by repeated injections of the glycin regeneration solution. Less plasma components were adsorbed to MUA, but multiple regeneration injections could also not completely remove the adsorbed plasma components. The reason for the observed lower adsorption is probably caused by the more-ordered and closer structure of the MUA SAM due to the presence of long alkyl chains. This ordered structure apparently generates a better barrier for protein adsorption and denaturation than is obtained with the shorter AcCys SAM.

Complete regeneration using glycin was possible for both MEA-multilayer assemblies and the dextran modified MUA surfaces. The plasma components adsorbed to dextran-modified MUA surfaces could even be regenerated with a single injection of the glycin solution. Non-specific binding caused by plasma components was also the lowest for these latter surfaces, although the three MUA-dextran variants showed a considerable variation in plasma protein adsorption. As surface regeneration is possible and protein adsorption on these non-modified surfaces is relatively low, only the dextran-modified MUA surfaces (MUA-CMD, MUA-HMD and MUA-Dex) were further investigated.

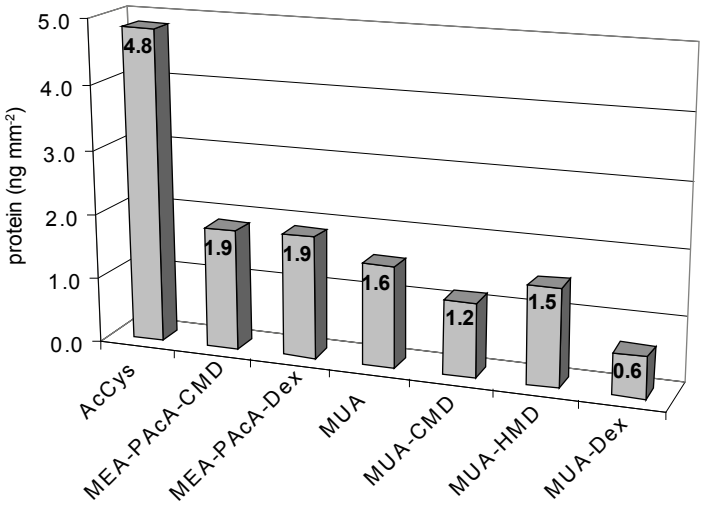

Figure 2: Adsorption caused by 4x-diluted bovine plasma upon contact with various, unmodified SPR coatings. Abbreviations can be found in Experimental.

3.3.2. IFN-γ interaction with dextran-modified sensor surfaces

The dextran-modified MUA surfaces were activated with the appropriate reagent and MD-2 antibody was attached to the dextran hydrogels (see Experimental). An example of antibody immobilisation is presented in Figure 3, showing a typical sensorgram for the immobilisation of MD-2 on a CMD surface. In this graph the injection steps, activation, antibody attachment and subsequent blocking of remaining activated groups, are shown. In this case the MD-2 immobilisation level obtained was 7.5 ng mm^{-2}. This can be calculated from the obtained angle shift [32] as 120 m° is equivalent to an amount of attached protein of 1 ng mm^{-2}. The mean immobilisation levels of MD-2 obtained on the three coatings that were tested, and the amounts of IFN-γ that were bound to the these MD-2 modified surfaces upon injection of a 1 µg mL^{-1} IFN-γ in HBS-ET, are summarised in Figure 4. The highest antibody immobilisation levels were obtained with MUA-CMD surfaces, the lowest with MUA-HMD surfaces. As can be observed in this graph, the amount of IFN-γ that is bound to the surfaces during sample injection, increases with increasing amount of immobilised antibody. The non-specific plasma-protein adsorption from a 100x-diluted plasma solution does not significantly differ for these surfaces.

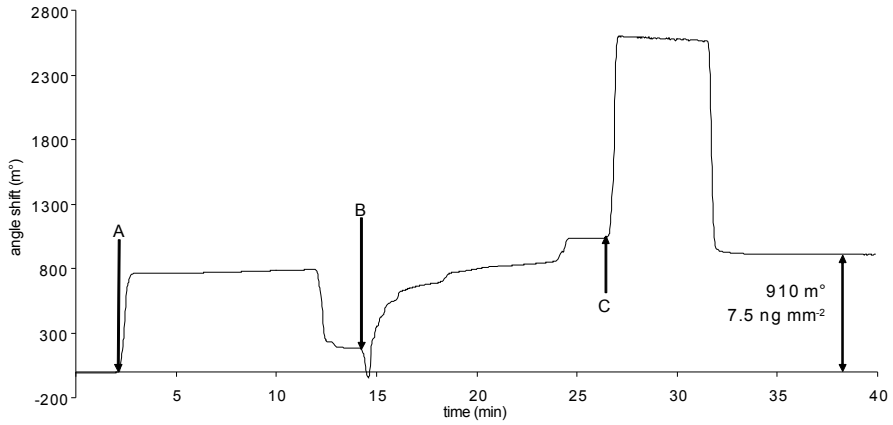

Figure 3: MD-2 immobilisation on a CMD-coated sensor. Subsequent injections of EDC-NHS (A), MD-2 (B) and ethanolamine (C). The antibody MD-2 immobilised gives rise to a response of 910 m° which is equivalent to an amount of 7.5 ng mm^{-2}.

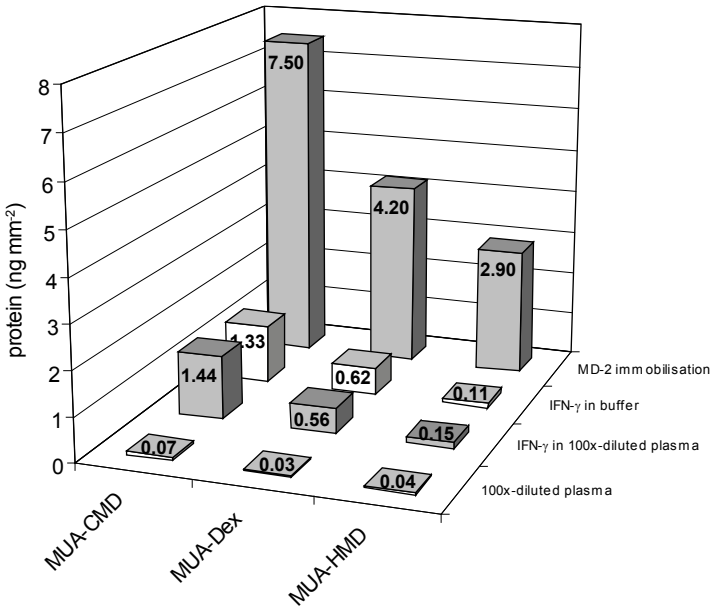

Figure 4: Amount of MD-2 immobilised on dextran-modified MUA surfaces and the amount of protein bound to the surfaces upon injections of 1 µg mL^{-1} IFN-γ in running buffer or 100x-diluted bovine plasma and non-spiked 100x-diluted bovine plasma.

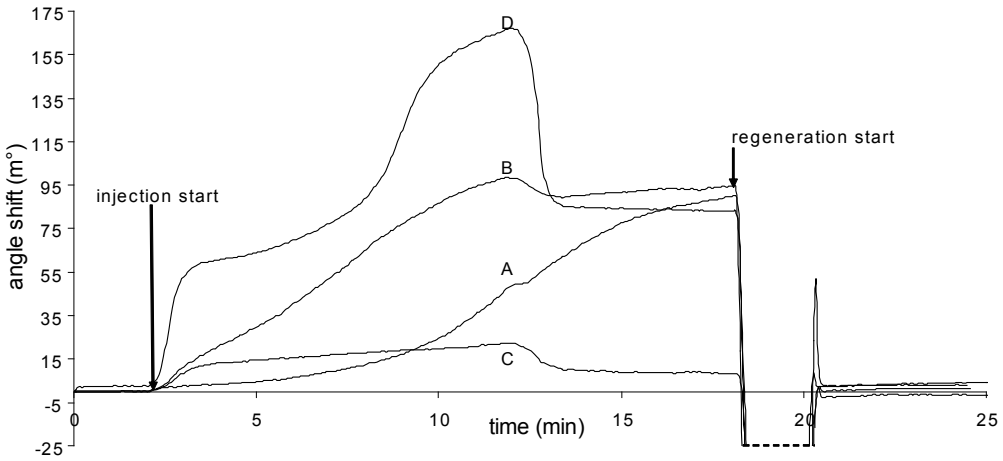

Figure 5: SPR sensorgrams of the IFN-γ interaction with MD-2 modified CM-dextran surfaces. The start of the sample-injection and regeneration are indicated with arrows. A: 500 ng mL^{-1} IFN-γ in HBS-ET buffer B: 500 ng mL^{-1} IFN-γ in 100x-diluted bovine plasma. C: 100x-diluted bovine plasma. D: 500 ng mL^{-1} IFN-γ in HBS-ET buffer containing 4 mg mL^{-1} BSA.

For MD-2 modified MUA-CMD surfaces, a difference in interaction behaviour between IFN-γ in buffer and diluted plasma was observed, especially after the injection was finished. As shown in Figure 5, curve A, during the injection of IFN-γ in buffer an increase of the binding rate seemed to occur that only gradually slowed down after the injected sample plug left the flow cell. The injection of IFN-γ in diluted plasma (Figure 5, curve B) did not show this anomalous behaviour. The binding rate is constant and seemed to decrease at the end of the injection. After the sample plug left the flow cell, the response seemed constant. Only a slight refractive index change at the start and end of the injection and a minor adsorption of plasma protein which was also observed for non-spiked injections, 0.08 ng mm^{-2}, was seen (Figure 5, curve C). Figure 5, curve D, shows the interaction of IFN-γ spiked in a 4 mg mL^{-1} BSA solution. Apart from the bulk refractive index changes at the start and end of the injection due to the high protein content of the solution, the presence of BSA seemed to have no influence on the interaction between the IFN-γ and the antibody-modified surface. Non-specific interaction of BSA was not observed on these surfaces.

The differences observed in the interaction between MD-2 modified CMD coating and IFN-γ spiked in buffer or in 100x-diluted bovine plasma indicate that CMD surfaces are not suited for the quantification of IFN-γ. This is especially true because of the increasing response after the injection of IFN-γ in buffer has been finished and no ligand enters the flow-cell. Likely these differences in behaviour are caused by the characteristics of both the protein and the sensor surface. The isoelectric point of the IFN-γ is high (pI = 9.6) and therefore the protein interacts with the net negatively charged CMD surface. The effect was also observed on non-derivatised CMD surfaces where 30 min incubation of a 1 µg mL^{-1} IFN-γ solution in HBS-ET resulted in 0.4 ng mm^2 IFN-γ adsorbed after the sample was removed and the surface was flushed with buffer, whereas no protein remained after an incubation of a reference sample containing 4 mg mL^{-1} BSA solution (pI = 5.5). The carboxyl groups remaining in the CMD hydrogel after antibody immobilisation will interact electrostatically

with affinity bound IFN-γ as well, causing a time-dependent surface density and hence a refractive index change. MUA-CMD surfaces that were incubated in bromoacetic acid for a shorter period of time (see Experimental) and hence contained a smaller amount of carboxyl functions showed a similar behaviour. Only a lower antibody immobilisation and hence less IFN-γ binding capacity was obtained. As can be observed in Figure 5 this effect was not observed when IFN-γ was present in diluted bovine plasma. This may be caused by a blocking effect of the ionic groups by plasma components present in the solution.

Influence of charged groups present on dextran was also observed by Griesser and coworkers [27]. They found that various proteins in plasma were adsorbed to carboxyl-modified dextran (CMD) surfaces, depending on the amount and density of carboxyl groups present. Small positively charged proteins were adsorbed in high amounts when dextran was highly substituted with carboxyl functions whereas only lower amounts of larger, slightly positive molecules were adsorbed. These results also emphasise the contribution of electrostatic attraction on the non-specific adsorption. Decrease of the amount of charged groups on the dextran coating without affecting the hydrophylic character and immobilisation yield, may avoid or lower non-specific protein adsorption and the effects observed with IFN-γ.

In order to prevent surface-charge effects, the carboxyl groups in CMD were activated with EDC and blocked with hydrazine, thus creating hydrazide groups that are uncharged at neutral pH. The amount of antibody immobilised on these surfaces was relatively low compared to the CMD surfaces (see Figure 4). This is probably due to the absence of electrostatic attraction (the "preconcentration" effect) which plays an important role in the high immobilisation yields obtained with CMD surfaces. The interaction of 1 µg mL^{-1} IFN-γ on an antibody modified sensor showed no difference in the form of the sensorgrams of IFN-γ in buffer and IFN-γ in 100x-diluted bovine plasma. Blanc plasma, however, gives response levels that are relatively large compared to the responses caused by IFN-γ binding (Figure 4). Therefore these surfaces were not further investigated.

Residual charged groups on dextran can also be avoided by coupling MD-2 antibody to dextran using hydroxyl-activating agents like CDI. This immobilisation method is generally used for the direct coupling of proteins to, for example, cross-linked agarose in chromatography [33]. In Figure 4 typical immobilisation values for the immobilisation of MD-2 to the sensor surface are shown. Figure 6 shows the interaction of 500 ng mL^{-1} IFN-γ with the sensor surface. There is no real difference between the interaction of IFN-γ in buffer and IFN-γ in diluted bovine plasma except for a refractive index change at the start and finish of the injection. After the sample plug left the flow-cell, some of the bound material desorbed from the surface and a minute amount of plasma protein adsorption was observed (0.03 ng mm^{-2}). The adsorption of diluted plasma components is half that of CMD surfaces operated under similar conditions (see Figure 4). These observations confirm the results obtained with unmodified surfaces incubated with 4x-diluted plasma where the non-specific response equals 1.2 ng mm^{-2} for CMD surfaces and 0.6 ng mm^{-2} for a native dextran coating (Figure 2). Compared to other SPR-based methods using receptor-modified dextran-hydrogels for the quantification of proteins in real samples, the sensor surface performs satisfactory. One such study used normal sheep serum as a model matrix and found 0.2 ng mm^{-2} protein adsorption on

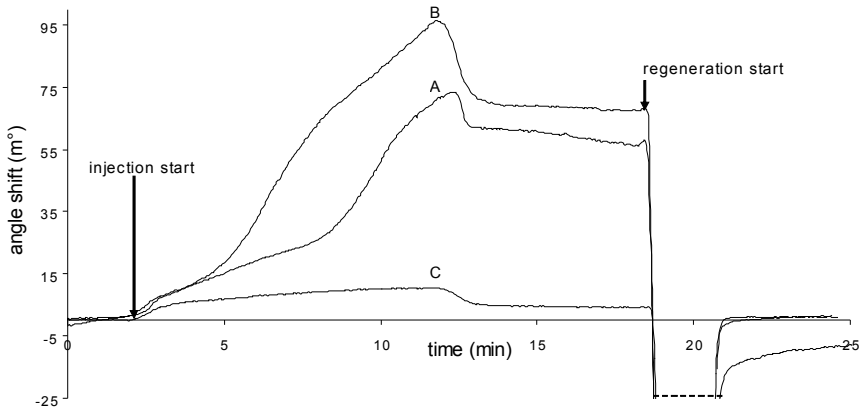

Figure 6: SPR sensorgrams of the IFN-γ interaction with MD-2 modified dextran surfaces. A: 500 ng mL^{-1} IFN-γ in HBS-ET buffer. B: 500 ng mL^{-1} IFN-γ in 100x-diluted bovine plasma. C: 100x-diluted bovine plasma.

an antibody-immobilised CM5-sensor surface [28]. Quantification of an insulin-analogue in culture-media on a dextran, modified with a chemical receptor, resulted in 0.6 ng mm^{-2} protein adsorbing non-specifically [34].

Most likely the non-charged character of this dextran modified sensor surface is causing this low non-specific response. This assumption is confirmed by the non-specific responses for non-modified dextran surfaces. Both 4 mg mL^{-1} BSA and 1 µg mL^{-1} IFN-γ in buffer gave no response after 30 min of incubation and washing with buffer, this in contrast to observations made with CMD surfaces discussed above.

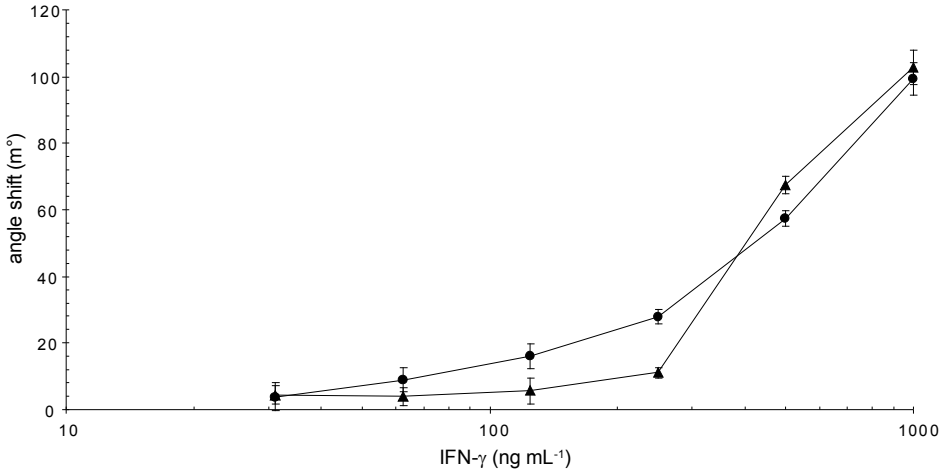

Figure 7: Dose-response curves of the IFN-γ interaction with MD-2 modified dextran surfaces; duplicate runs on a MD-2 modified MUA-Dex surface. (●) IFN-γ in HBS-ET buffer, (▲) IFN-γ in 100x-diluted bovine plasma.

A study with XPS on the differences in protein-adsorption characteristics between dextran modified surfaces with low and high degrees of carboxylation showed that dextran with few carboxyl-functions resulted in a more open structure that is more easily penetrated by relatively large molecules than dextrans with large amounts of carboxyl-functions showing charge rejection [27]. Nevertheless, the amount of protein that was (electrostatically) adsorbed was higher on the latter surface and this was also observed when comparing the protein adsorption from a 4x-dissolved bovine plasma on MUA-CMD and MUA-Dex. However, the nature of the adsorbed proteins is yet unclear and might be different for each of the surfaces.

In Figure 7 the dose-response curves are shown for the interaction of IFN-γ samples with a MD-2 modified MUA-Dex surface. Concentrations of 250 ng mL^{-1} and higher of IFN-γ in 100x-diluted plasma can be determined. Injections of these IFN-γ samples result in similar response levels for both buffered and diluted bovine-plasma samples. The lower responses observed when injecting diluted plasma samples compared to buffered samples may be caused by the interaction of plasma components and the IFN-γ.

Higher antibody immobilisation levels should be obtained in order to make the antibody-modified MUA-Dex coatings more efficient in the binding of antigen and thus even more suitable for quantification and especially the isolation of proteins from complex samples like plasma. As was also observed with the HMD, the electrostatic attraction (preconcentration) that CMD offers during the immobilisation of an antibody, is not present in underivatised dextran. This results in lower antibody immobilisation levels. Also in acetone, dextran aggregates and precipitates. It is therefore likely that CDI activation in acetone of the dextran hydrogel is less efficient than reported for the activation of crosslinked oligosaccharides [33]. Activation should thus be either accomplished in a solvent accommodating the both activating agent CDI and the dextran hydrogel, or a reagent should be used that is stable in an aqueous environment. These possible approaches will be studied in future.

3.4. Conclusions

Several SPR coatings were tested for their ability to prevent non-specific adsorption from plasma components. The best results with respect to plasma adsorption and surface regenerability for the tested coatings are obtained with dextran modified MUA SAMs. When MD-2 is covalently coupled to these surfaces, a different behaviour of the various substituted dextrans is observed upon injection of the analyte IFN-γ. On CMD coatings an acceleration in binding-rate is observed during the injection, which continues after the injection is finished. This binding-behaviour is not observed with IFN-γ in diluted

plasma. The difference in interaction between buffer and plasma dissolved IFN-γ and the antibody-derivatised coating is not observed with the non-charged hydrogels, HMD and underivatised dextran. The response upon injection of IFN-γ on a MD-2 HMD surface is low compared to the non-specific adsorption of diluted plasma, making this coating unsuited for the detection of IFN-γ. The non-specific interaction of diluted plasma is lowest on MUA-Dex coatings, whereas the specific interaction between immobilised MD-2 and IFN-γ results in a relatively high response. This makes non-modified dextran-coated SPR sensor disks best suited for the detection of IFN-γ in complex matrices like plasma with a lower detection limit of 250 ng mL^{-1}. Future research will be directed at obtaining higher immobilisation levels using alternative coupling chemistry and hydrogel preparation in order to make the dextrane coating more suitable for quantification and especially the isolation of proteins from complex samples like plasma.

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3.5. Literature

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4.

Development of an open-tubular trypsin reactor for on-line digestion of proteins

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Abstract

A study was initiated to construct a micro-reactor for protein digestion based on trypsin-coated fused-silica capillaries. Initially, surface plasmon resonance was used both for the optimisation of the surface chemistry applied in the preparation, and for monitoring the amount of enzyme that was immobilised. The highest amount of trypsin was immobilised on dextran-coated SPR surfaces which allowed the covalent coupling of 11 ng mm^{-2} trypsin. Fused-silica capillaries were modified in a similar manner and the resulting open-tubular trypsin-reactors having a pH optimum of pH 8.5, display a high activity when operated at 37°C and are stable for at least two weeks when used continuously. Trypsin auto-digestion fragments, sample carry-over and loss of signal due to adsorption of the protein were not observed.

On-line digestion without prior protein denaturation, followed by micro-LC separation and photodiode array detection, was tested with horse-heart cytochrome C and horse skeletal-muscle myoglobin. The complete digestion of 20 pmol μL^{-1} horse cytochrome C was observed when the average residence time of the protein sample in a 140 cm x 50 µm capillary IMER was 165 s. Mass spectrometric identification of the injected protein on the basis of the tryptic peptides proved possible. Protein digestion was favourable with respect to reaction time and fragments formed when compared with other on- and off-line procedures. These results and the easy preparation of this micro-reactor provide possibilities for miniaturised enzyme-reactors for on-line peptide mapping and inhibitor screening.

4.1. Introduction

A demand for smaller enzyme reactors emerged in recent years, as a consequence of the ongoing miniaturisation in biochemical and analytical sciences. These micro-reactors have been used in biocatalysis and biosensing. In the field of proteomics, the reactors are a tool in peptide mapping, in which proteins are identified via peptide fragment identification after proteolysis. Currently, in spite of its limitations, most of these analyses are conducted by means of 2D gel electrophoresis followed by digestion of the proteins, liquid chromatographic (LC) separation and mass spectrometric (MS) identification of the peptides [1-3]. The most time-consuming step in this procedure is the protein digestion using a protease. In general, every protein to be investigated is individually incubated with the protease in a concentration of approximately 1 - 2% protein weight for 2 to 18 h at an elevated temperature (typically 37 °C). In addition to the long incubation time needed, a certain level of auto-digestion of the protease can be expected. To reduce sample handling, digestion time and the risk of sample contamination, methods for the on-line digestion of proteins have been developed that use proteases immobilised on a solid support.

Immobilised enzyme reactors have been developed and used over the years for several industrial and analytical purposes [4-6]. An obvious benefit for immobilising biocatalysts is the fact that the enzyme can be used in several catalytic cycles and that both catalyst and reaction mixture can easily be separated. Moreover, immobilised enzymes generally show an improved stability even at more extreme reaction conditions. Several procedures have been developed for the immobilisation of enzymes, e.g. by adsorption or encapsulation in a matrix or membrane. Alternatively, and more often used, is the covalent attachment of biocatalysts to carrier materials, which allows the immobilisation of a large amount of enzyme for a high activity per surface area. Generally, particulate large-pore carrier materials are used, such as controlled-pore glass (CPG) [7, 8], silica [9] or polymers like the commercially available poroszyme [10-12]. Current research in the production of immobilised enzymes is focussed on the use of monolithic materials as they enable efficient

fragmentation of proteins [13-17]. Although both commercially available and self-prepared reversed-phase capillary monolithic columns have shown to successfully pass the reproducibility assessment [18, 19], the synthesis of monoliths, suited for small scale enzyme reactors, can still be troublesome. Materials suited for the fabrication of larger-scale enzyme reactors, are commercially available from BIA Separations (Ljubljana, Slovenia).

Although it is possible to apply an immobilised enzyme reactor (IMER) positioned after the separation column [20], most papers dealing with on-line digestion of protein-samples position the IMER upstream of the separation column. In these cases the sample is first digested and the resulting peptide fragments are separated and identified by LC-MS. This approach is often employed in multi-dimensional LC methods [17, 21, 22], and has also found application in peptide-mapping using capillary electrophoresis [23, 24]. Alternatively, as recently shown by Zhao *et al.* [25] and Krenkova *et al.* [26] who covalently coupled trypsin to the wall of fused-silica nanoelectrospray emitters, a protein sample can be analyzed by direct infusion into a mass spectrometer.

The present paper describes the development of trypsin-modified open-tubular micro-reactors. The chemistry was controlled and optimised using surface plasmon resonance (SPR), a technique allowing sensitive and real-time monitoring of surface reactions such as protein binding [27]. The surface modification resulting in the highest enzyme immobilisation yield, was used to covalently immobilise the trypsin on the inside wall of a fused-silica capillary. The constructed trypsin micro-reactor, which is compatible with micro- and nano-LC, was further characterised. The influence of reaction time, pH, temperature and reactor stability were investigated with the model substrate insulin B-chain. The reactor was also applied to the digestion of the proteins cytochrome C and myoglobin. The produced peptides were analysed with liquid chromatography-mass spectrometry.

4.2. Experimental

4.2.1. Materials

The SPR equipment used was from IBIS Technologies (at present available from Eco Chemie, Utrecht, The Netherlands) equipped with a 200-µL polycarbonate cuvet. The gold-sensor disks, purchased from SSENS (Hengelo, The Netherlands), were positioned on the IBIS-prism using index-matching oil from R.P. Cargille Laboratories Inc (Cedar Grove, USA). PEEK nuts, unions, tubing and loops were from Upchurch Inc (Santa Monica, USA). Manual injections during the preparation of the reactors were performed using a Rheodyne 7010 injector (Inacom, Veenendaal, The Netherlands) equipped with a 1-mL PEEK loop. The model 10AD*vp* HPLC pumps from Shimadzu (Kyoto, Japan) were used for reactor preparation and activity determinations. The water used for washing and to prepare buffers was produced by a Sartorius Arium 611 ultrapure water system (Nieuwegein, The Netherlands, conductivity > 18.2 MΩ cm). The model ABS759A UV absorbance detector was equipped with a capillary flow-cell (75-µm i.d.) and was obtained from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands).

On-line digestion experiments with micro-HPLC separations were conducted using LC-Packings instruments and columns (Amsterdam, The Netherlands). The equipment consisted of an injector (Famos), nanovalve column switcher (Switchos), nanopump (Ultimate) and a photodiode array detector (PDA) equipped with a micro flow cell (45 nL). The reversed-phase pre-columns were 5x0.3 mm with 5-µm 100 Å C18 particles. The 150x0.3 mm reversed-phase micro-column contained 3-µm 100 Å C18 PepMap particles. The mass spectrometer was an Agilent LC/MSD XCT ion trap (Amstelveen, Netherlands).

Acetic acid, boric acid, calcium chloride ($CaCl_2$), ethanol, ethanolamine (EA), hydrochloric acid (HCl), hydrogen peroxide, sodium dihydrogen phosphate, sodium chloride, sodium hydroxide (NaOH), and sulphuric acid were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile and

ethanol were from Biosolve (Valkenswaard, The Netherlands). 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), tris-hydroxymethyl-aminoethane (TRIS), urea and acetone were purchased from Acros Organics (Geel, Belgium). Benzamidin, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), benzoyl-arginine ethyl ester (BAEE), iodoacetamide, *N*-hydroxysuccinimide (NHS) and carboxyl-modified dextran (CMD) were purchased from Fluka (Buchs, Switzerland). Horse-heart cytochrome C, insulin B (oxidised), polyoxyethylenesorbitan monolaurate (Tween 20) and porcine pancreas trypsin were purchased from Sigma (St Louis, USA). The specific activity of trypsin was determined according to the method of Schwert and Takenaka [28] using BAEE as substrate and turned out to be 14700 U mg^{-1}. Aminopropyltriethoxysilane (APTES), carbonyl diimidazole (CDI), glycidoxy-propyltrimethoxysilane (GOPS) and mercaptoethanol (ME) were from Aldrich Chemical Company (Milwaukee, USA). Amino-modified dextran (AMD) was from Unavera ChemLab (Mittenwald, Germany). The standard polyimide coated fused-silica capillaries (50 µm and 75 µm ID, 150 µm OD) were purchased via Bester (Amstelveen, Netherlands). Piranha solution was prepared by mixing 1 part of 30% hydrogen peroxide and 6 parts of concentrated sulphuric acid. TRIS digestion buffer consisted of 50 mM TRIS and 1 mM $CaCl_2$ set to pH 8.2 using a 1 M HCl solution.

4.2.2. Preparation of dextran-coated SPR sensor disks

The SPR sensor disks were extensively cleaned in a freshly prepared piranha solution. After 1 h the disks were thoroughly rinsed with water, dried in a stream of nitrogen gas and immediately incubated for 6 h in a 10 mM solution of ME in ethanol in order to produce a self-assembled monolayer (SAM) containing hydroxyl functionalities. After SAM formation, the disks were washed with ethanol and water, and dried with nitrogen gas. The resulting ME disks were incubated for 1 h with a 10% GOPS solution in 98% ethanol after which the disks were washed with ethanol, dried with nitrogen gas and stored overnight at 50 °C.

Dextran-modified sensors were prepared by incubating GOPS-modified sensor disks for 20 h at room temperature with a 10% AMD solution in a 50 mM borate buffer pH 9.5. After removing the solution the disks were washed with water, ethanol and water, dried in a gentle flow of nitrogen gas and stored at room temperature in a closed-off box until use. Alternatively, ME disks were incubated for 1 h with a 10% APTES solution in acetone. After washing, drying and storing overnight in the oven, similar to the GOPS disks, the surfaces were incubated for 1 h with a solution containing 5% CMD in water containing 200 mM EDC and 50 mM NHS. After removing the solution, the disks were washed with water and ethanol, and incubated for 15 min in a solution containing 100 mM CDI in acetone. These activated surfaces were washed with ethanol, blown to dryness with nitrogen and incubated overnight with a solution of 10% AMD in water. After removing the solution the disks were washed with water, ethanol and water, dried in a gentle flow of nitrogen gas and stored at room temperature in a closed-off box until use.

4.2.3. SPR experiments

The GOPS-modified SPR sensor disks were incubated overnight with 200 µL of a solution containing 2.5 mg mL^{-1} trypsin and 50 µg mL^{-1} benzamidin in 50 mM borate buffer pH 9.5 (see Figure 1A). Both types of dextran-modified sensor-disks were activated for 60 min with 200 µL 100 mM CDI in dry acetone. After washing the surface with water to remove the last traces of acetone, the sensors were incubated overnight with 200 µL 2.5 mg mL^{-1} trypsin and 50 µg mL^{-1} benzamidin in 50 mM borate buffer pH 8.5 (see Figure 1B and 1C, resp.). The remaining esters were inactivated by incubating the disks for 10 min with 1 M EA in 50 mM borate buffer pH 8.5. The successive steps in the immobilisation were monitored with SPR and the amount of covalently coupled enzyme was calculated from the recorded angle shift. During the experiments the SPR system was thermostatted at 25.0 °C.

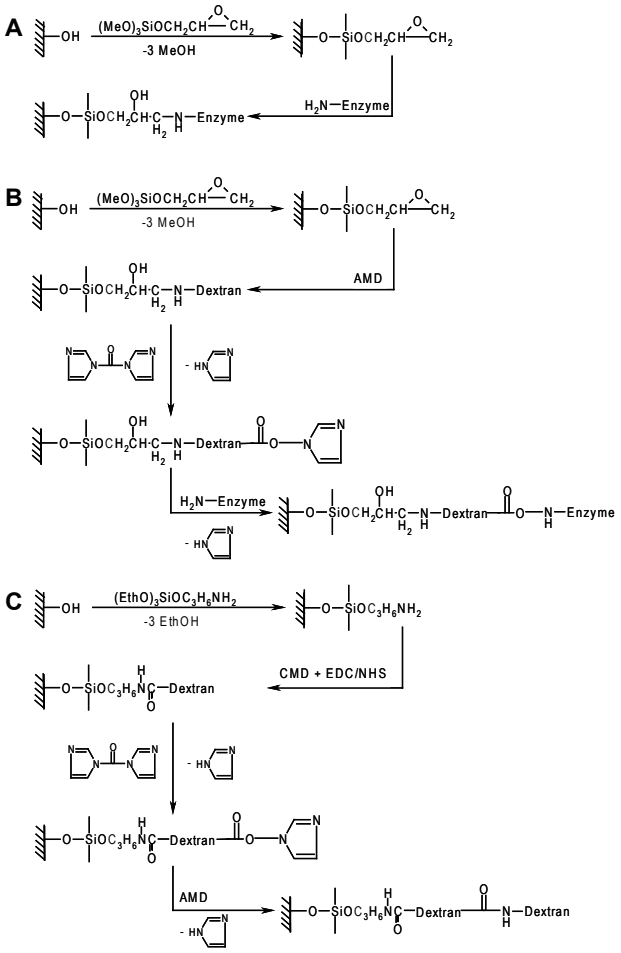

Figure 1: Surface modifications used to immobilise the enzyme in fused-silica capillaries. A: GOPS-modified; B: GOPS/AMD-modified; C: APTES/CMD/AMD-modified surface. In the latter case the CDI activation and enzyme (H_2N-enzyme) coupling are as in Figure 1B.

4.2.4. Preparation of dextran-coated fused-silica capillaries

In order to generate a proper surface for silanisation, fused-silica capillaries were cleaned for 30 min with a 2 M NaOH solution at a flow-rate of 5 µL min^{-1}. Then the capillary was washed for 30 min with 0.1 M HCl, for 5 min with water and finally for 5 min with ethanol. In order to prepare dextran-coated capillaries, the capillaries were flushed for 60 min with a 10% GOPS solution in ethanol. After this step, the capillaries were closed off with silicon plugs and dried overnight at 50°C. After silanisation, the capillaries were

flushed with methanol at a flow-rate of 10 µL min^{-1} after which the capillaries were chemically modified in flow, using injections with a Rheodyne 7010 manual injector equipped with a 1-mL PEEK loop, in a way similar as described above in the SPR section. Washing steps were conducted at flow-rates of 10 µL min^{-1}, overnight incubations at a flow rate of 1 µL min^{-1}. All chemistries are outlined in Figure 1.

4.2.5. Enzyme activity determination

Insulin B chain was used as a substrate to determine the activity of trypsin. Injections of 1 µL of a concentration of 20 µmol l^{-1} in digestion buffer were introduced into the reactor that was kept at the indicated temperature using a water bath. To determine the enzyme activity in solution, trypsin was incubated in these solutions as well (trypsin end concentration 0.4 µg mL^{-1}). At time intervals from 0.5 to 30 min, 100 µL samples were taken and the activity was stopped by the addition of 5 µL 20% TFA in water. Buffers that were used to determine the effect of pH on activity were MES (pH 5.5 to 6.5), MOPS (pH 6.5 to 7.8), TRIS (pH 7.5 to 9) and CHES (pH 8.6 to 10.1). The buffers were set to pH using a 1 M NaOH solution and were prepared at a 50 mM concentration, also containing 5 mM $CaCl_2$. To determine the effect of temperature, the temperature during incubation was varied between 10 °C and 60 °C. Both the on-line and off-line samples were analysed using micro-HPLC with PDA detection as outlined below. The insulin B conversion of both immobilised trypsin and the enzyme in solution was calculated from the peak areas of substrate and products.

4.2.6. On-line protein digestion in micro-HPLC

For the on-line peptide and protein digestion experiments, the trypsin-modified capillaries were positioned between the LC-injector and a 10-port valve as shown in Figure 2 and were immersed in a thermostatted water bath set at 37 °C unless mentioned otherwise. Protein samples were prepared in digestion buffer and were transported through the capillary towards a 10-port

valve using a 5% acetonitrile solution containing 0.05% TFA. The peptide fragments formed during digestion were concentrated on a RP-trapping column (TC) and salts and other buffer components present in the sample were removed. By switching the valve, the trapping column is in series with the RP-micro column and an acetonitrile gradient started. The gradient was composed of two solutions: (A) 5% acetonitrile in water containing 0.05% TFA and (B) 80% acetonitrile in water containing 0.04% TFA. In 30 min the gradient changed linearly from 0 to 50% B, followed by 10 min at 90% B and 20 min at 0% B. The eluent was monitored with the PDA detector in the range from 200 to 595 nm.

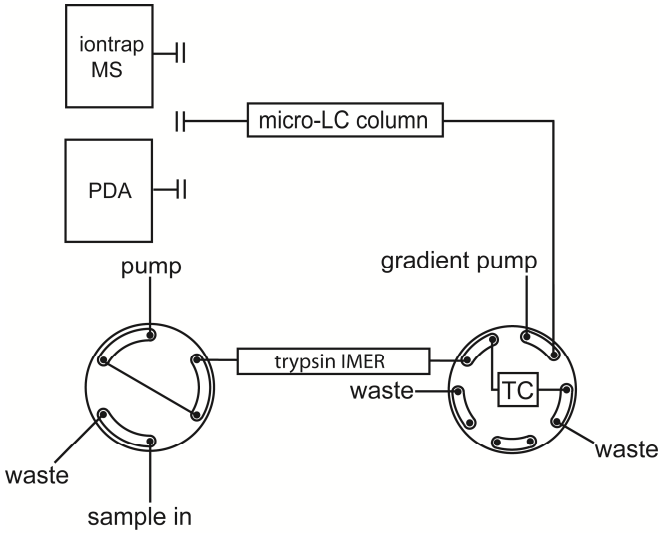

Figure 2: Setup used for on-line protein digestion using a trypsin-modified fused-silica capillary. For detection a PDA or an ion-trap MS has been used.

ESI-MS was conducted in the positive ion-mode with the capillary voltage set at 3500V. The flow rate and temperature of the nitrogen drying gas were 5 L min^{-1} and 325 °C, respectively. The sequence of the peptide fragments was determined by using the mass spectrometer in auto-MS/MS mode fragmenting the two peptides that were most abundantly present when the signal reached threshold. The MS/MS result was analysed by a Mascot database search (www.matrixscience.com).

4.3. Results and discussion

4.3.1. Surface chemistry and enzyme immobilisation

As shown before [29], the modification of a SPR sensor surface with a dextran hydrogel leads to less non-specific adsorption of proteins compared to unmodified surfaces. The presence of such a layer also enhances the immobilisation capacity of biomolecules compared to monolayer based coatings. Due to the flexible nature of the dextran chains, the accessibility is often improved as well compared to molecules immobilised on a flat surface. As the amount of protein present in a capillary after immobilisation cannot be determined easily, SPR sensors were used as a model to investigate the effect of the different surface modifications on the amount of trypsin that could be attached covalently. Hydroxyl functionalities necessary to enable silanisation are introduced using mercaptoethanol (ME), but all other surface modifications are carried out in exactly the same way, both on the SPR sensor surfaces and in the fused-silica capillaries.

Trypsin immobilisation after silanisation with GOPS by the reaction of the trypsin primary amines and the glycidyl function of GOPS results in an SPR angle shift of 350 ± 20 m° (n=3), which equals an amount of trypsin of 2.9 ± 0.2 ng mm^{-2} covalently attached to the surface, which is close to monolayer coverage of trypsin. When the GOPS-silanised surface is modified with AMD, resulting in a dextran hydrogel, the amount of trypsin that can be immobilised after CDI activation increases to 9.5 ± 0.3 ng mm^{-2} (n=3). To further increase the amount of trypsin, an intermediate dextran layer was added. Therefore, fused-silica capillaries were APTES silanised and modified with CMD and AMD, subsequently. The amount of trypsin that could covalently be attached to these layers was determined with SPR and proved to be 11.1 ± 0.5 ng mm^{-2} (n=2). As this amount is significantly more than obtained with the GOPS/AMD-surface, all further experiments have been conducted with fused-silica capillaries of which the surface is modified with dextran in this way.

Assuming that the investigated surface modification allows a similar amount of trypsin to be immobilised per surface area in fused-silica capillaries, the quantity of enzyme immobilised on the dextran-modified capillaries is more than five times larger per surface area compared to other open-tubular, trypsin micro-reactors described in literature [24, 30-32]. These reactors, first described by Amankwa and Kuhr [32], are based on immobilised avidin and at saturation, 6.5 pmol biotinylated trypsin was immobilised in a 50-cm long capillary with a 50-µm i.d.. This amount equals 2 ng trypsin mm^{-2} of capillary surface, which is less than monolayer coverage.

4.3.2. Characterisation of the enzyme reactor

Fused-silica capillaries were modified with dextran as described above and the activity and characteriststics of the trypsin micro-reactor were assessed with the oligopeptide insulin B chain, a 30 amino acids long insulin fragment. When insulin B is exposed to the enzyme present in the reactor, hydrolysis at the arginin present on position 22 and, to a lesser extent, at the lysine on position 29 is observed. Using the aforementioned substrate insulin B, the pH optimum was determined for the enzyme in solution and the immobilised enzyme. As can be observed in Figure 3A, the optimum pH values for both free and immobilised trypsin are pH 8.5. Therefore, all further experiments are conducted at this pH value.

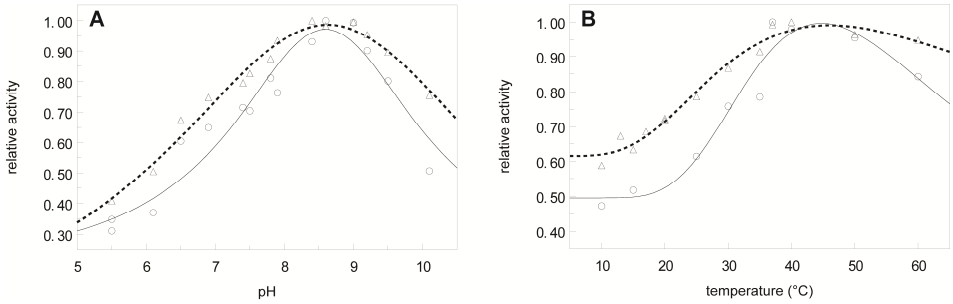

Figure 3: The effect of (A) pH and (B) temperature on the relative activities of (○) trypsin in solution and(Δ) trypsin immobilised on an APTES/CMD/AMD-modified fused-silica surface. The maximum insulin B conversion rate determined under experimental conditions is 4.41±0.02 pmol min^{-1} $µg^{-1}$ for the enzyme in solution and 12.32±0.35 pmol for a 3 µL, 50 µm i.d. microreactor operated at a flow rate of 1 µL min^{-1}.

Also the effect of temperature on the activity of both the immobilised trypsin and the enzyme in solution was determined in the range from 10 °C to 60 °C. As can be observed in Figure 3B, the activity of trypsin increases with temperature to reach a maximum around 40 °C and decreases at higher temperatures. The immobilised enzyme shows a higher activity compared to the enzyme in solution, which may be due to an often observed higher stability of immobilised enzymes, also under more extreme conditions. From the data the activation energy can be determined. For the immobilised enzyme a value of 12.2 ± 0.3 kJ mol^{-1} can be calculated and for the enzyme in solution a value of 17.2±0.5 kJ mol^{-1}. This means that for the immobilised enzyme the temperature has less effect on the activity than for the enzyme in solution. As the highest activity is observed at 37 °C, all further experiments are conducted at that temperature.

A specific activity is determined of 4.41 ± 0.02 pmol min^{-1} μg^{-1} at a concentration of 20 pmol μL^{-1} insulin B and pH 8.5 and 37°C for the enzyme in solution. Under similar conditions, in a microreactor of 3 µL with a 50 µm i.d. and operated at a flow rate of 1 µL min^{-1}, an amount of 12.32 ± 0.35 pmol insulin B is converted. In such a reactor a total of 2.67 µg trypsin might be present taking the earlier SPR observations into account, which means that the immobilised enzyme is both accessible and still active after immobilisation. The specific activity after immobilisation is higher than observed in recent papers for the protease pepsin immobilised on beaded chitosan [33, 34] and trypsin photoimmobilised in a fused-silica capillary [34].

The stability during operation of the open-tubular reactor was tested at 37°C and at pH 8.5. Activity tests using the model substrate show that the activity of a reactor that is continuously in operation is constant for at least two weeks. The enzyme trypsin dissolved in digestion buffer and incubated for 24 h at 37°C looses 60% of its activity, and after three days no activity remains.

4.3.3. On-line digestion of proteins

The on-line digestion of horse cytochrome C is accomplished as described in the Methods section. Samples are submitted to on-line digestion in a 22-cm long trypsin-modified APTES/CMD/AMD coated capillary with an i.d. of 75 µm (total volume 1 µL) or with an i.d. of 50 µm and a length of 51 cm (1 µL) or 140 cm (2.75 µL). When the protein is not reduced, the heme-moiety will remain covalently attached to the peptides containing the protein sulfhydryls. By monitoring the heme containing peptides at 395 nm, the progress of the digestion and the amount of undigested protein can be determined. The results are summarised in Table 1.

Table 1: Summary of the on-line digestion experiments for cytochrome C. The experiments were conducted at pH 8.5 and 37°C (n=3).

capillary ID (µm)	reactor volume (µL)	amount injected (µg mL^{-1})	(pmol)	digestion time (s) (s)	% undigested protein (SD)
75	1	248	20	60	65.9 (4.8)
75	1	124	10	60	35.8 (3.4)
75	1	12.4	10	60	23.6 (1.3)
50	1	248	20	60	14.0 (1.9)
50	1	124	10	60	2.3 (2.0)
50	2.75	248	20	165	0
50	2.75	124	10	165	0
50	2.75	12.4	10	165	0

The experiments were conducted at pH 8.5 and 37 °C (n=3)

As expected when a limited amount of enzyme activity is present in a reactor (22 cm x 75 µm), for increasing concentrations cytochrome C a larger amount of protein is undigested. Nevertheless, still many tryptic peptides are generated. As can be expected, an increase in exposure time of the substrate with the immobilised enzyme will result in an improved digestion yield. A longer contact time is achieved by increasing the reactor volume by using a longer enzyme-modified capillary. Additionally, the enzyme-to-substrate ratio is increased which is accomplished by changing the surface-to-volume ratio by using a capillary with a smaller internal diameter. As can be observed in Table 1, decreasing the i.d. of the capillary leads to an improved digestion for a

reactor of equal volume due to a higher surface-to-volume ratio and hence a higher amount of enzyme. The use of a 140 cm x 50 µm capillary allows the complete digestion of up to 20 pmol (248 µg mL^{-1}) of cytochrome C in less than 5 min including the sample concentration and removal of salts by the trapping column.

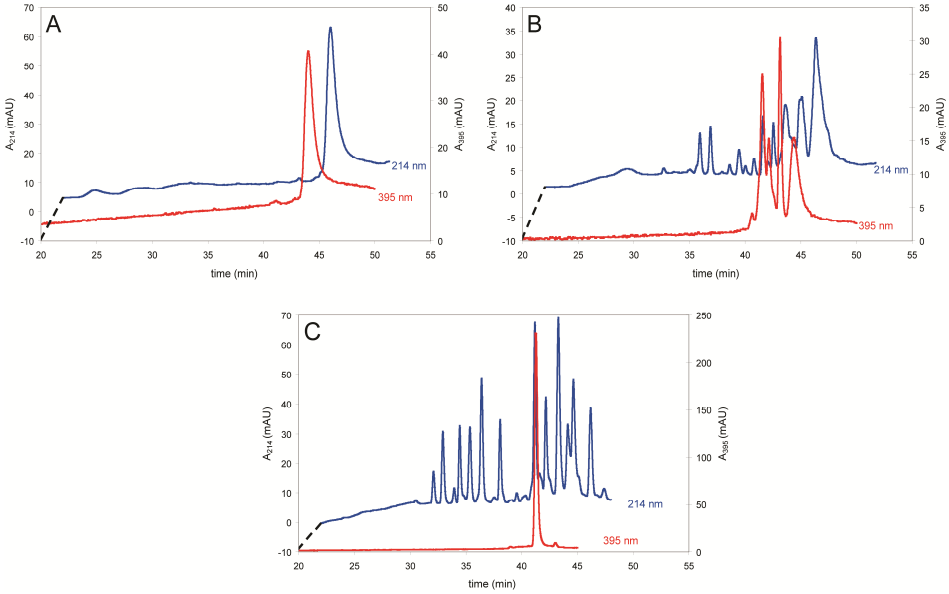

Figure 4: Chromatograms of the injection of 10 pmol horse cytochrome C in capillary digestion systems monitored at 214 nm and 395 nm. The experiments were conducted with an APTES-CMD-AMD derivatised fused-silica capillary of (A) 510x0.050 mm not containing trypsin operated at 1 µL min^{-1} (blank); (B) 1400x0.050 mm, trypsin-modified operated at 5 µL min^{-1} (average sample residence time 33 s); (C) as (B) but operated at 1 µL min^{-1} (average sample residence time 165 s). For clarity the beginning of the chromatogram displaying the 214 nm signal is offset as indicated.

Chromatograms for the on-line digestions obtained with capillaries with an i.d. of 50 µm are shown in Figure 4. Figure 4A shows a blank run in a capillary containing no enzyme and Figures 4B and 4C present on-line digestions for a micro-reactor of 2.75 µL operated at 5 µL min^{-1} and 1 µL min^{-1}, respectively. As discussed above, an incomplete digestion of the injected cytochrome C will lead to the presence of multiple peptide-fragments containing the heme group as visible in Figure 4B. The digestion is complete when the sample exposure time is 165 s (flow rate 1 µL min^{-1}) as both intermediate products and the undigested protein (retention time 44 min in Figure 4A),

which are visible at a wavelength of 395 nm as outlined above, are no longer observed. An injection of off-line digested cytochrome C showed a similar chromatogram as is shown in Figure 4C.

Table 2: General overview of the on-line digestion experiments for cytochrome C (n=3).

flow rate (µL min^{-1})	digestion time (s)	sequence coverage (%)	total no peptides matched	Mascot score	undigested protein (%)
10	17	66	11	347	60.3
5	33	66	21	501	31.8
2	83	66	23	649	18.5
1	165	87	27	833	0

The on-line digestion of horse cytochrome C is also monitored with mass spectrometry. The effect of flow rate and hence incubation time on the digestion of the protein and the number of peptides identified with a Mascot database search is determined and summarised in Table 2. In this table the undigested amounts of protein which have been determined using the PDA detector are also shown. At a flow rate of 1 µL min^{-1} the protein digestion is complete and many peptides are matched resulting in high sequence coverage and Mascot score. With higher flow, and hence decreasing incubation time, the amount of protein that remains undigested increases and consequently less peptides are produced and observed. However, even at relatively high flow rates still an adequate amount of peptides is formed and the protein can be identified on basis of the fragments present. Nevertheless, the ion intensity is low and some peptides are not retrieved as they are below the threshold for auto-MS/MS. Table 3 summarises the peptides observed and matched using the MS/MS-data and a database search of proteins digested at a flow rate of 1 µL min^{-1}. A base-peak chromatogram (BPC) of the digestion of cytochrome C under these conditions is shown in Figure 5A. The MS/MS fragmentation of one of the peptides is presented in Figure 5B.

In further experiments horse myoglobin was digested on-line. This protein is generally regarded difficult to digest [15, 35]. When 1 µL of a 10 µmol L^{-1} solution in buffer is injected at a flow rate of 1 µL min^{-1} (165 s exposure time), the injected protein is completely digested as was observed with UV detection

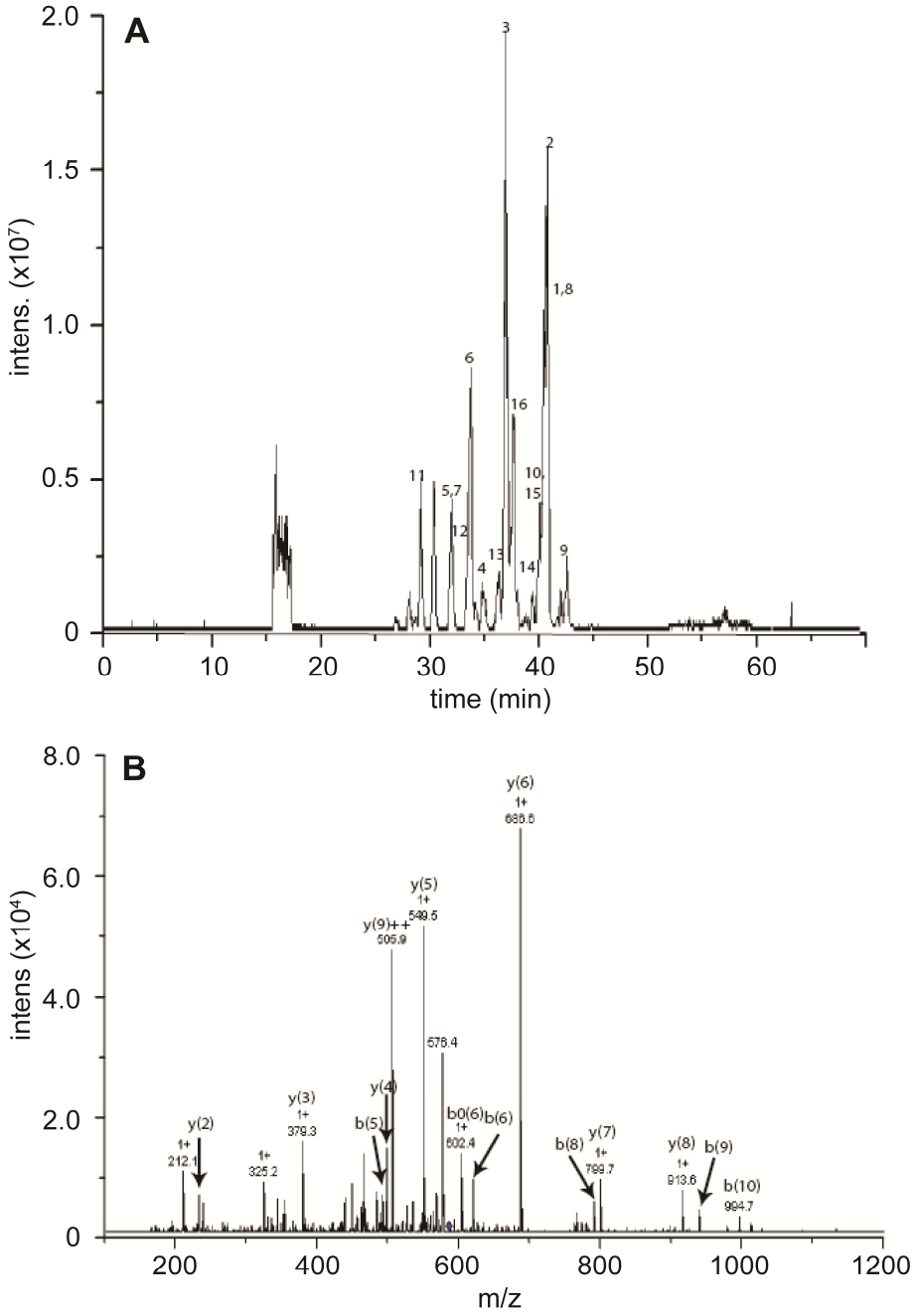

Figure 5: On-line digestion of 10 pmol cytochrome C at a flow rate of 1 µL min^{-1}: (A) Base Peak Chromatogram, the numbers correspond with the matched peptides in Table 3, and (B) MS/MS fragmentation of the peptide TGPNLHGLFGR with m/z 584.9 showing several of the matched fragment ions.

(data not shown). Using mass spectrometric analysis, 13 different peptides are observed and matched using the MS/MS-data and a Mascot database search, resulting in a sequence coverage of 88%. The matched peptides are summarised in Table 3 and a BPC of the on-line digestion of myoglobin is shown in Figure 6A and a MS/MS spectrum of one of the tryptic peptides. Both the degree of digestion and the sequence coverage are adequate compared to other systems that often use a high percentage of modifier to enhance digestion as the absence of denaturating agents during digestion leads to little or no digestion of myoglobin [35]. Therefore these reactors are generally used for direct infusion into MS or off-line protein digestion as the presence of high concentrations of methanol or acetonitrile in the digestion buffer will seriously impede on-line protein digestion in combination with RP-LC.

Table 3: Peptide fragments observed with MS in the on-line digestion of cytochrome C and myoglobin The experiments were conducted at pH 8.5 and 37°C at a flow rate of 1 µL min^{-1} using a 2.75 µL trypsin reactor.

Peptide sequence	Theoretical m/z	Experimental m/z	Position	Missed cleavage
Cytochrome C (sequence coverage 87%)				
1....IFVQKCAQCHTVEK	1632.8	816.9 (2+)	9–22	1
2....HKTGPNLHGLFGR	1432.8	717.1 (2+)	26–38	1
3....TGPNLHGLFGR	1167.6	584.6 (2+)	28–38	0
4....TGPNLHGLFGRK	1295.7	648.6 (2+)	28–39	1
5....KTGQAPGFTYTDANK	1597.8	533.4 (3+)	39–53	1
6....TGQAPGFTYTDANK	1469.7	735.6 (2+)	40–53	0
7....TGQAPGFTYTDANKNK	1711.8	571.4 (3+)	40–55	1
8....GITWKEETLMEYLENPKK	2208.1	736.8 (3+)	56–73	2
9....EETLMEYLENPK	1494.7	748.0 (2+)	61–72	0
10..EETLMEYLENPKK	1622.8	812.1 (2+)	61–73	1
11..YIPGTK	678.4	678.1 (1+)	74–79	0
12..MIFAGIKK	906.5	454.0 (2+)	80–87	1
13..KTEREDLIAYLKK	1477.8	739.6 (2+)	88–99	2
14..TEREDLIAYLKK	1349.7	675.5 (2+)	89–99	1
15..EDLIAYLK	963.5	482.6 (2+)	92–99	0
16..EDLIAYLKK	1091.6	546.6 (2+)	92–100	1
Myoglobin (sequence coverage 88%)				
1....GLSDGEWQQVLNVWGK	1814.9	908.7 (2+)	1–16	0
2....VEADIAGHGQEVLIR	1605.8	803.6 (2+)	17–31	0
3....VEADIAGHGQEVLIR	1605.8	536.1 (3+)	17–31	0
4....LFTGHPETLEK	1270.7	636.0 (2+)	32–42	0
5....HLKTEAEMK	1085.6	543.5 (2+)	48–56	1
6....HGTVVLTALGGILK	1377.8	689.7 (2+)	64–77	0
7....HGTVVLTALGGILKK	1505.9	502.7 (3+)	64–78	1
8....KKGHHEAELKPLAQSHATK	2109.1	703.7 (3+)	78–96	2
9....KGHHEAELKPLAQSHATK	1981.0	661.1 (3+)	79–96	1
10..GHHEAELKPLAQSHATK	1853.0	618.8 (3+)	80–96	0
11..YLEFISDAIIHVLHSK	1884.0	628.8 (3+)	103–118	0
12..HPGNFGADAQGAMTK	1500.7	751.5 (2+)	119–133	0
13..ALELFRNDIAAK	1359.8	680.6 (2+)	134–145	1
14..YKELGFQG	940.5	471.0 (2+)	146–153	1

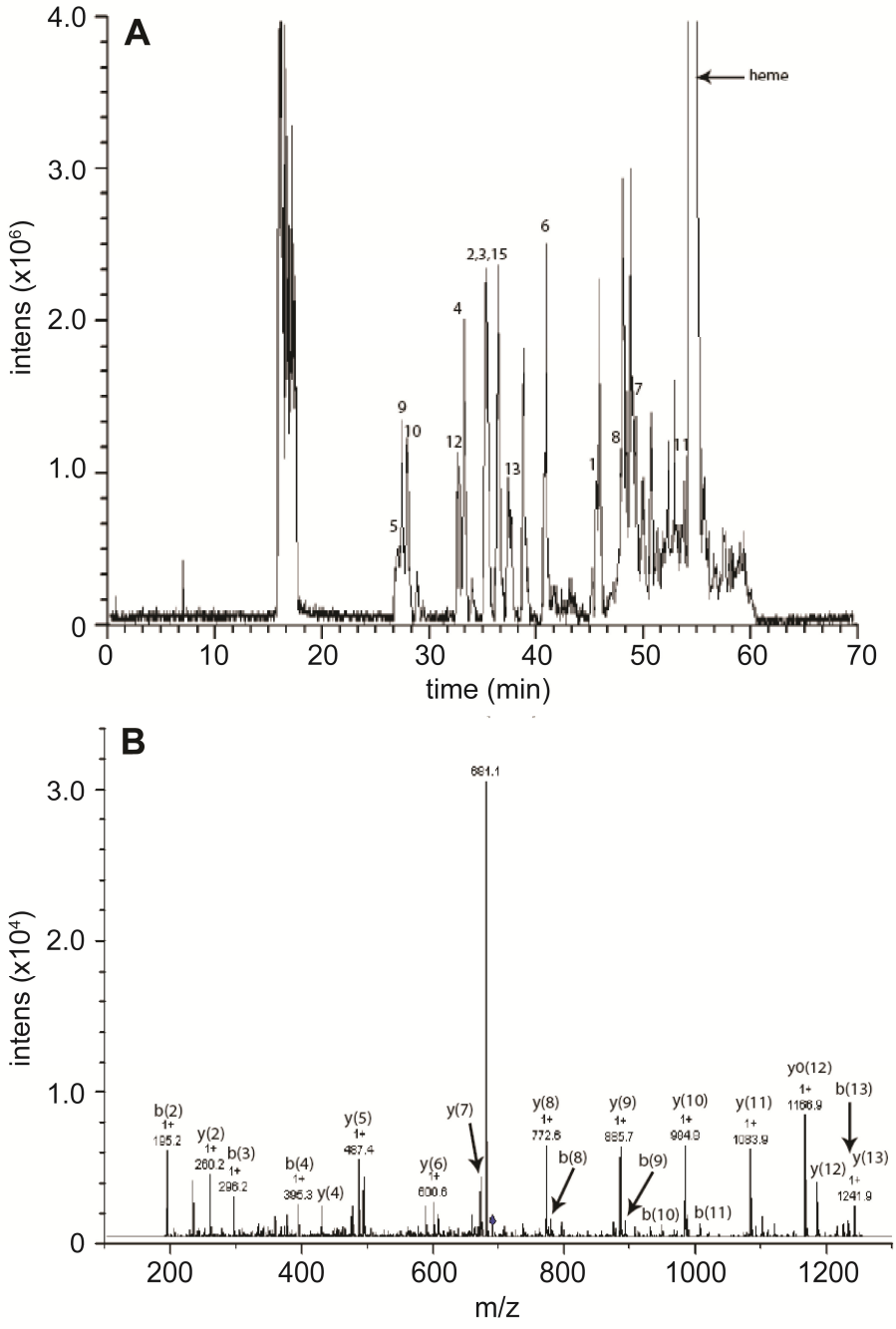

Figure 6: Results of the on-line digestion of 10 pmol myoglobin at a flow rate of 1 µL min^{-1}: (A) Base Peak Chromatogram, the numbers correspond with the matched peptides in Table 3, and (B) the MS/MS spectrum of the peptide HGTVVLTALGGILK with m/z 689.7.

Amankwa and Kuhr [32] reported that proteins and peptides adsorb to the capillary surface of their trypsin reactor. Reactor fouling, and as a result sample carry-over, is also observed when trypsin-modified particulate polymers or monoliths are used [36]. Adsorption of proteins and peptides to these relatively hydrophobic columns is prevented by the addition of up to 20% methanol to the digestion buffer [10, 36]. For dextran-coated surfaces, it is known that the non-specific adsorption of protein is minimal and depends on the character of the oligosaccharide layer [29, 37, 38]. During protein digestion experiments on SPR sensor surfaces that are modified identical to the capillary reactors no adsorption during incubation is observed (data not shown). Similarly, during on-line digestion using the dextran coated capillaries, sample adsorption and carry-over is not observed as repeated sample injections result in identical chromatograms and blank buffer injections do not show peptide fragments. On basis of the latter result and the long lifetime of the reactor, trypsin auto-digestion is probably absent.

The developed IMER has been applied for the digestion of horse cytochrome C and myoglobin in buffer without prior pre-treatment followed by separation and MS/MS sequencing. The time needed to obtain complete protein digestion and sample trapping is 5 min with an average sample residence time of 165 s. The total analysis time, digestion and LC-separation, is 65 min. The developed micro-reactor is competitive when compared with other systems used for on-line protein digestion [16, 17, 39-41]. In the capillary IMERs described by Amankwa and Kuhr [32] and Bossi *et al.* [34] the complete digestion of native proteins was only obtained when the protein resided in the trypsin-coated capillary for 15-25 min. The peptide separation was carried out with CE in these latter cases.

Many reactors use a protein denaturation step to make the protein more susceptible to proteolytic action. The digestion efficiency can be enhanced by partial denaturation through the addition of 35-45% acetonitrile to the buffer [11, 15, 35, 36] or by sonication [42, 43]. However, the latter has not yet been used in an automated setup and the presence of high amounts of modifier will

make reversed-phase chromatography in a subsequent step for the separation of the fragments produced during protein digestion very difficult. The described reactors were efficient in terms of time needed for digestion and the digestion result, but were as a rule used for direct infusion into the MS or used for off-line digestion.

Alternatively, protein denaturation is accomplished using chemicals such as SDS, guanidine HCl or urea. A recent example is the miniaturised on-line proteolysis-capillary LC system as described by Samskog *et al.*,[10] who employed a 10-µL column packed with poroszyme. The sample contained guanidine·HCl for protein denaturation, and was diluted with a buffer, also containing methanol, prior to injection. The total time needed for digestion, trapping and the removal of the high concentration of salt was 15 min. The system needed periodical regeneration to counteract the effects of residual salts in the analyte.

4.4. Conclusions

To study the immobilisation of the protein trypsin in a fused-silica capillary, a number of surface modifications was tested. In order to investigate the amount of protein that can covalently be attached to these surfaces, SPR sensor disks are modified with these coatings to mimick the capillary surface. The SPR measurements show that the best results were obtained using a dextran coating with an intermediate layer. The resulting open-tubular trypsin-reactors having a pH optimum of pH 8.5, display a high activity when operated at 37 °C and are stable for at least two weeks when used continuously.

The capillary reactors show flow-dependent catalysis. For a capillary IMER, the conversion of the insulin B chain increases with decreasing flow and hence a longer residence time. The same is observed for the digestion of horse cytochrome C. The complete digestion of 20 pmol μL^{-1} horse cytochrome C is observed without the need of protein denaturation, reduction or alkylation when the average residence time of the protein sample in a 140 cm x 50 µm

capillary IMER is 165 s. For the proteins used in this study trypsin-reactors were described that were capable of faster digestion using denaturating agents like acetonitrile to enhance the digestion process. However, the presence of such agents would seriously hamper direct analysis of the peptides formed using RPLC and MS. Identification of the proteins cytochrome C and myoglobin is possible by the tryptic peptides that are on-line produced, separated by micro-RPLC and analyzed using mass spectrometry with auto MS/MS.

The open-tubular reactor can be produced easily, reproducibly and inexpensively, and can be used for other applications such as enzyme-inhibitor studies. Future research will focus on the development of miniaturised multi-dimensional analysis systems employing on-line digestion using these IMERs. The capillary enzyme reactors show no backpressure and seem promising for coupling to other analytical techniques such as capillary electrophoresis and surface plasmon resonance.

4.5. Literature

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5.

Pepsin immobilised in dextran-modified fused-silica capillaries for on-line protein digestion and peptide mapping

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Abstract

On-line protein digestion of proteins under acidic conditions was studied using micro-reactors consisting of dextran-modified fused-silica capillaries with covalently immobilised pepsin. The proteins used in this study differed in molecular weight, isoelectric point and sample composition. The injected protein samples were completely digested in 3 min and the digest was analysed with micro-HPLC and tandem mass spectrometry (MS/MS). The different proteins present in the samples could be identified with a Mascot database search on basis of auto-MS/MS data. It proved also to be possible to digest and analyse protein mixtures with a sequence coverage of 55 and 97% for the haemoglobin β- and α-chain, respectively, and 35-55% for the various casein variants. Protease auto-digestion, sample carry-over and loss of signal due to adsorption of the injected proteins were not observed. The backpressure of the reactor is low which makes coupling to systems such as Surface Plasmon Resonance biosensors, which do not tolerate too high pressure, possible. The reactor was stable for at least 40 days when used continuously.

5.1. Introduction

In proteomic research an important and unavoidable step in the elucidation of the amino acid sequence of the protein(s) under investigation is a digestion step using a proteolytic enzyme. The traditional digestion protocol is often a time-consuming step as, in general, 2 to 18 h of incubation is necessary for a reasonable amount of peptides to be formed [1-3]. Moreover, since the protease is in solution together with the protein to be digested, a certain amount of auto-digestion can be expected. To eliminate the risk of sample contamination, methods for the (on-line) digestion of proteins have been developed that use proteases immobilised on a solid support, thereby reducing the sample handling as well as the digestion time, and simplifying the separation of the catalyst and solution, containing substrate(s) and product(s). Immobilised enzyme reactors (IMERs) have been used over the years for both industrial and analytical purposes [4-6]. When the enzyme is covalently attached to the carrier surface, the protein's lysine residues are generally coupled to surfaces containing e.g. epoxy- or carbodiimide-activated carboxyl groups. As a support for protease immobilisation, both particulate materials such as controlled-pore glass [7, 8], silica [9] and polymers [10-13], as well as monoliths [14-17] have been used.

The protease, that is generally used, is trypsin, which is preferred for its high selectivity. However, the optimal pH for trypsin activity is slightly basic and for purposes where a low operational pH is a prerequisite, pepsin may be the protease of first choice. This is, for example, the case in H/D exchange experiments which are conducted at acidic condition and rather low temperatures, conditions to which pepsin is relatively tolerant [18-21]. The sample that is digested may also be present in an acidic solution during multi-dimensional separations, for instance, after an immuno-affinity clean-up or after sample fractionation using a cation-exchange column. Finally, due to the prevention of non-specific interaction, capillary electrophoresis (CE) of proteins and peptides in bare fused-silica capillaries is often conducted at acidic

conditions. Therefore, in-capillary digestion prior to CE analysis may be performed at these conditions as well [22].

The present research describes the use of pepsin micro-reactors consisting of pepsin immobilised in a fused-silica capillary. The capillary is first derivatised with a dextran hydrogel to passivate the silica surface. Surfaces with such a hydrogel provide the possibility to immobilise larger amounts of biomolecules and additionally show improved interaction capabilities compared to a flat surface [23, 24]. Due to the small volume of the resulting reactors, they are compatible with micro- and nano-HPLC and can be operated both in buffers and in aqueous solutions containing a modifier. The influence of substrate exposure time on the formation of peptides was investigated. Further, the applicability of the reactor was studied by the digestion and peptide mapping of proteins differing in molecular weight, isoelectric point and post-translational modifications, showing the usefulness of the described system for on-line protein digestion at low pH.

5.2. Experimental

5.2.1. Materials

On-line digestion experiments with micro-HPLC separations were conducted using LC-Packings equipment, tubing, sleeves and columns (Amsterdam, The Netherlands). The equipment consisted of an injector (Famos), nanovalve column switcher (Switchos), nanopump (Ultimate) and a photodiode array (PDA) detector equipped with a micro-flow cell (45 nL). The nanotrap reversed-phase pre-columns were 5x0.3 mm with 5 µm 100 Å PepMap C18 particles. The 150x0.3 mm reversed-phase analytical micro-column contained 3 µm 100 Å PepMap C18 particles. The nanotrap column was connected to the Switchos valve with the provided PEEKsil tubing. The micro-HPLC column was attached directly to the valve, whereas the integrated transfer tube was joined to the PDA using a PTFE sleeve as described by the

supplier. Upon mass spectrometry (MS), the transfer tube was connected to the electrospray needle using the aforementioned sleeve and a PEEK nut to. The XCT ion trap-MS equiped with an electrospray ionisation (ESI) unit was from Agilent (Amstelveen, The Netherlands).

The standard polyimide coated fused-silica capillaries used for the preparation of the micro-reactors (50 µm ID, 150 µm OD) were purchased via Bester (Amstelveen, The Netherlands). Manual injections for the modification of fused-silica capillaries were performed using a Rheodyne 7010 injector obtained from Inacom (Veenendaal, The Netherlands). The model 10AD*vp* HPLC pumps from Shimadzu (Kyoto, Japan) were used for modification and immobilisation procedures. The ultrapure water used for washing and to prepare buffers was produced by a Sartorius Arium 611 UV (Nieuwegein, The Netherlands).

Acetic acid, boric acid, calcium chloride ($CaCl_2$), ethanol, ethanolamine (EA), glycine, hydrochloric acid (HCl), sodium dihydrogen phosphate, sodium chloride, sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile and ethanol were from Biosolve (Valkenswaard, The Netherlands). 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), tris-hydroxymethylaminoethane (TRIS), urea and acetone were purchased from Acros Organics (Geel, Belgium). Horse-heart cytochrome C, horse skeletal-muscle myoglobin, bovine casein sodium salt, bovine serum albumin, human haemoglobin, lactoglobulin, β-lactalbumin A, polyoxyethylenesorbitan monolaurate (Tween 20), and porcine pepsin were purchased from Sigma (St Louis, USA). Recombinant human interferon-γ was from U-Cytech (Utrecht, The Netherlands). Aminopropyl triethoxysilane (APTES), carbonyl diimidazole (CDI), glycidoxypropyltrimethoxysilane (GOPS) and mercaptoethanol (ME) were from Aldrich Chemical Company (Milwaukee, USA). Benzamidine, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), iodoacetamide, *N*-hydroxysuccinimide (NHS) and carboxyl-modified dextran (CMD) were purchased from Fluka (Buchs, Switzerland). Amino-modified dextran (AMD) was from Unavera ChemLab (Mittenwald, Germany).

5.2.2. Preparation of dextran-coated fused-silica capillaries

In order to generate a proper surface for silanisation, fused-silica capillaries with an internal diameter of 50 µm were cleaned at a flow rate of 5 µL min^{-1} with subsequently 2 M NaOH for 30 min, 0.1 M HCl for 30 min, water for 5 min and finally with ethanol for 5 min. To prepare dextran-coated capillaries, the capillaries were incubated for 60 min with a 10% APTES solution in ethanol. After this step, the capillaries were flushed at a flow rate of 5 µL min^{-1} with ethanol for 5 min and dried overnight at 50 °C.

To modify the surface with dextran, the capillaries were incubated for 30 min with a solution of 5% CMD in water also containing 200 mM EDC and 50 mM NHS. After 30 min they were flushed for 5 min at 5 µL min^{-1} with water, filled with a solution of 10% AMD in water and incubated overnight. The resulting dextran-modified capillaries were washed at a flow rate of 5 µL min^{-1} with both water and methanol for 5 min, after which 100 mM CDI in acetone was injected for 30 min. After this activation step, they were flushed with ethanol and water for 5 min. A solution containing 2.5 mg mL^{-1} pepsin in 50 mM formic acid was injected at a flow rate of 2 µL min^{-1} and the enzyme coupling was allowed to proceed for 8 h. The reaction was stopped by injection of 1 M glycin pH 3. Finally, the pepsin micro-reactors were flushed with water and used immediately or stored in a closed box at 4 °C until use. All reactions were conducted at room temperature except for the drying step following APTES incubation. The reactors were 153 cm long (total volume 3 µL). The chemistry is summarised in Fig. 1.

5.2.3. Sample preparation

Sample solutions containing cytochrome C, myoglobin, human interferon-γ, haemoglobin and casein were prepared as 10 µM in digestion buffer consisting of 50 mM glycin pH 1.3. Solutions containing either BSA, ovalbumin or β-lactoglobulin B were prepared in a concentration of 5 mg mL^{-1} in 100 mM ammonium bicarbonate buffer to which DTT was added for reduction and iodoacetamide for alkylation. After removing the excess reagents

by ultrafiltration, the proteins were redissolved in digestion buffer at a concentration of 10 µM.

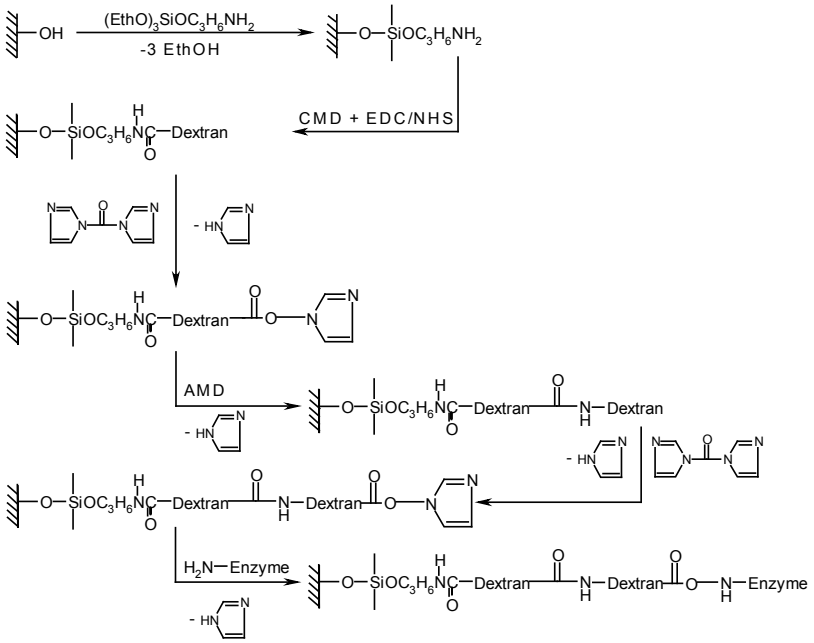

Figure 1: Scheme of the surface modification and enzyme immobilisation in fused-silica capillaries (see Experimental section for exact conditions).

5.2.4. On-line protein digestion in micro-HPLC

For the on-line protein digestion, the protease-modified capillaries with a total reactor volume of 3 µL were positioned between the HPLC-injector and a 10-port valve as shown in Fig. 2. Protein samples were prepared in digestion buffer and transported through the capillary towards a 10-port 2-way valve using a 2% acetonitrile solution containing 0.05% TFA. The flow rate during digestion was 1 µL min^{-1} unless otherwise mentioned, resulting in an average sample residence time of 3 min. The peptide fragments formed during digestion were concentrated on a RP-trapping column (TC). After placing the trapping column in-line with the analytical micro-column a gradient started. The gradient was composed of two solutions: (A) 5% acetonitrile in water containing 0.05% TFA and (B) 80% acetonitrile in water containing 0.04% TFA. In 30 min the

composition changed from 0 to 50% B, followed by 10 min 90% B and a 20-min period at 0% B. The flow rate was 4 µL min^{-1} and the eluent was monitored in the range from 200 to 595 nm with the PDA detector. In peptide-mapping experiments the column exit was connected to an electrospray interface and ESI-MS was conducted in the positive ion mode. The capillary voltage was set at 3500V. The flow rate of heated dry gas was maintained at 6 L min^{-1} (temperature 325°C). The spectrometer was scanned from m/z 400 to 2200. Tandem MS (MS/MS) data was obtained by operating the ion trap in the auto-MS/MS mode. The digestion capillary was kept at 37°C. The MS/MS data were analyzed using a Mascot database search (www.matrixscience.com).

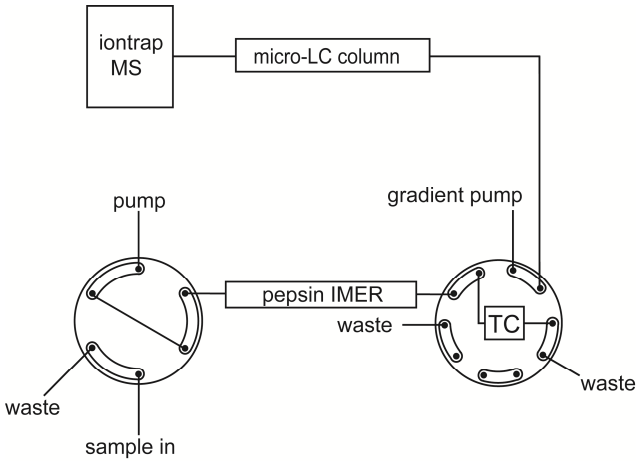

Figure 2: Setup for on-line digestion using pepsin-modified fused-silica capillaries (see Experimental section).

5.3. Results and discussion

5.3.1. Characterisation of the pepsin reactor using UV-detection

As shown before [23, 24], the modification of a surface with a dextran hydrogel (see Fig. 1) leads to less non-specific adsorption of proteins compared to an unmodified surface. Moreover, the amount of biomolecules that can be immobilised per surface area can be increased significantly [24]. The efficiency of

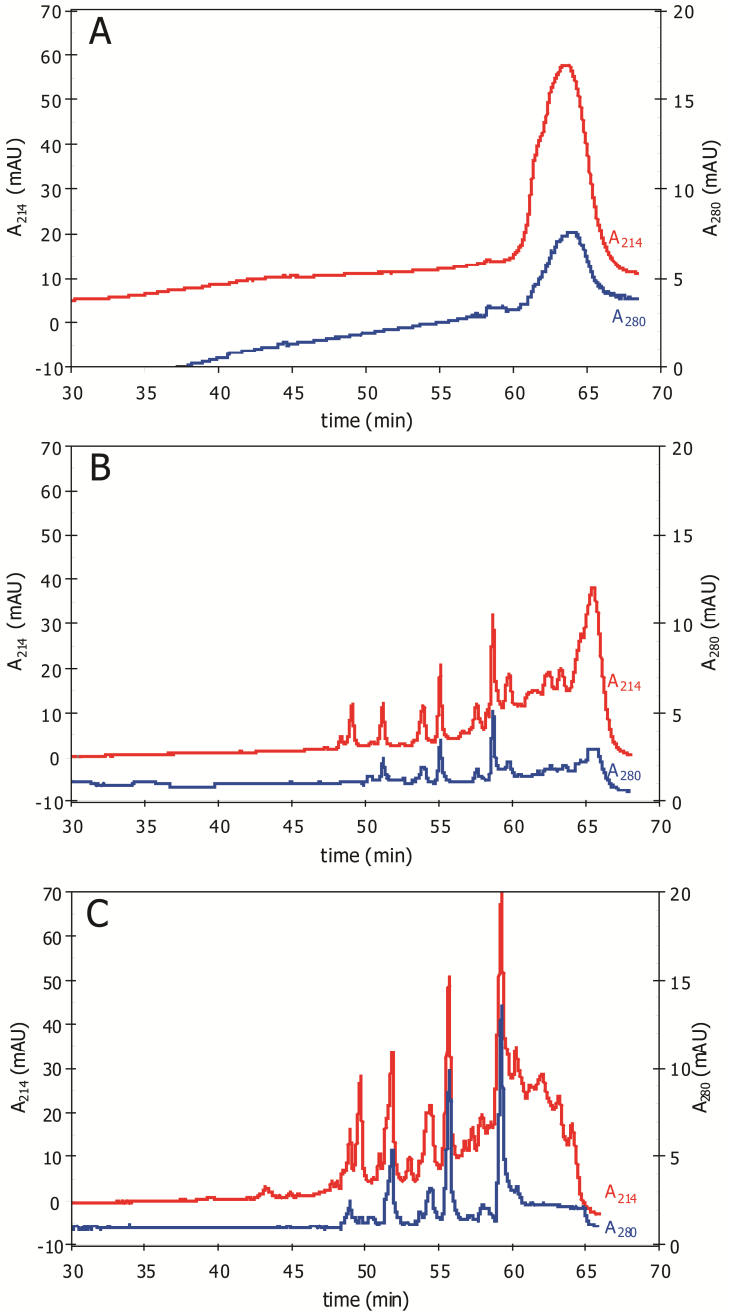

Figure 3: Chromatograms of the injection of 10 pmol haemoglobin in the capillary digestion systems monitored at 214 nm and 280 nm. The experiments were conducted with (A) a capillary not containing pepsin operated at a digestion flow rate of 1 µL min^{-1} (blank); (B) a pepsin-modified capillary operated at 10 µL min^{-1} (average sample residence time 18 s); (C) as (B) but operated at 1 µL min^{-1} (average sample residence time 180 s).

digestion and the time necessary for protein digestion using the pepsin IMER was investigated by varying the digestion flow and hence the incubation time. The pepsin activity is usually expressed by the suppliers as the change in absorbance at 280 nm, measured as TCA-soluble products using haemoglobin as substrate. The assay is carried out by the digestion of the protein, followed by the addition of acid to precipitate undigested protein and absorbance determination of the peptides present in solution [25]. The efficiency of and the time necessary for protein digestion using the pepsin IMER was investigated using human haemoglobin. This protein is a heterotetrameric complex with a molecular weight of 64.5 kD and pI 6.8, comprising four polypeptide chains (two α chains and two β chains). The flow during proteolysis was varied from 1 to 10 µL min^{-1} resulting in incubation times between 180 to 18 s. The chromatograms, monitored at 214 and 280 nm, are shown in Fig. 3. Fig. 3a shows the chromatogram of a blank run, Figs 3b and 3c show the on-line digestion of 1 µL haemoglobin (10 pmol total protein injection) at a flow rate of 10 µL min^{-1} and 1 µL min^{-1}, respectively.

As visible in Fig. 3a, the undigested protein elutes 60 min after injection. As can be observed in Figs 3b and 3c, the pepsin micro-reactor is capable of digesting the injected protein resulting in the production of many proteolytic fragments visible at 214 nm. The number of peptide fragments produced during proteolysis and detected at 280 nm is low, elute within 60 min after injection. Fig. 4 shows the total area of the proteolytic peptides determined at 280 nm as a function of the contact time. At a flow rate of 1 µL min^{-1} the injected protein is not observed any more at 280 nm and the total area of the peptides reaches a plateau value, but even at an incubation time of only 18 s a conversion of 30% of the injected protein can be calculated.

Because complete digestion of the protein is observed at a flow rate of 1 µL min^{-1} with an average substrate contact time of 3 min, this flow rate is used for all further experiments. Regular on-line digestion of haemoglobin at this flow rate during continuous operation for 40 days, results in complete digestion of the injected substrate and hence reproducible values for the A_{280}

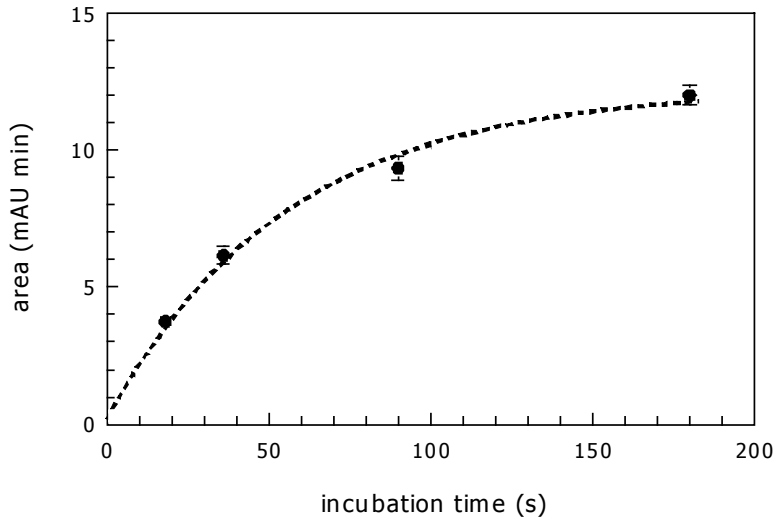

Figure 4: Total area of the peptide peaks observed at 280 nm for the on-line digestion of 10 pmol haemoglobin using pepsin-modified capillaries at different enzyme contact times. The error bars show the standard deviation (n = 5).

area of the produced fragments. The same result was obtained with a reactor stored at 4°C for one month. The reactor can therefore be used and stored for a longer period.

To further examine the pepsin reactor, two other protein substrates were submitted to fragmentation. The first is horse myoglobin, a single-chain globular protein containing a heme prosthetic group. The protein has an isoelectric point (pI) of 6.9 and a molecular weight of 16.9 kD. The protein contains no cystein residues, but is generally considered difficult to digest [26, 27]. The second protein is bovine serum albumin (BSA). This single polypeptide molecule has a pI of 5.3, a molecular weight of 66 kD protein and contains 17 disulfide bridges and one sulfhydryl group. The chromatograms of the digestion of BSA are shown in Fig. 5. Fig. 5a shows an injection of 10 pmol BSA using a dextran-modified capillary which has not been derivatised with pepsin. Fig. 5b shows the chromatogram of BSA using the described pepsin reactor. As is clear from these chromatograms, the injected protein is completely digested using a 3-min residence time. Under the same conditions, myoglobin can be completely digested as well (data not shown). This means that the reactor might be a useful tool for protein sequencing using mass spectrometric analysis.

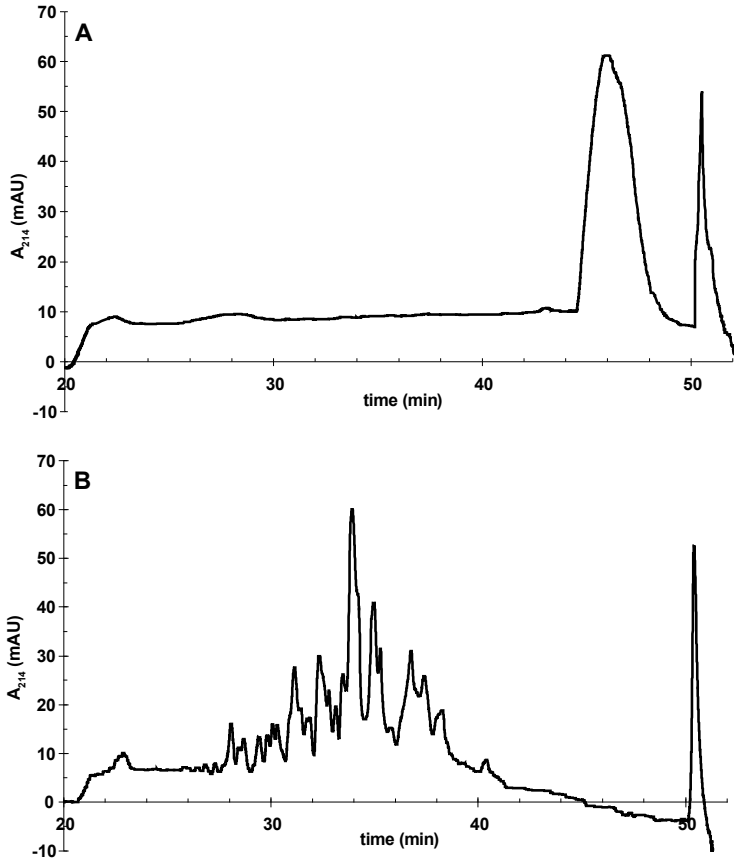

Figure 5: Chromatograms of the injection of 10 pmol BSA in the capillary digestion systems monitored at 214 nm. The digestion experiments were conducted at 1 µL min^{-1} using (A) a non-derivatised capillary and (B) a pepsin-modified micro-reactor. The average sample residence time of the sample in the reactor was 180 s; the analysis was performed as described in Experimental.

5.3.2. On-line digestion with MS/MS detection

To investigate the applicability of the reactor for sequencing and identification of proteins with MS, the analytical column is attached to the micro-ESI sprayer of an ion trap (see Fig. 2). Various proteins, differing in molecular weight and composition, are subjected to on-line digestion and HPLC-MS as described in the experimental section. A first group of proteins consisted of horse myoglobin and recombinant human interferon-γ (rhIFN-γ). The rhIFN-γ is a single-chain protein with a molecular weight of 16.9 kD and a pI of 9. Analysis of the MS/MS spectra resulting from the 3-min digestion of the injected

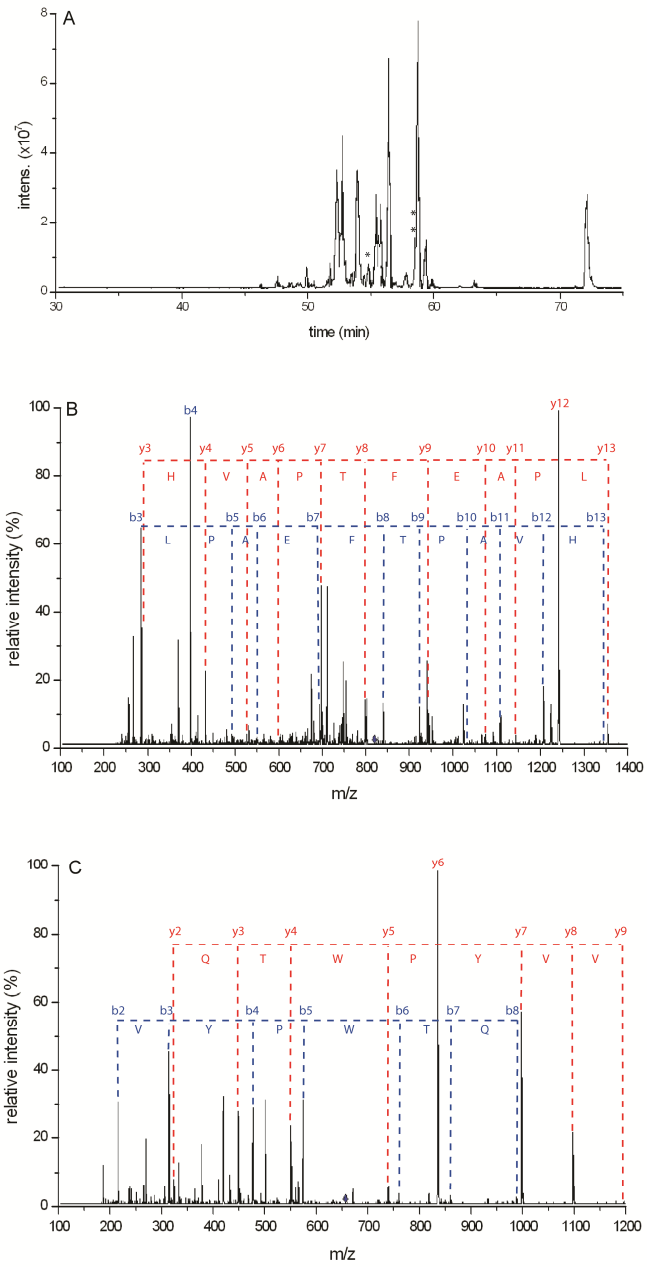

Figure 6: Results of the on-line digestion of 10 pmol haemoglobin at a flow rate of 1 µL min^{-1} using a pepsin micro-reactor. (A) Chromatogram of a MS/MS run, (B) the MS/MS spectrum of the peptide AAHLPAEFTPAVHASL with m/z 816.1 from the haemoglobin α-chain, (C) the MS/MS spectrum of the peptide LVVYPWTQRF with m/z 654.6 originating from the haemoglobin β-chain. The position of the peptides is indicated in the chromatogram using a single and double asterisk, respectively.

proteins using a Mascot database search, results in 11 identified peptides for myoglobin (sequence recovery 58%) and 7 peptides for rhIFN-γ (sequence recovery 69%).

The proteins α-lactalbumin, β-lactoglobulin A and BSA contain several disulfide bridges and are therefore more rigid and difficult to digest. The protein α-lactalbumin contains 4 disulfide bridges. This metalloprotein has a molecular weight of 14.2 kD and a pI of approximately 4.5. The protein β-lactoglobulin contains two disulfide bridges and a free sulfhydryl and has a molecular weight of 18.4 kD and a pI of 5.1. Several peptide fragments were matched by a Mascot database search on the MS/MS spectra. For α-lactablumin the sequence recovery is 62% (18 peptides), for bovine β-lactoglobulin A 67% (29 peptides) and BSA 48% (30 peptides).

Human haemoglobin is subjected to on-line digestion using the pepsin reactor followed by reversed-phase HPLC and MS/MS analysis. An MS/MS base-peak chromatogram of a haemoglobin digestion is shown in Fig. 6a. Fig. 6b shows the spectrum of an ion with m/z 816.1 which is identified as the peptide AAHLPAEFTPAVHASL present in the haemoglobin α-chain. Similarly, Fig. 6c presents the spectrum of the peptide LVVYPWTQRF originating from the haemoglobin β-chain with a m/z 654.6. The analysis of the MS/MS spectra using a Mascot database search resulted in 21 peptides matched for the haemoglobin α-chain (sequence coverage 97%). For the β-chain 14 peptides are identified (coverage 55%). The identification of both chains on basis of the peptides formed during pepsin digestion means that when different protein chains are present in a sample, the digestion and further HPLC-MS analysis is not influenced.

The applicability of the reactor was further investigated by subjecting the micro-reactor to the digestion of a protein mixture. To this end casein was selected which is composed of α-s1-, α-s2-, β- and κ-casein. The casein was dissolved in buffer and submitted to digestion immediately followed by reversed-phase chromatography with auto-MS/MS analysis. Table 1 shows the peptides that were matched by the Mascot database search of the data from

Table 1: Peptides identified in the on-line digestion of casein using pepsin micro-reactors and MS/MS analysis.

Peptide sequence	Theoretical m/z	Experimental m/z	Calculated	Position
κ-Casein, 36% sequence coverage				
FSDKIAKYIPIQYVL	1796.41	599.81	1797.01	39–53
VLSRYPSYGLN	1267.66	634.64	1267.27	52–62
SRYPSYGLN	1055.50	528.54	1055.07	54–62
YYQQKPVAL	1108.60	555.06	1108.11	63–71
VESTVATL	818.44	819.13	818.12	160–167
EASpPEVIESPPEINT (phosphoserine)	1690.73	846.08	1690.15	168–182
VQVTSTAV	803.44	402.51	803.01	183–190
α-s2-casein – bovine, 35% sequence coverage				
KNMAINPSKEN	1244.62	622.78	1243.56	24–34
YQKFPQY	972.47	487.01	972.02	89–95
YQKFPQYLQY	1376.68	689.12	1376.24	89–98
YQKFPQYLQYL	1489.76	745.67	1489.34	89–99
LYQGPIVLNPWDQVKRNAVPITPTL	2831.57	944.89	2831.67	99–123
VYQHQKAMKPWIQPKTKVIPYVRYL	3113.74	779.41	3113.64	183–207
α-s1-casein – bovine, 50% sequence coverage				
RPKHPIKHQGLPQEVLNE	2119.17	707.12	2118.34	16–33
RPKHPIKHQGLPQEVLNENL	2346.29	783.13	2346.37	16–35
NENLLRF	904.48	453.11	904.21	32–38
FVAPFPEVF	1051.54	526.66	1051.31	39–47
FVAPFPEVFGKE	1365.70	683.64	1365.27	39–50
FVAPFPEVFGKEKVNE	1835.95	918.58	1835.15	39–54
AYFYPEL	901.42	902.13	901.12	158–164
FRQFYPEL	1000.51	501.16	1000.30	165–171
YYVPLGTQ	939.47	470.61	939.21	180–187
YYVPLGTQYTDAPSF	1720.80	861.19	1720.37	180–194
YTDAPSF	799.05	800.06	799.33	188–194
SDIPNPIGSENSEKTTMPLW	2215.31	1108.66	2215.05	195–214
β-casein, 55% sequence coverage				
RELEELNVPGE	1283.31	642.66	1283.64	16–26
QDKIHPFAQTQSL	1511.25	756.63	1511.77	61–73
PVVVPPFLQPEVM	1450.79	726.19	1450.37	98–108
TLTDVENLHLPLPLL	1686.96	844.22	1686.43	141–155
TDVENLHLPLPLL	1472.82	737.22	1472.43	143–155
TDVENLHLPLPLLQS	1687.91	844.72	1687.43	143–157
DVENLHLPLPLLQS	1586.87	794.15	1586.29	144–157
SLSQSKVLPVPQKAVPYPQRDMPIQA	2876.56	959.60	2875.78	179–204
LYQEPVLGPVRGPFPIIV	1993.14	997.29	1992.57	207–224
YQEPVLGPVRGPFPIIV	1880.06	940.77	1879.53	208–224
PVLGPVRGPFPIIV	1459.89	730.76	1459.51	211–224

the auto-MS/MS fragmentation of the peptides formed during the digestion of casein. Peptides originating from all the proteins present in casein are found after pepsin digestion. The sequence coverage of these proteins ranges from 35% for α-s2-casein to 55% for β-casein. The sequence recoveries obtained for an in-solution digestion of β-casein with pepsin [28] or digestion of this protein with a trypsin micro-reactor [29] are with 39% and 35%, respectively, both lower than the recovery realised with this pepsin reactor for the analysis of whole casein. The relatively lower sequence coverages for both α-s2-casein and

κ-casein compared to the β-casein may be caused by post-translational modifications of these proteins and the low proportion of these proteins in the sample (typically up to 12% of the casein present in skim milk) [30, 31]. Additionally, the peptides containing the cysteine residues present in κ-casein were not observed in the database search due to the fact that no reduction and alkylation was carried out.

The results for the digestion at low pH of proteins differing in molecular weight and isoelectric point, and the observation that a complex sample like casein can be digested efficiently with good sequence recovery, show the potential of the pepsin micro-reactor. As observed during UV experiments, complete protein digestion can be realised within 3 min, but even in 18 s 30% of the injected protein is digested. During the MS experiments that were conducted, sample carry over, due to protein or peptide adsorption to the reactor wall, was not observed. Peptide fragments originating from pepsin auto-digestion were also not detected during the mass spectrometric analysis of the digested proteins.

When the obtained data on sequence coverage are compared with results from other protease micro-reactors used for on-line protein digestion, it becomes clear that for some trypsin reactors higher sequence coverages can be obtained [24,32]. This may be caused by the fact that trypsin will hydrolyze peptide bonds on the C terminus of arginin and lysine only whereas, depending on the pH of operation, pepsin will preferentially cut most positions where either tyrosine, tryptophan, phenylalanine or leucine is present. This will result in several smaller fragments that may not be retrieved in standard HPLC-MS/MS operation. This is confirmed by the fact that, although the sequence recovery is slightly lower, the injected protein is completely digested as could be observed by UV-VIS detection. A combination of both a pepsin and a trypsin reactor, operated at different pH values, may be useful to elucidate the full sequence of unknown proteins.

Compared to pepsin reactors described in literature, the reactor performs competitively, both in the time needed to completely digest the

protein subjected to proteolysis, as well as the sequence recovery. In the pepsin reactors developed for MS analysis of protein samples the time needed for protein digestion ranges from a few minutes for an electrospray pepsin-capillary [32] and a packed-bed pepsin-reactor [21] to hours for a reactor consisting of pepsin adsorbed to a PEEK transfer tube [33]. The sequence recoveries based on MS/MS data that were reported were 35% [32] and 56% [21] for cytochrome C. The protein lysozyme subjected to on-line digestion using a sol-gel column [22] is only partly digested, yet 70% of the amino acid sequence of the protein was recovered on basis of the fragments observed. Similarly, a peptide map with a coverage of 83% for myoglobin and 30% for BSA was obtained for a 1 h digestion using a reactor consisting of PEEK adsorbed pepsin [33]. For the digestion of haemoglobin using this reactor an incubation time of 4 h was needed.

5.4. Conclusions

The applicability of a pepsin micro-reactor for the on-line digestion of proteins is tested with a number of proteins varying in molecular weight, isoelectric point and sample composition. The reactor consists of pepsin covalently immobilised in a dextran-modified fused-silica capillary. The open-tubular reactors show a flow-dependent digestion, which was demonstrated for native haemoglobin. Complete digestion of human haemoglobin, horse myoglobin and bovine serum albumin is observed for a digestion time of 3 min with on-line micro-HPLC with UV-detection. The protein sequence of these and other proteins can largely be retrieved with a database search on the MS/MS data which make the reactors an interesting tool for the characterisation of unknown proteins.

The pepsin micro-reactors are stable during continuous operation for 40 days and show no sample carry over. The open-tubular pepsin reactor is easily and reproducibly prepared from relatively inexpensive materials. Future research will focus on the development of multi-dimensional analysis systems employing on-line protein digestion using these pepsin IMERs.

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6.

Development of an on-line SPR-digestion-nanoLC-MS/MS system for the quantification and identification of interferon-γ in plasma

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Abstract

An automated, on-line system for protein quantification and identification, employing Surface Plasmon Resonance (SPR), enzymatic protein digestion, nanoLC and tandem-MS (MS/MS), has been developed. For the experiments recombinant human interferon-γ (rhIFN-γ) in buffer or diluted bovine plasma was used as a model protein. Upon injecting 90 µL of a 1 µg mL^{-1} solution of rhIFN-γ in diluted plasma at a flow rate of 10 µL min^{-1}, 320 fmol of protein was reproducibly bound to the sensor surface. After desorption of the isolated protein from the SPR surface using 10 mM glycine pH 1.3, on-line digestion, nanoLC and MS/MS analysis, rhIFN-γ could be identified on basis of peptide masses and MS/MS fragmentation data. A sequence recovery of 66% was found when a pepsin micro reactor was used. For a trypsin micro reactor the sequence recovery was 50%. In the latter case, the desorbed protein solution was mixed with a TRIS buffer for optimal enzyme activity. With the identified trypsin- and pepsin-produced peptides and because parts of their amino acid sequences overlap, the protein sequence can be largely elucidated showing the potential for the analysis of unknown proteins. The SPR-digestion-nanoLC-MS/MS platform provides unattended analysis of a sample within 60 min.

6.1. Introduction

Molecular interaction plays an important role in biological processes and signal transduction. To understand the relation between a binding event and the occurrence of a trigger or reaction, molecular interaction research has evolved over the years as a subject of study, for example, in the fields of proteomics, pharmaceutical research and (biomarker) screening. Various methods can be used to monitor biological interactions, but due to the ease of use and its flexibility, Surface Plasmon Resonance (SPR) is an often employed technique for the study of protein-protein interactions.

An important feature of SPR instruments is the possibility to obtain real-time data on the interaction of a ligand to its receptor allowing kinetic data to be determined [1-3]. Moreover, the amount of material necessary to perform the experiments is less, labelling is not required and variation in surface chemistries is possible, allowing various immobilisation strategies and interaction experiments. As there exists a linear relation between the amount of bound protein and the sensor response, an accurate determination of picogram amounts of material present on the SPR sensor surface is possible [4]. Due to this high sensitivity, nM-concentration determinations in a direct fashion can be performed for a protein [5-7]. When a signal enhancement is implemented using a secondary antibody e.g. with a liposome label, even low pM concentrations can be determined [8, 9].

In standard SPR experiments the selectivity of the sensor is established by the character and build up of the sensor surface, for example, the receptor molecule used. As the response is merely an account of the mass present at the sensor surface, there is no absolute certainty about the nature of the ligand interacting with the receptor. To identify a ligand, and discriminate between proteins interacting (a-)specifically, a second, identifying, technique is necessary. A possible technique to carry out this identification is mass spectrometry (MS). In proteomics mass spectrometry is generally employed for the identification of proteins. This is often accomplished on the basis of the molecular weight of the whole protein, as well as the protein peptides obtained

by proteolysis and the sequencing of these peptides by fragmentation in tandem-MS.

The combination of SPR molecular interaction monitoring and MS is explored and reported by several groups. Apart from the use of the SPR as a screening device followed by separate MS identity confirmation in case of a positive sample [10-12], SPR instruments have also been employed as micropurification units to isolate and quantify the material analysed and identified by MS. Originally, MS analysis was performed "on chip" using Matrix Assisted Laser Desorption/Ionisation – Time-of-Flight (MALDI-ToF) MS to determine the molecular weight of the protein bound to the receptor [13-17]. Alternatively, a transfer-pin which is an exact copy of the sensor surface can be positioned near the sensor and used for MALDI-MS analysis [18]. Elution of the surface bound ligand, collecting the sample for further handling and MS analysis has been described as well. This strategy is by now the most popular implementation for studies in which SPR is successfully combined with MS [19-22]. In the combinations of SPR and MS, MALDI-ToF is the most often used method for MS analysis as it is both sensitive and accurate, and is relatively forgiving for minor contaminants present in a sample like salt residues originating from the various non-volatile buffers used in SPR [15]. Alternatively, Liquid Chromatography Electrospray Ionisation-MS (LC-ESI-MS), a system often used in proteomics research, has been increasingly employed in combination with SPR [22-26].

In the present study, an automated, on-line method for protein quantification based on SPR with identification using ion-trap MS is developed. The combination of SPR and MS includes on-line protein digestion using an IMER, consisting of either a trypsin- or a pepsin-modified open-tubular reactor, and a nanoLC reversed-phase separation. The platform enables the isolation and quantification of a protein from a plasma sample, followed by elution and digestion of the protein. Identification of the proteolytic peptides is performed using nanoLC and auto-MS/MS followed by a database search of the results. The feasibility of the system will be demonstrated by the isolation,

quantification and identification of recombinant human interferon-γ (rhIFN-γ) spiked in bovine plasma which is used as a model system.

6.2. Experimental

6.2.1. Materials

The SPR equipment used was from IBIS Technologies equipped with a 0.5-µL flow-cell. The gold sensor disks, purchased from SSENS (Hengelo, The Netherlands), were positioned on the IBIS-prism using index-matching oil from R.P. Cargille Laboratories Inc (Cedar Grove, USA). PTFE spacers of 50 µm (BASi, Kenilworth, Warwickshire, UK) into which a rectangular hole of 1.25 x 8 mm was punched, were used to create a SPR-flow cell that was further defined by the sensor disk and a home-made polycarbonate cylinder with PEEK in- and outlets to enable liquid flow-through. PEEK nuts, unions, tubing, and loops were from Upchurch Inc (Santa Monica, USA). Fused-silica capillaries used for the preparation of the micro reactors were purchased from Bester (Amstelveen, Netherlands). Manual injections during the preparation of the IMER were performed using a Rheodyne 7010 injector (Inacom, Veenendaal, The Netherlands) equipped with a 1-mL PEEK loop. Model 10ADvp HPLC pumps, the SIL-10ADvp injector and the SCL-10 Avp controller were obtained from Shimadzu (Kyoto, Japan). Ultrapure water was produced by a Sartorius Arium 611 ultrapure water system (Nieuwegein, The Netherlands; conductivity > 18.2 MΩ cm).

A reversed-phase 1 mm x 500 µm Styros 2R PEEK Nano Trap column was purchased from OraChrom, Inc (Woburn, Ma, USA). The 150 mm x 75 µm reversed-phase analytical nano-column containing 3 µm 100 Å C_{18} PepMap particles was obtained from LC Packings (Amsterdam, The Netherlands). The XCT ion trap-MS equipped with a nano-ESI ion source was from Agilent (Amstelveen, The Netherlands).

Amino-modified dextran (AMD) was from Unavera ChemLab (Mittenwald, Germany). Acetic acid, boric acid, calcium chloride ($CaCl_2$), ethanol, ethanolamine (EA), formic acid (FA), glycine, hydrochloric acid (HCl), sodium dihydrogen phosphate, sodium chloride, sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile and ethanol were from Biosolve (Valkenswaard, The Netherlands). Acetone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), trifluoro acetic acid (TFA), tris-hydroxymethylaminoethane (TRIS), and urea were purchased from Acros Organics (Geel, Belgium). rhIFN-γ, polyoxyethylene-sorbitan monolaurate (Tween 20), porcine pepsin, bovine pancreas trypsin and sulfo-*N*-hydroxysuccinimide (s-NHS) were purchased from Sigma (St Louis, USA). The monoclonal mouse human anti-interferon-γ antibody (MD-2) was a generous gift from dr. P.H. van der Meiden (U-Cytech, Utrecht, The Netherlands). Aminopropyl triethoxysilane (APTES), carbonyl diimidazole (CDI), glycidoxypropyl trimethoxysilane (GOPS) and mercapto ethanol (ME) were from Aldrich Chemical Company (Milwaukee, USA). Carboxyl-modified dextran (CMD) and *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC) were purchased from Fluka (Buchs, Switzerland).

6.2.2. Preparation of SPR-sensor surfaces and immobilised enzyme reactors

CMD-sensor surfaces were prepared according to a published method [6]. For antibody immobilisation, the CMD sensors were placed in the SPR and a 0.5-µL flow-through cell was positioned on top of the surface. Antibody immobilisation was performed by three 9-min injections of 200 mM EDC and 50 mM NHS in water, 50 µg mL^{-1} MD-2 in 10 mM acetic acid (pH 4.7) and 1 M ethanolamine pH 8.5 in a 10 µL min^{-1} HBS-ET buffer flow, respectively.

The preparation of dextran-coated fused-silica capillaries and the modification thereof with either trypsin or pepsin was performed according to earlier published methods [27, 28]. The resulting IMERs (internal diameter 50 µm, length 1.53 m, volume 3 µL) are positioned between the SPR and a 6-port 2-way valve containing a trapping column as shown in Figure 1.

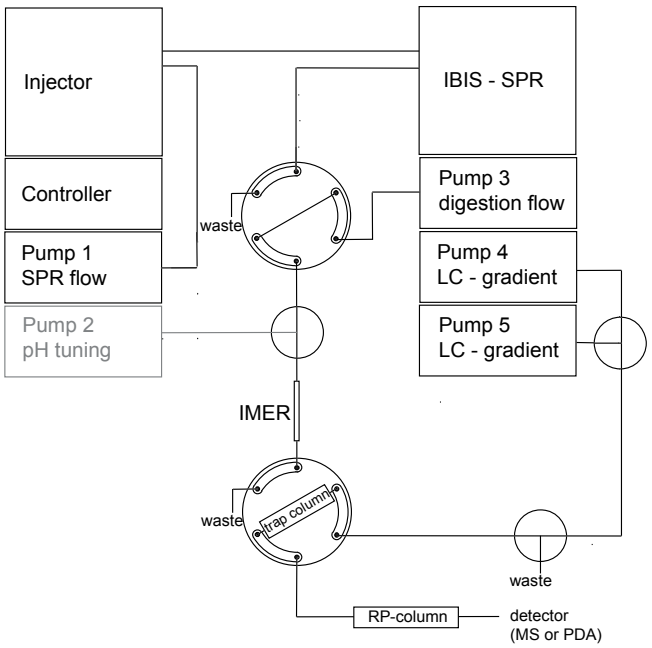

Figure 1: Experimental set-up used for the on-line SPR, digestion, nanoLC trapping and separation followed by ESI-MS/MS using an ion trap. For more information on equipment and consumables, see Experimental section.

6.2.3. System control and SPR analysis

The various instruments in the platform (see Figure 1), the data acquisition and the events triggered with an A/D converter were controlled through the Class VP (version 5) software run on a Windows 98 (II) computer. Additionally, the SPR sensor response was monitored using IBIS Data Analysis software. During the SPR interaction analysis experiments, 9-min sample injections were followed by a 3-min buffer flow to remove the sample and unbound components from the sensor surface and the flow-channel. The bound protein was desorbed by using a 1-min injection of 10 mM glycine pH 1.3, preceded by a 1-min injection of 10 mM acetic acid, pH 4.5 to remove unwanted salts and buffer components which might impede both digestion and analysis. The collection valve was switched to retain the liquid containing the desorbed ligand upon arrival of low pH regeneration solution, which was calculated on basis of the flow rate and tubing volume and checked by the pH change. The total volume of the retained sample proved to be 15 µL. The SPR

experiments were conducted at a flow rate of 10 µL min^{-1} and a controlled temperature of 25 °C.

6.2.4. Protein digestion using the IMERs

During on-line protein digestion, the eluted ligand present in the low pH regeneration solution was transported through the IMER using a 2% acetonitrile solution containing 0.05% TFA. For trypsin digestion, the pH of the solution containing the desorbed ligand was raised to pH 8 by adding a compensation buffer, 200 mM TRIS pH 10, at the same flow as the sample. For pepsin digestion, pH correction was not necessary and the samples were digested at pH 1.3 without any further treatment. The total flow rate during all digestions was 2 µL min^{-1}, resulting in an average sample residence time of 1.5 min. After maintaining this digestion flow for 15 min, the flow rate was changed to 10 µL min^{-1} to concentrate and wash the peptides trapped on the RP-trapping column (TC) prior to analysis.

6.2.5. Protein digest analysis using nanoLC-MS/MS

An acetonitrile gradient was started upon positioning the TC, containing the concentrated and desalted digest, in-line with the analytical nano-column. The gradient was composed of two solutions: (A) 2% acetonitrile in water also containing 0.1% FA and 0.01% TFA and (B) 80% acetonitrile in water containing 0.08% FA and 0.01% TFA. In 30 min the composition changed linearly from 0 to 50% B, followed by 10 min at 90% B and finally a 20-min period at 0% B. The flow rate was 250 nL min^{-1} and the column outlet was connected to a nano-electrospray interface positioned perpendicular towards the Agilent XCT ion-trap inlet. The ESI-MS was conducted in the positive-ion mode with the capillary voltage set at 1000 V. Flow rate and temperature of the nitrogen drying gas were 5 L min^{-1} and 150°C, respectively. The peptide fragment sequences were determined in the auto-MS/MS mode. All experiments were conducted at room temperature. The resulting data was analyzed using FindPept (www.expasy.org) and Mascot (www.matrixscience.com).

6.3. Results and discussion

6.3.1. System development

In "ligand fishing" experiments the need exists to immobilise an adequate amount of "bait", that is the structure to bind or interact with the biomolecule of interest, to obtain enough ligand to allow mass spectrometric analysis. In order to bind sufficient ligand during the experiments a large surface area is needed. To accomplish this, a rectangular hole with an area of 10 mm^2 was punched manually in 50 µm PTFE sheet spacers. These spacers were sandwiched between the sensor disk and a polycarbonate block with a liquid in- and outlet hence creating a flow cell with a total volume of 500 nL. Mass-transfer testing using a 5-min injection of 0.1% glycerol showed that, at the operational flow rate of 10 µL min^{-1} used for the experiments described here, the sample enters and leaves the flow cell as a plug.

Apart from the sensor surface area available for interaction, also the amount of receptor molecules present is of importance. Research has shown that modification of surfaces with a hydrogel such as dextran, leads both to less non-specific interaction as well as to higher immobilisation values compared to random antibody adsorption or coupling to self-assembled monolayers [6]. Compared to carboxyl-modified dextran surfaces (CMD) less non-specific adsorption of protein was observed on non-derivatised dextran surfaces. Nevertheless, due to the observed high antibody immobilisation levels obtained and, consequently, high ligand binding, CMD sensor surfaces are used for antibody immobilisation in this study.

On the prepared CMD surfaces 7.9 ng mm^{-2} mouse anti-interferon-γ antibody is immobilised (942 ± 35 m°, n = 3). The observed amount equals a total of 52 fmol mm^{-2} of antibody being present on the sensor surface taking into account that an angle shift of 120 m° equals a total of 1 ng mm^{-2} of protein, and a molecular weight of 150 kDa for an IgG molecule. Upon repeated exposure of the antibody-modified surface to 100x diluted bovine plasma, a non-specific response of 6.2 ± 1.8 m° (n=6) was observed. This corresponds to

0.05 ng mm^{-2} protein adsorbed non-specifically to the surface, which is less than earlier observed for these surfaces. It can be attributed to a more effective activation and blocking of the surface using the modified immobilisation-method.

For the digestion of proteins, protease micro reactors have been developed which are capable of efficient digestion of the injected proteins as shown earlier [27, 28]. The sequence of the protein subjected to proteolysis can be largely retrieved on basis of the mass of the proteolytic peptides and MS/MS fragmentation analysis thereof. As shown in Figure 1, the reactor is positioned directly after the SPR sensor, only separated by a valve containing a loop in which the protein removed from the surface during sensor regeneration is collected prior to digestion. In order to produce the peptides, it is important that the enzyme reactor is active in the regeneration solution used to remove the bound ligand from the sensor surface. Since the mouse monoclonal antibody applied here is regenerated with 10 mM glycine, pH 1.3, the pepsin reactor can be employed without further pH correction. However, when the trypsin IMER is applied, compensation of the low pH buffer solution containing the protein removed from the sensor surface is necessary for optimal turnover. For that purpose, a 200 mM TRIS solution, pH 10, is pumped at the same flow rate as the regeneration solution and mixed prior to being transferred to the IMER.

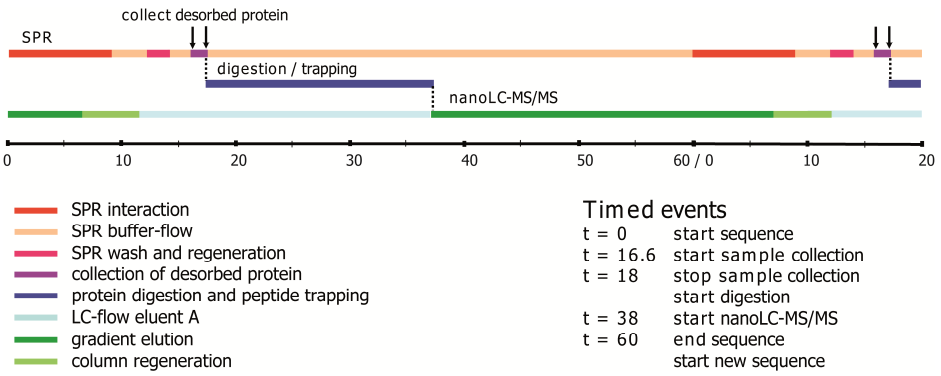

Figure 2: Schematic representation of all events in time during operation of the SPR-digestion-nanoLC-MS/MS system. Events are separated in three different instrumental sections: SPR, ligand digestion and chromatography with MS detection. The various actions during these events are indicated.

Figure 2 presents the sequence of events and time necessary to conduct all the actions including the digestion and chromatography. Upon the start of the sample injection, the system program starts and in time a number of triggers is generated to start an action, such as the collection of the regenerated ligand, the digestion thereof and the start of the nanoLC-MS analysis of the proteolytic peptides. The total run-time of the program is 60 min after which a new sample injection is started. At that time the nanoLC-MS analysis and column regeneration have not finished yet, meaning that the experimental cycles partly overlap thus saving time.

6.3.2. System operation

A net response of 65 ± 3 m° just prior to regeneration is generated upon the interaction of 1 µg mL^{-1} rhIFN-γ in 100x diluted plasma. In order to avoid interference of running-buffer components during digestion and chromatography, the running buffer is removed from the flow cell using a 1-min injection of an acetic acid buffer (10 mM, pH 4.5). Repeated injection of this washing buffer did not lead to a change in response, meaning that no rhIFN-γ is removed from the surface during exposure to this buffer. For regeneration of the surface, a solution containing 10 mM glycine, pH 1.3 is used which proved effective during an earlier study [6]. After the regeneration step, executed immediately following the washing step, the sensorgram returns to the baseline level demonstrating complete desorption of the protein bound to the sensor surface (see Figure 3). This result is repeatedly obtained, illustrating that the regeneration procedure does not damage the receptor-modified sensor surface, allowing for a new detection cycle. The observed net response corresponds to 5.4 ng rhIFN-γ. Taking into account both the complete removal of the bound protein due to the regeneration step and the molecular weight of the protein (16.9 kDa), the amount of protein regenerated from the sensor surface is calculated to be 320 fmol.

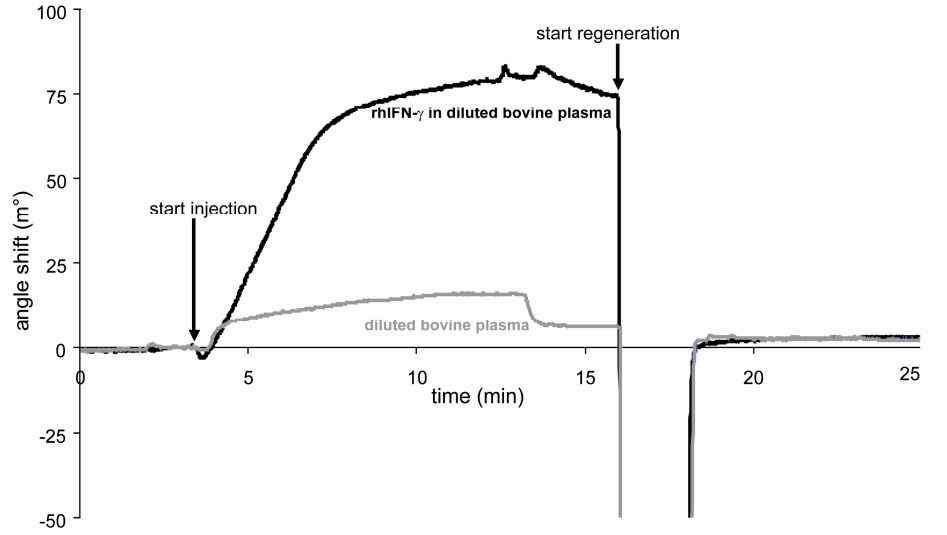

Figure 3: SPR sensorgrams showing the interaction of 1 µg mL^{-1} rhIFN-γ in 100x-diluted bovine plasma and 100x-diluted bovine plasma containing no rhIFN-γ with MD-2 modified CM-dextran surfaces. The start of the sample-injection and regeneration is indicated with arrows.

To test the applicability of the developed setup, the rhIFN-γ bound to the sensor surface was desorbed and digested using the trypsin IMER with pH compensation as described. After digestion the produced peptides were concentrated and desalted on a trapping column. In the following nanoLC-ESI-MS/MS analysis, several ions were automatically fragmented and identified as peptide fragments originating from rhIFN-γ. Figure 4 presents the MS/MS fragmentation and analysis result for the peptide AIHELIQVMAELSPAAK with an experimental mass of 1821.0, showing several of the matched fragment ions. Apart from this peptide other peptides, summarised in Table 1, can be repeatedly identified on the basis of the observed mass and fragmentation result as originating from rhIFN-γ isolated from buffer or plasma. Unfortunately, database identification using fragmentation data is not always feasible for all proteolytic peptides due to the low intensity of some of the observed fragments. However, in runs of diluted bovine plasma containing no rhIFN-γ none of the summarised peptides are observed and for blanc buffer injections no significant responses are observed. On basis of the data presented in Table 1, a sequence coverage of 50% is calculated.

Similarly, the nanoLC ESI-MS/MS analysis of the rhIFN-γ, isolated with the SPR and digested using the pepsin IMER, show several ions that are repeatedly observed. These ions can be identified on basis of MS analysis and MS/MS fragmentation as proteolytic peptides originating from the protein rhIFN-γ. In Figure 5 the fragmentation result of the peptide NVQRKAIHELIQVM with a mass of 1678.4 is shown. The observed peptides are also summarised in Table 1. As mentioned earlier, database analysis of fragmentation data does not necessarily lead to peptide identification. Nevertheless, as repeated blank injections do not show any of the observed masses identified as peptide fragments and the observed masses correspond with peptides produced by pepsin digestion of the protein rhIFN-γ, the fragments summarised in Table 1 irrefutably originate from the isolated protein. The protein sequence coverage on basis of all the observed peptides is 66%.

Combining the results of the respective trypsin and pepsin digestions allow 90% of the protein amino acid sequence to be retrieved. This is caused by overlap in fragments produced by the respective proteases with their own specificity and the fact that occasionally the sequences complement each other. With respect to the proteases used in this study, trypsin is a highly specific protease that hydrolyzes peptide bonds on the C terminus of arginin and lysine only. On the other hand, depending on the pH of operation, pepsin will preferentially cleave at tyrosine, tryptophan, phenylalanine or leucine. Also alanine, glutamic acid and glutamine have been reported as cleaving sites. This will result in many different and often overlapping peptide fragments. The difference in specificity between the two proteases makes it possible to compare the amino acid sequences of the peptide fragments and fit them together. Therefore, the combination of the results obtained with both a pepsin and a trypsin reactor may be useful to elucidate the sequence of isolated unknown proteins.

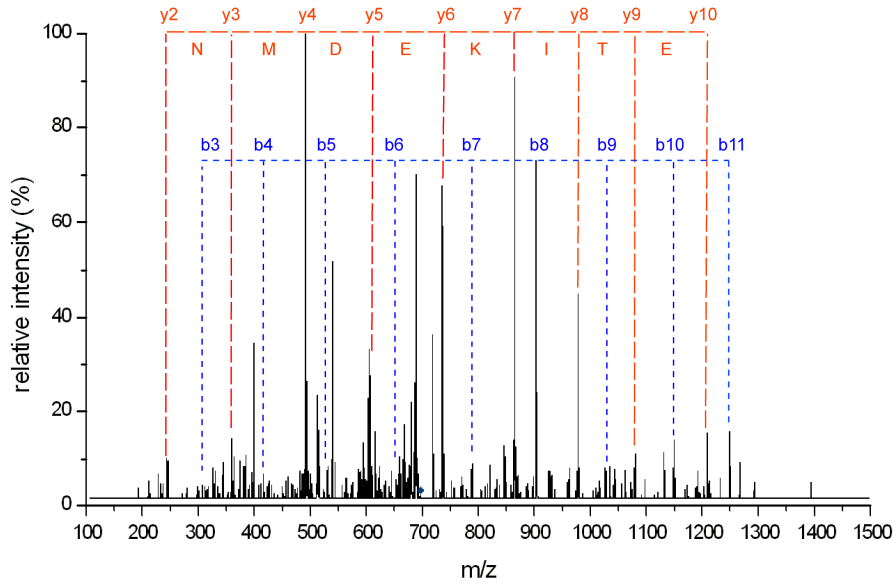

Figure 4: MS–MS fragmentation of the peptide AIHELIQVMAELSPAAK with experimental mass 1821.0, obtained by trypsinolysis of rhIFN-γ released from the SPR sensor chip, showing several of the matched fragment ions.

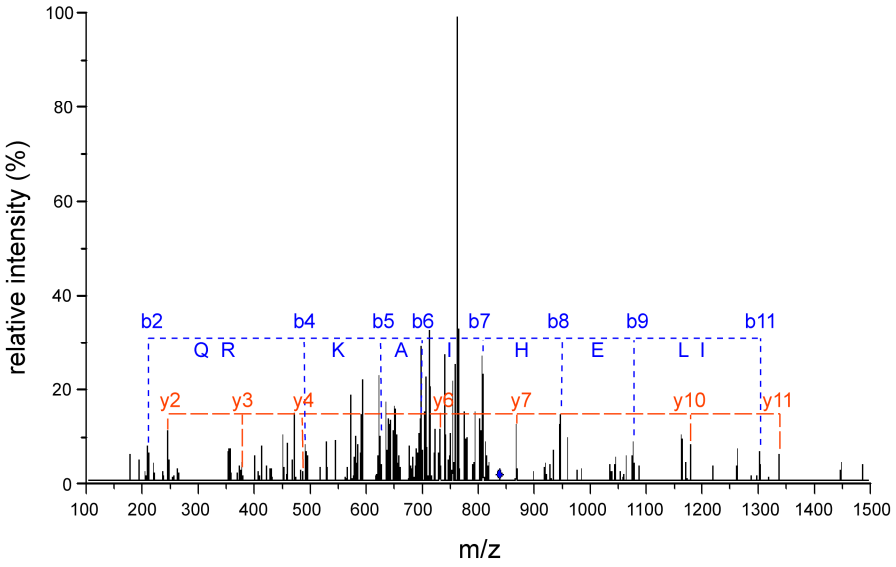

Figure 5: MS–MS fragmentation of the peptide NVQRKAIHELIQVM with an experimental mass 1678.4 obtained by pepsin digestion of the rhIFN-γ released from the SPR sensor surface, showing several of the matched fragment ions.

Table 1: Peptides observed by MS in the on-line SPR-digestion-nanoLC runs of rhIFN-γ in diluted bovine plasma.

Exp. mass (Da)	DB mass (Da)	Δ mass (Da)	Peptide	Position
On-line trypsin digested				
880.35	880.42	0.07	MQDPYVK	1–7
1610.69	1610.85	0.17	LFKNFKDDQSIQK	56–68
2596.70	2596.29	−0.41	NFKDDQSIQKSVETIKEDMNVK	59–80
1392.41	1392.70	0.30	SVETIKEDMNVK	69–80
735.78	735.33	−0.45	EDMNVK	75–80
1473.15	1472.68	−0.46	EDMNVKFFNSNK	75–86
1729.05	1728.87	−0.17	EDMNVKFFNSNKKK	75–88
1884.67	1884.98	0.31	EDMNVKFFNSNKKKR	75–89
2569.07	2569.34	0.27	KKRDDFEKLTNYSVTDLNVQR	87–107
809.32	809.38	0.06	RDDFEK	89–94
1820.97	1820.99	0.03	AIHELIQVMAELSPAAK	109–125
2547.95	2547.46	−0.49	AIHELIQVMAELSPAAKTGKRKR	109–131
On-line pepsin digested				
802.17	802.43	0.26	VKEAENL	6–12
812.16	812.47	0.30	NLKKYF	11–16
585.25	585.26	0.01	GHSDVA	19–24
779.33	779.39	0.06	DNGTLFL	25–31
704.05	704.37	0.32	KNWKE	35–39
1538.55	1538.80	0.25	SDRKIMQSQIVSF	41–53
1767.77	1767.95	0.18	SQIVSFYFKLFKNF	48–61
775.59	775.40	−0.19	IVSFYF	50–55
1016.84	1016.58	−0.26	IVSFYFKL	50–57
570.11	570.33	0.22	YFKL	54–57
753.19	753.36	0.17	DMNVKF	77–82
912.62	912.43	−0.19	TNYSVTDL	97–104
1678.41	1677.93	0.47	NVQRKAIHELIQVM	105–118
1053.26	1053.58	0.31	IHELIQVMA	111–119
888.26	888.45	0.19	VMAELSPAA	117–125
1404.17	1403.83	−0.34	AKTGKRKRSQML	125–136

As reported above, a slight offset of 6 m° is observed after bovine plasma injection caused by plasma protein adsorption that is not removed by the running buffer flow. As a result, apart from the peptides originating from rhIFN-γ, two additional fragments are repeatedly found that can be attributed to BSA present in the plasma which is non-specifically bound to the sensor. Although complete prevention of non-specific interaction of plasma proteins is difficult to achieve, the selective removal of these proteins without removing the isolated ligand will be a subject of later study. Nevertheless, the currently obtained results show that rhIFN-γ present in diluted plasma can be isolated on an antibody-modified sensor surface, efficiently removed from the surface using a glycine regeneration solution, on-line digested and analysed by nanoLC-MS/MS. Although the concentration of the model protein rhIFN-γ is higher than present in human plasma, the current system could be readily used in e.g.

fermentation control for both concentration determination and identification of the pharmaceutically employed recombinant protein [29].

The regeneration step, using an acidic glycine solution, completely desorbed the bound protein ligands. Using this strategy, the sensor chip can be used for a new analysis which is certainly not possible when MS analysis is performed on-chip [13, 17]. The desorbed protein in the regeneration buffer can be digested immediately in the protease IMER, thus minimizing the risk of sample loss and contamination by antibodies or other receptor proteins used to capture the ligands of interest which might occur when performing on-chip digestion. When an on-chip digestion is performed [25], there is the risk of partial digestion of the receptor molecule which will bias later SPR analyses.

Apart from the sensor surface reusability, also the time needed for a complete experiment using the on-line SPR-digestion-nanoLC-MS system is less compared to the off-line [25] and automated on-line [24] SPR nanoLC-MS approaches described elsewhere. The main cause for this is the time needed for the digestion of the SPR-isolated protein which varies from 2 to 10 h, respectively. The off-line system also needed manual intervention as the nanoLC-ESI/MS/MS analysis was performed apart from the SPR analysis and protein digestion, and a single run took approximately 3 h to complete. In contrast, the system described in this paper is capable of starting a fully automated experiment consisting of SPR interaction analysis and desorption, digestion, trapping and desalting, followed by nanoLC and MS/MS every 60 min.

Compared to existing methods that combine SPR analysis and MS/MS identification, efficient transfer of the isolated ligand is realised by coupling the SPR and the chromatographic system via the IMER, thus creating the fully automated, on-line system described in this paper. Consequently, sample handling which might lead to sample loss using an off-line approach, is minimised. Moreover, the time needed for completing a single experimental cycle is lower than with other reported systems combining SPR and MS/MS. This means that an increased throughput can be realised. A further gain in time may be accomplished by trapping and concentrating the protein sample

desorbed from the sensor surface prior to digestion. Using such an approach might lower the time needed for digestion considerably. Also an optimisation in chromatography might save time and further increase the throughput.

6.4. Conclusions

SPR biosensors are versatile devices that have been employed successfully in interaction analysis, screening, quantification, epitope mapping, and micropurification of proteins and peptides. Nevertheless, for identification, the ligand bound to the sensor has to be analysed using another technique, such as mass spectrometry (MS). Many strategies have been investigated such as on-chip MS or the use of the sensor as micropurification unit in the analysis of (pooled) samples. Although the advantages of combining SPR with MS are clear, the possibilities for direct coupling and the creation of an on-line system are limited due to the fact that most SPR instruments are equipped with a flow-through cell that does not tolerate backpressure. Consequently, to bring SPR on-line with MS a low pressure sample transfer method is necessary. Additionally, for protein quantification and identification an efficient digestion approach is required,

In the described experiments the ligand, desorbed from the SPR sensor surface, is collected in a loop and immediately directed to an on-line digestion reactor after which the formed peptides are accumulated and desalted on a trapping column prior to liquid chromatography (LC) and MS detection. The system is automated and programmed in a sequence of timed events that can easily be adapted to meet particular requirements. The total runtime is limited compared to current methods for SPR-MS due to efficient sample collection and the on-line digestion and the possibility to start a new interaction analysis experiment while finishing the nanoLC-MS/MS in a running cycle. The mid-femtomole protein sensitivity is comparable with values for SPR-MS/MS observed in other studies. The results show that the automated, on-line system for SPR-digestion-nanoLC-MS/MS is able to isolate and identify proteins present

in a complex sample. The developed system could be a relevant tool in proteomics, residue analysis, biomarker identification and (orphan) receptor screening. Future experiments using the SPR-digestion-nanoLC-MS/MS system will focus on the analysis of real biological samples as well as on increasing the sample throughput.

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6.5. Literature

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7.

Quantification and identification of β_2-microglobulin in urine using an on-line SPR-digestion-nanoLC-MS/MS system

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Abstract

A fully automated setup combining Surface Plasmon Resonance (SPR), enzymatic protein digestion, nanoLC and tandem-MS (MS/MS) was used to develop a method for quantification and identification of the protein β_2-microglobulin (B2M) in urine. Protein concentrations ranging from 100 ng mL^{-1} to 10 µg mL^{-1} were prepared by adding B2M to urine, and 90 µL of these samples were introduced in the system at a flow rate of 10 µL min^{-1}. After desorption of the isolated protein from the SPR surface using 10 mM glycine pH 2.5, on-line digestion with pepsin and nanoLC-MS/MS analysis, B2M could be identified on basis of peptide masses and MS/MS fragmentation data. For B2M the limit of detection was 100 ng mL^{-1}. The lowest concentration in urine allowing identification based on mass and fragmentation of the proteolytic peptides was 200 ng mL^{-1}. An injection of this concentration resulted in the isolation of 50 fmol of protein for which a sequence coverage of 12% was obtained. The developed method allows the quantification and identification of B2M present in diluted urine samples.

7.1. Introduction

SPR biosensors have obtained a firm position in protein research since their commercial introduction in the early 1990s [1-3]. An important reason for this is the possibility to acquire kinetic information from the real-time data on, for example, the interaction between a ligand and receptor [4-6]. Also an accurate determination of the amount of material present on the sensor surface is possible as there exists a linear relation between the amount of bound protein and the sensor response [7]. Additionally, the various surface chemistries that can be employed, the possibility to regenerate the sensor surface between measurement cycles, and the fact that labelling of the interactants is not required, make SPR a versatile tool allowing various interaction studies such as the screening for potential drug candidates [8, 9] or protein concentration determinations. The latter can be accomplished using a kinetic [10, 11] or an equilibrium approach [12-14] allowing nM protein-concentrations to be determined. However, accurate quantification of even low pM concentrations of a protein is possible by amplification of the primary response using a (labelled) secondary antibody [15, 16].

Although the amount of an active protein in a sample can be determined effectively with SPR, it is important to consider the fact that the immobilised receptors used in SPR recognise an epitope of the ligand and not the ligand as a whole. Therefore, as the SPR response is merely the determination of the refractive index change at the sensor surface caused by (non-)specific binding of a substance, the identity of an interacting protein has to be established using another strategy. Currently, the main technique for protein identification is mass spectrometry (MS), which is often used in proteomics. In these cases, the protein is identified on basis of the molecular weight of the protein and/or peptides obtained after proteolysis. Fragmentation of the latter during a tandem-MS experiment and sequencing may provide the required additional information.

Several groups have explored the combination of SPR interaction monitoring and MS for the quantification and identification of proteins. "On

chip" SPR-MS using Matrix Assisted Laser Desorption and Ionisation – Time-of-Flight (MALDI-ToF) MS to determine the molecular weight of the protein bound to the receptor [17-21] was first reported. This approach was refined using a transfer-pin positioned near the sensor surface. The pin is an exact copy of the sensor surface and is used for further MALDI-MS analysis of isolated ligands [22]. Currently, the most often implemented strategy for off-line SPR-MS is the desorption and collection of the SPR isolated protein for further study using various techniques used in protein research [23-26].

In the combinations of SPR and MS, MALDI-ToF is the method generally used for MS analysis as it is both sensitive and accurate, and is relatively tolerant for minor contaminants present in a sample such as salt residues originating from the various non-volatile buffers used in SPR [19]. Nevertheless, Electrospray Ionisation-MS (ESI-MS) can also be performed as was shown in papers describing the on-chip isolation and digestion of proteins, followed by LC-ESI-MS/MS analysis of the peptides [27-29]. Recently a system for on-line SPR-digestion-LC-MS/MS was developed [30]. The fully automated platform was used for the isolation and quantification of the protein interferon-γ from a diluted plasma sample, the on-line digestion using either a trypsin or a pepsin micro-reactor, and identification of the peptides using LC-MS. A new analysis cycle could be started every 60 min without manual intervention [30].

In the various SPR-MS studies summarised above, protein quantification only means that the amount subjected to MS analysis is known. The commonly employed destructive method of either on-chip MS or digestion prior to MS analysis makes repetitive concentration determinations using the same sensor chip unfeasible. Furthermore, due to the small sensor dimensions of most commercial SPR instruments, surface saturation was shown necessary in order to allow protein ligand identification during LC-MS analysis [27, 29]. This generally means that the SPR response is out of the linear calibration range. SPR sensors with larger surface areas capable of isolating sufficient ligand to allow MS identification at lower concentrations have not been used for on-line SPR-LC-MS purposes, although the use of such sensors has been described before for

micropurification purposes [31, 32]. Similarly, the BIACORE off-line surface preparation unit (SPU) has been occasionally used for ligand isolations [33].

The present study describes an application of an SPR-MS system in which quantification, digestion and identification of a protein in a biological matrix is performed repetitively in a single run. The concentration and the identity of the protein β_2-microglobulin (B2M) in urine is determined in a biologically relevant range using the aforementioned SPR-digestion-LC-MS/MS system and SPR sensors with a relatively large surface area. Since the method of operation is non-destructive, calibration and concentration determination can be carried out reproducibly on a single chip. The identity of the isolated protein is established by MS analysis using an ion-trap MS. Apart from the SPR biosensor and the MS, the developed on-line system also includes on-line protein digestion using a pepsin-modified open-tubular reactor and a reversed phase nanoLC for the separation of the peptides produced during proteolysis prior to MS analysis. The feasibility of the on-line SPR-digestion-LC-MS/MS system for the isolation, quantification and identification of a ligand from urine over a range of concentrations is studied.

7.2. Experimental

7.2.1. Materials

The SPR equipment used was from IBIS Technologies (Hengelo, The Netherlands) and was equipped with a home-made 0.5-µL flow-through cell. The gold sensor disks, purchased from SSENS (Hengelo, The Netherlands), were positioned on the IBIS prism using index-matching oil from R.P. Cargille Laboratories Inc (Cedar Grove, USA). PEEK nuts, unions, tubing, and loops were from Upchurch Inc (Santa Monica, USA). Model 10AD*vp* HPLC pumps, a SCL10 A*vp* controller, Class VP software (version 5) and an A/D converter were obtained from Shimadzu (Kyoto, Japan). The injector consisted of a Gilson 231 sample injector and a Gilson Dilutor 401 syringe pump (Den Haag, The Netherlands). Cheminert CN2 six port - two position valves and actuators were

from VICI (Schenkon, Switzerland). Fused-silica capillaries used for the preparation of the microreactors were purchased from Bester (Amstelveen, The Netherlands). Manual injections during the preparation of the immobilised enzyme reactor (IMER) were performed using a Rheodyne 7010 injector (Inacom, Veenendaal, The Netherlands) equipped with a 1-mL PEEK loop. Water was produced by a Sartorius Arium 611 ultrapure water system (Nieuwegein, The Netherlands; conductivity > 18.2 MΩ cm). The reversed-phase trapping columns were 1 mm x 500 µm Styros 2R PEEK Nano Trap columns and were obtained from OraChrom, Inc (Woburn Ma, USA). The 150 mm x 100 µm Chromolith CapRod RP18E column was obtained from Merck (Darmstadt, Germany). The XCT iontrap-MS was from Agilent (Amstelveen, The Netherlands).

Acetic acid, boric acid, calcium chloride ($CaCl_2$), ethanol, ethanolamine (EA), formic acid (FA), glycine, hydrochloric acid (HCl), sodium dihydrogen phosphate, sodium chloride, sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile and ethanol were from Biosolve (Valkenswaard, The Netherlands). Acetone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), trifluoro acetic acid (TFA), tris-hydroxymethylaminoethane (TRIS), and urea were purchased from Acros Organics (Geel, Belgium). Human β_2-microglobulin, polyoxyethylenesorbitan monolaurate (Tween 20), porcine pepsin and sulfo-*N*-hydroxysuccinimide (s-NHS) were purchased from Sigma (St Louis, USA). The monoclonal mouse anti-human β_2-microglobulin antibody (clone B2M-01) was purchased from Alexis (Axxora, Raamsdonksveer, The Netherlands). Aminopropyl triethoxysilane (APTES), carbonyl diimidazole (CDI) and mercapto ethanol (ME) were from Aldrich Chemical Company (Milwaukee, USA). Carboxyl modified dextran (CMD) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) were purchased from Fluka (Buchs, Switzerland). Amino modified dextran (AMD) was from Unavera ChemLab (Mittenwald, Germany). Urine samples were collected from three subjects, centrifuged at 10000 g and filtered through a Minisart RC15 Syringe Filter (pore size 0.2 µm, Sartorius Nieuwegein, The Netherlands).

HEPES buffered saline-EDTA Tween 20 (HBS-ET) consisted of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% Tween 20 set to pH 7.4 using 2 M NaOH.

7.2.2. Preparation of the SPR surfaces and pepsin IMER

The CMD modified sensor surfaces were prepared according to a method published earlier [13]. For antibody immobilisation, a CMD sensor was placed in the SPR and a 0.5-µL flow-through cell was positioned on top of the surface. The immobilisation of the antibody was performed by three 9-min injections of 200 mM EDC and 50 mM NHS in water, 50 µg mL^{-1} antibody clone B2M-01 in 10 mM acetic acid (pH 4.7) and 1 M ethanolamine pH 8.5 in a 10 µL min^{-1} HBS-ET buffer flow, respectively.

The preparation of a pepsin IMER in dextran coated fused silica capillaries was performed according to an earlier published method [34]. The IMER (internal diameter 50 µm, length 1.53 m, volume 3 µL) was positioned between the SPR and a 6-port 2-way valve containing a trapping column as shown in Figure 1 and used for on-line protein digestion at a flow rate of 2 µL min^{-1} using a 2% acetonitrile solution containing 0.05% TFA for sample transport.

7.2.3. System control and SPR analysis

The instruments in the platform (as shown in Figure 1) and the data acquisition were controlled through an A/D converter and the Class VP software (version 5). Additionally, the SPR sensor response was also monitored using IBIS Data Analysis software installed on the same computer. In the Class VP software a number of timed events was programmed. Upon the injection of the sample, the system program is started and in time a number of triggers is generated to set off an action, such as the collection of the regenerated ligand, the digestion and the commence of the nanoLC-MS analysis of the produced peptides. The total run time of the program is 55 min. The sequence of events and time necessary to conduct the actions including the digestion and chromatography is presented in Figure 2.

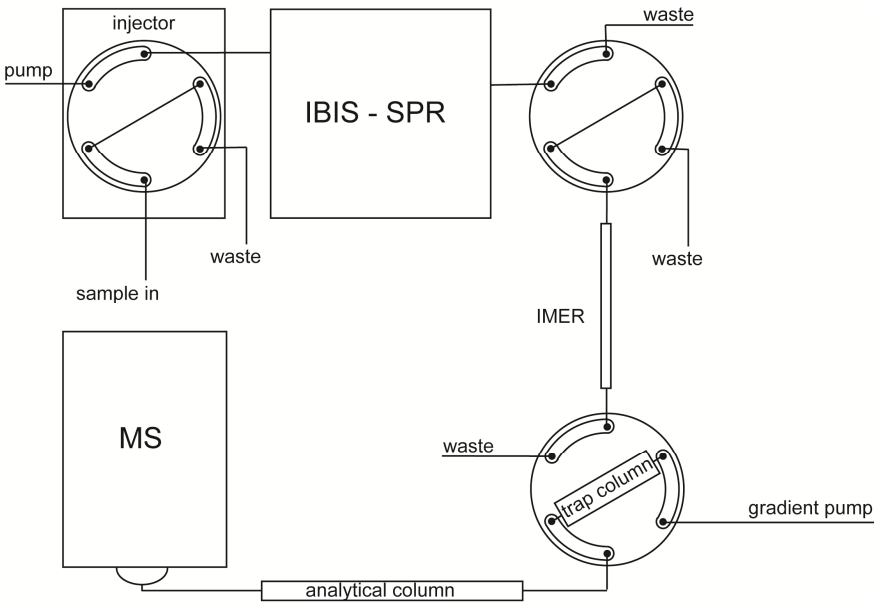

Figure 1: Experimental set-up used for the on-line SPR, digestion, nanoLC trapping and separation followed by ESI-MS/MS using an ion trap.

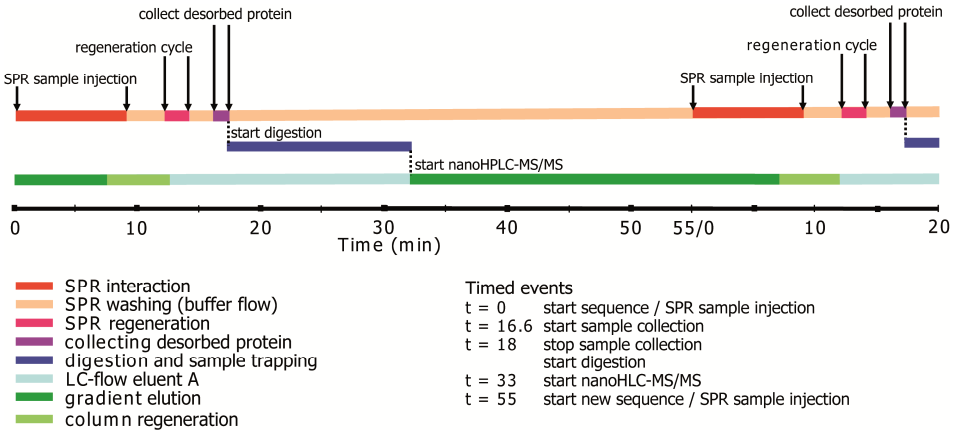

Figure 2: Schematic representation of the events as executed by the automated SPR – digestion - LC-MS/MS platform.

During the SPR interaction analysis experiments, the 9-min sample injections were followed by a 3-min HBS-ET buffer flow to remove any unbound material from the sensor surface and the flow channel. For the regeneration a 1-min plug of 10 mM acetic acid pH 4.5 was injected to remove any running buffer components which might impede both digestion and analysis. This was immediately followed by a 1-min injection of 10 mM glycine pH 2.5 for ligand desorption. The collection valve was switched to retain the liquid containing the eluted ligand upon arrival of low pH regeneration solution, which was calculated on basis of the flow rate and tubing volume and checked by the pH change. All SPR experiments were conducted at a flow rate of 10 µL min^{-1} and a controlled temperature of 25 °C. The on-line digestion and nano-LC separation were conducted at room temperature (typically 23 °C).

7.2.4. Sample analysis using nanoLC-MS

At the start of the acetonitrile gradient, the trapping column (TC) containing the concentrated and washed digested protein sample, was positioned in-line with the analytical nanoLC-column. The LC gradient was composed of two solutions: (A) 2% acetonitrile in water containing 0.1% FA and 0.01% TFA and (B) 80% acetonitrile in water containing 0.08% FA and 0.01% TFA. In 30 min the composition changed from 0 to 50% B, followed for 10 min by 90% B and eventually for a 20-min period by 0% B. The flow rate was 300 nL min^{-1} and the column outlet was connected to a nano-electrospray interface positioned perpendicular towards the Agilent XCT iontrap inlet. The ESI-MS was conducted in the positive ion mode with the capillary voltage set at 1000 V. Flow rate and temperature of the nitrogen drying gas were 5 L min^{-1} and 150 °C, respectively. The peptide fragment sequences were determined using the iontrap MS in the auto-MS/MS mode. All experiments were conducted at room temperature. The resulting data was analyzed using FindPept (www.expasy.org) and/or Mascot (www.matrixscience.com).

7.3. Results and discussion

7.3.1. SPR set up and β_2-microglobuline quantification

For the SPR concentration determination in a biological matrix prior to LC/MS identification, an antibody - antigen pair was used consisting of the protein human β_2-microglobulin (B2M) and the mouse-monoclonal antibody anti-human β_2-microglobulin clone B2M-01. The protein, a single polypeptide chain of 99 amino acids with a molecular weight of 11.6 kDa, is present on the cell surface of mainly myeloid and lymphoid cells and is identical to the light chain of the human leukocyte antigens. The concentration of urinary low molecular weight proteins such as B2M is generally determined using an immunoassay e.g. ELISA [35]. In healthy adults B2M may be found in blood serum and urine in low amounts (less than 2 µg mL^{-1} and 0.8 µg mL^{-1}, respectively). Elevated levels are observed in some forms of cancer, AIDS and renal failure due to e.g. diabetic complications [36-40]. During the experiments conducted in this study, B2M was isolated from urine.

For the SPR experiments, a flow-through cell was constructed that consisted of the sensor disk, a polycarbonate block with in- and outlet and a 50-µm Teflon spacer with a punched oval perforation creating a 10 mm^2 sensor surface. Using the immobilisation protocol described above, an antibody immobilisation level of 7.6 ng mm^{-2} is obtained (angle shift 910 ± 7 m°, n = 3). Sensorgrams of blank injections and injections containing 1 µg mL^{-1} B2M are shown in Figures 3A and 3B, respectively. As shown in these figures, upon the injection of 1 µg mL^{-1} B2M in buffer, a response of 51 ± 2 m° (n=6) is observed just before regeneration. Upon the injection of a 10x-diluted urine sample containing 1 µg mL^{-1} B2M, an angle shift of 48 ± 2 m° is observed (n=6, data not shown). Similarly, the injection of 1 µg mL^{-1} B2M in 2x-diluted urine results in an angle shift of 49 ± 2 m° (n=3), which is equal to the response generated in buffer and 10x-diluted urine. During the injection of the latter samples a large refractive index change is observed due to the salts and other constituents present in these samples. However, after the injection of the spiked urine sample is finished, the response

immediately returns to the level observed for a concentration of 1 µg mL^{-1} B2M in buffer. When a diluted urine sample containing no B2M is injected, no protein binding is detected. These observations indicate that non-specific adsorption from the sample is not detectable. For regeneration a solution of 10 mM glycin, pH 2.5, is used which shows effective desorption of the ligand without affecting the binding characteristics of the receptor molecule. Consequently, at least 50 cycles of interaction between ligand and antibody followed by regeneration can be performed without loss of response.

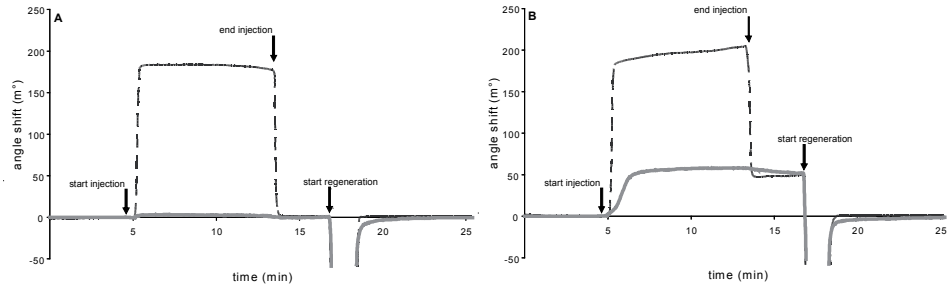

Figure 3: Sensorgrams of the interaction of immobilised mouse-anti B2M and B2M present in the sample. (A) buffer (grey) and 2x-diluted urine (black, dashed) and (B) buffer (grey) and 2x-diluted urine (black, dashed) each containing 1000 ng mL^{-1} B2M.

In a series of injections, several antigen concentrations were introduced on the antibody-modified SPR sensors. The B2M samples were prepared both in buffer as well as in 10x-diluted urine, which is a dilution generally used for ELISA analyses, and in 2x-diluted urine. The described SPR concentration determination, which can be performed in approximately 15 min including sensor regeneration, features a detection range of 100 ng mL^{-1} to 5000 ng mL^{-1} B2M present in the injected sample. On basis of the observations, the response curve shown in Figure 4 can be constructed. As shown in this figure, both 2x- and 10x-diluted urine samples spiked with B2M gave a response equal to B2M in buffer. In a study of biomarkers for septic acute kidney injury [41], normal urine B2M values have been reported to vary between 16 and 760 ng mL^{-1}. This means that elevated concentrations of B2M observed in urine, attributed to several physical disorders [36-40], can be quantified using the described method.

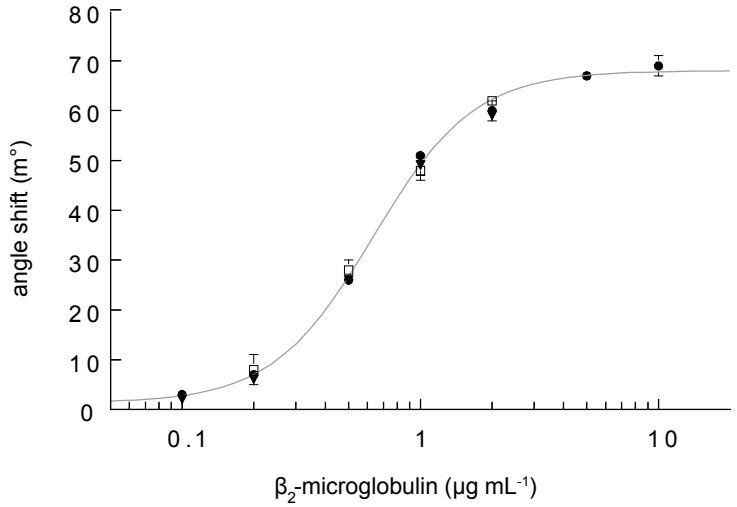

Figure 4: The response curve of B2M as a function of the injected concentration; (●) B2M in buffer, (□) B2M in 10x-diluted urine and (▼) B2M in 2x-diluted urine.

Moreover, when lower concentrations have to be determined using the described SPR assay, response amplification using a secondary antibody as described elsewhere [15, 16] might be performed. The need of a second specific antibody and the presence of this antibody during proteolysis and MS identification of the SPR bound protein as described below, makes this approach less attractive.

7.3.2. Proteolysis and MS identification

In contrast to the immunoaffinity methods such as ELISA which are generally employed for B2M determinations, the SPR method can be combined with a second analysis method aimed at protein identification. In the present system this is accomplished by combining the SPR with on-line digestion and nanoLC-MS as described earlier [30]. For the on-line digestion of proteins, protease microreactors have been developed [34, 42], which have shown to be capable of efficient digestion of proteins. Since the mouse monoclonal antibody used during the experiments is regenerated using acidic solutions, a pepsin reactor is employed during this study which, in contrast to trypsin, shows high proteolytic activity at the pH value of the buffer used for protein desorption.

Consequently, the solution containing the desorbed B2M needs no pH correction. As shown in Figure 1, this reactor is placed between the valve in which the loop for collecting the desorbed ligand is positioned, and the reversed phase trapping column.

In order to avoid interference of running buffer components during pepsin digestion and subsequent chromatography, the buffer HBS-ET is removed from the flow-cell using a 1-min injection of an acetic acid buffer (10 mM, pH 4.5), immediately followed by the regeneration solution. As repeated injection of only the washing buffer after a B2M injection did not lead to a change in response, obviously the protein bound to the antibody-modified sensor surface is not removed during exposure to this buffer. Upon the arrival of the desorbed protein noticeable by the pH change caused by the regeneration buffer, the position of the aforementioned valve is changed for the collection of the sample. When the sample is collected the valve is changed to its original position and digestion commences. The identity and sequence of the desorbed protein subjected to proteolysis is then retrieved on basis of both the mass of the peptides and MS/MS fragmentation and analysis.

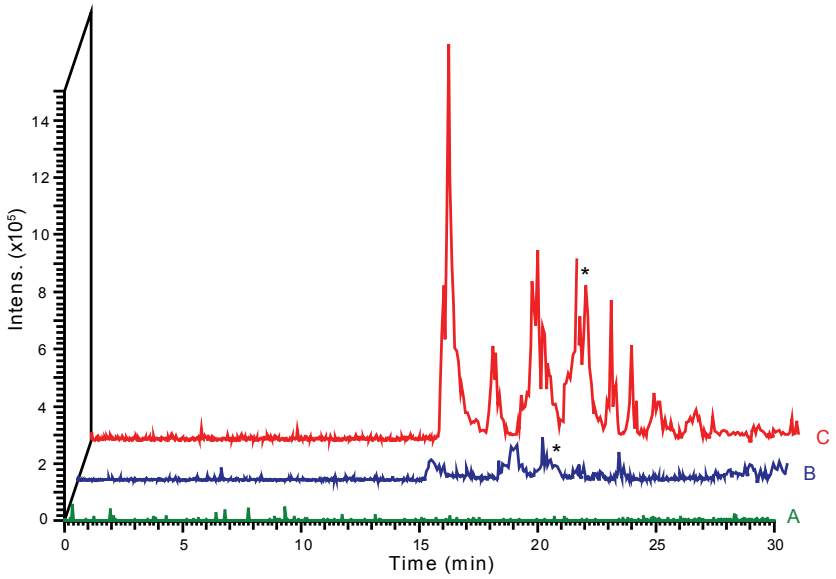

Figure 5: Base peak chromatograms for the MS/MS analysis of 2x-diluted urine containing (A) 0 ng/mL^{-1}, (B) 200 ng mL^{-1} and (C) 2 µg mL^{-1} B2M.

The system for on-line SPR-digestion-LC-MS/MS was applied to the isolation and identification of B2M present in urine. The protein bound on the antibody modified sensor surface was desorbed, digested, and concentrated and desalted on a trapping column as described above. In the following nanoLC-ESI-MS/MS analysis, a number of peptides was fragmented and identified as digested fragments from the isolated B2M. In Figure 5 base peak chromatograms of the analysis of diluted urine to which B2M is added, are shown. The asterisk in both graphs is positioned at the peak of the proteolytic peptide (L)/SFSKDWSF/(Y). In blank urine no significant signal is observed and in a database search no peptides are found. In the spiked urine samples several peaks can be observed that can be attributed to peptides originating from B2M. Figure 6A presents the MS/MS fragmentation and Mascot analysis result of the aforementioned peptide (L)/SFSKDWSF/(Y) with m/z 502.4, retrieved from a sample containing 2 µg mL^{-1} B2M. Similarly, Figure 6B shows the matched fragment ions of the same peptide from a urine sample with 200 ng mL^{-1} B2M. As can be observed in these figures, both the ion intensity compared to background ions and thus the number of identified y- and b-fragment ions are lower for the lower concentration of B2M.

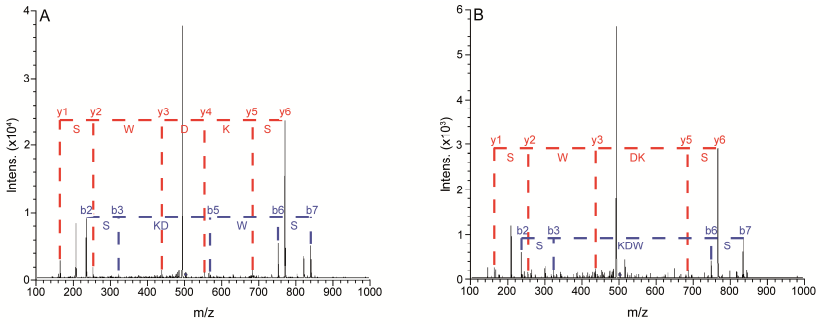

Figure 6: MS/MS fragmentation of the peptide SFSKDWSF with m/z 502.4 showing several of the matched fragment ions. The peptide originates from the protein B2M injected in a concentration of (A) 2 µg mL^{-1} and (B) 200 ng mL^{-1} both in 2x-diluted urine (see text).

As is shown in Figures 6A and 6B, identification of a protein becomes increasingly difficult when less material is present due to the low intensity of the ions formed during MS/MS fragmentation of the proteolytic peptides.

Nevertheless, unambiguous protein identification on the basis of peptide masses as well as on fragmentation results is still possible for injected concentrations of 200 ng mL^{-1} B2M. Upon the injection of this concentration the peptides (Y)/VSGF/(H), (L)/KNGE/(R) and (L)/SFSKDWF/(Y) have been retrieved in a database search of the fragmentation results, which corresponds to a sequence coverage of 12%. The SPR response generated by the injection of this concentration corresponds with a total amount of protein of 50 fmol. When higher concentrations of B2M in urine or buffer are injected the protein coverage increases to approximately 70% for concentrations of 2 µg mL^{-1}. Upon the injection of a urine sample containing 100 ng mL^{-1} occasionally fragments with a m/z value are observed that correspond to peptides found at higher concentrations. However, a database search did not result in peptide identification at these concentrations. In runs with urine containing no B2M no m/z signals were observed in the MS analysis that were above threshold.

The results described above obtained for the lower concentration are comparable with SPR-MS protein identification described elsewhere allowing the identification of multiple peptides [27-29]. However, the presented automated system allows the analysis of a range of concentrations thus making protein concentration determination over a relevant range possible. An important advantage of the developed method is that the sensor surface can be reused for a new analysis as the receptor molecule is not affected due to either on-chip digestion or on-chip MS. The on-chip MS methods, combining SPR with MALDI-ToF MS, detect the isolated protein on the basis of its total mass [17, 21] and for e.g. B2M detection limits of 700 amol total protein have been reported [19]. Still, for unambiguous protein identification accurate determination of the peptide masses is helpful, but tandem MS information is required. An approach that allows the unambiguous identification of an antibody isolated protein using on-chip SPR-MS was described in a recent study [43]. The on-chip digestion followed by MALDI-ToF MS/MS allowed protein identification at a level of 6 fmol mm^{-2} based on peptide mass and tandem MS result. However, due to performing the digestion on chip the sample also contains fragments of the

protease caused by autodigestion and partially digested receptor protein which is present on the sensor surface.

Secondly, besides sensor surface reusability, the time needed for a complete experiment using the on-line SPR-digestion-nanoLC-MS system is advantageous compared to other methods combining SPR and MS. The off-line and automated on-line SPR nanoLC-MS approaches described elsewhere [27, 29] take 3 to 11 h for a single run, respectively. The main cause for this is the time needed for the digestion of the SPR-isolated protein. The system described in this paper is capable of starting a fully automated experiment consisting of SPR interaction analysis and desorption, efficient digestion, trapping and desalting, followed by nanoLC and MS/MS every 55 min. Additionally, the off-line system needed manual intervention as the ESI-MS analysis was performed separately from the SPR isolation and protein digestion. Consequently, sample handling which might lead to sample loss using an earlier reported off-line approach [23] is minimised.

7.4. Conclusions

SPR biosensors are versatile devices that have often been used for micropurification and quantification of proteins and peptides. Nevertheless, for the identification of the ligand bound to the sensor another technique, such as mass spectrometry (MS), has to be employed. In the described experiments, SPR is used for the determination of the concentration of a protein in a biological matrix. The isolated protein is then desorbed from the SPR sensor surface, collected in a loop and immediately directed to an on-line digestion reactor after which the peptides, formed during the process of digestion, are accumulated and desalted on a trapping column prior to liquid chromatography (LC) and MS detection. The system is automated and programmed in a sequence of timed events that can easily be adapted to meet particular requirements. The total runtime is limited compared to current methods for SPR-MS due to efficient sample collection, the on-line digestion and the possibility to start a new SPR analysis while finishing the LC-MS/MS of the

previous sample. Optimisation of the chromatographic method and the digestion step, e.g. by trapping and concentrating the protein sample desorbed from the sensor surface prior to digestion, might save time and further increase the throughput.

Using the SPR-MS method, the concentration of B2M present in urine can readily be determined and B2M can be identified in a biologically relevant concentration range. Although the femtomole protein sensitivity is comparable with reported SPR-MS/MS methods, the proposed method allows protein quantification and identification in only 55 min for a full analysis. The results show that proteins present in a diluted sample can be isolated on an antibody modified sensor surface and efficiently removed from the surface using a glycine regeneration solution, allowing for subsequent acidic proteolysis using pepsin and MS identification of the fragments. Applying this strategy, the sensor chip can be reused for a new analysis cycle which is not possible when MS analysis is performed on-chip. The system allows the development of various antibody or receptor based applications aimed at protein binding monitoring and identification and future research will investigate the application of the developed system for orphan receptor screening.

7.5. Literature

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8.

Conclusions and perspectives

8.1. Introduction

In this thesis a system comprising a surface plasmon resonance (SPR) sensor, open-tubular proteolytic enzyme reactor and an ion-trap mass spectrometer (MS), has been developed and its potential for quantification and characterisation of a ligand interacting with an immobilised receptor has been demonstrated. In this chapter general comments and conclusions on the various aspects related to the developed surface chemistry, reactors, the system interfacing, setup and applicability are provided. Additionally, suggestions for future research and potential alternative systems will be given.

Although SPR-MS as a system for studying molecular interactions, e.g. ligand fishing, orphan receptor and drug screening, has been in use since 1997, several aspects remain to be improved. Among these are non-specific adsorption (NSA) and surface modification allowing protein coupling. These are related, as they concern sensor-surface coating allowing both the immobilisation of receptor molecules as well as preventing unwanted interactions. In the case of an on-line SPR-LC-MS/MS system, an interface between the SPR and the LC system is required. Additionally, there is need for a means of efficient digestion of isolated proteins in order to allow tandem-MS identification.

The topics that were studied in this thesis arose from these subjects. First surface-coating development was performed for both sensors and enzyme reactors. The developed sensors and reactors were characterised. Next, the integration of all necessary instruments was investigated, ultimately evolving into a proof of principle. Lastly, an application was developed in which the isolation, quantification and identity confirmation of β_2-microglobulin in urine was performed.

8.2. Surface chemistry for SPR sensors

The successful application of interaction monitoring depends both on the possibility of immobilizing a receptor molecule and on the prevention of non-specific and irreversible adsorption processes. Several coatings were tested

for their ability to prevent non-specific adsorption. As a matrix in this SPR-based study buffer-diluted plasma was used. The best results with respect to plasma adsorption and surface regenerability are obtained with dextran-based MUA SAMs. Three different variations of these dextran surfaces were tested, i.e. carboxyl-modified dextran, hydrazide-modified dextran and native dextran. Although low non-specific interactions were observed for all three modifications, the best results were obtained with native dextran coatings as the adsorption of plasma constituents is the lowest. Although less antibody molecules are immobilised on sensors coated with native dextran than on sensors with a carboxyl-modified dextran surface, the interaction between the immobilised MD-2 antibody and its ligand IFN-γ results in a relatively high response. This makes the non-modified dextran-coated SPR sensor disks well suited for the detection of IFN-γ in complex matrices like plasma with a lower detection limit of 250 ng mL^{-1}.

Hydrogels such as the different forms of carboxyl-modified dextran allow high immobilisation yields, but show non-specific adsorption of, especially, positively charged groups. Native dextran hydrogels allow antibody immobilisation with smaller loading but also with minimal non-specific adsorption. A different material known for its non-fouling characteristics is oligoethyleneglycol (OEG) such as 1-mercapto-11-undecyl tri(ethylene glycol). The highly ordered structure of the latter has proven to be efficient against adsorption of matrix components when applied on sensor surfaces. However, these OEG-surfaces only allow for a single layer of receptor molecules, meaning that the potential amount of ligand isolated from the matrix is limited compared to the three-dimensional structure offered by a hydrogel. The sensor surface area and the requirements for the downstream analysis method determine the choice of surface chemistry. In the case of on-line SPR-LC-MS/MS the use of hydrogel surfaces is essential to immobilise a large amount of antibody thus increasing the isolation capacity. In order to isolate an adequate quantity of ligand the choice was made to use carboxyl-modified dextran for the sensor surface. By extensive blocking of the carboxyl groups after antibody attachment

and using an additional washing step prior to desorption, non-specific adsorption during operation is minimised.

8.3. Protease-immobilised enzyme reactors

For unambiguous identification the mass determination of the antibody-isolated protein itself is generally not sufficient and tandem mass spectrometry (MS) information on peptide fragments obtained after proteolysis is required. Therefore open-tubular trypsin and pepsin reactors were developed that could be positioned between SPR and the nanoLC instrument. The requirements are high enzymatic activity to minimise the time needed for digestion and a small volume (diameter) of the device. The latter is accomplished by using fused-silica capillaries. To study the immobilisation of the protein trypsin, a number of silica surface modifications was tested. In order to investigate the amount of protein that can covalently be attached to these surfaces, SPR sensor disks are adapted with these coatings to mimick the capillary surface. The SPR measurements show that the highest quantities of trypsin could be immobilised using a two-layer dextran coating consisting of a silanised silica surface successively tailored with carbodiimide activated carboxyl-modified dextran and amino-dextran. Enzyme activity determinations show that the immobilised enzyme is fully active.

The digestion efficiency of the resulting trypsin capillary reactors depends on the internal diameter of the immobilised enzyme reactor (IMER) and the flow-rate indicating that diffusion is an important parameter in the reactor. The conversion of the insulin B chain improves with smaller i.d. capillaries and decreasing flow and hence a longer residence time. The same is observed for the digestion of horse cytochrome C. The complete digestion of horse cytochrome C is observed without the need of protein denaturation, reduction or alkylation. In a capillary IMER of 140 cm x 50 µm the average residence time of the protein sample is 165 s. The resulting open-tubular trypsin-reactors having a pH optimum of pH 8.5, display a stable high activity when operated at 37°C for at least two weeks when used continuously.

Identification of the proteins cytochrome C and myoglobin is possible by analysis of the tryptic peptides that are on-line produced using MS/MS.

Desorption of ligands from the receptor is often accomplished by injecting a low-pH buffer, which is not compatible with the protease trypsin. Although pH correction is feasible by infusing a neutralizing buffer, digestion at low pH is simpler in case of combining affinity-based isolation methods and nanoLC-MS/MS. Moreover, using different proteolytic enzymes with varying specificities might be useful when analysing unknown proteins. For that reason, a pepsin micro-reactor was produced in a fashion similar to the trypsin reactor for the on-line digestion of proteins. During characterisation of the pepsin IMER the reactors proved to be stable. The sequence recoveries of a number of proteins and protein mixtures subjected to proteolysis can largely be retrieved with a database search on the MS/MS data.

For the IMERs similar requirements with respect to NSA and protein/enzyme immobilisation apply as for the sensor surfaces. Several digestion methods employing immobilised trypsin exist that perform the digestion in buffers containing up to 40% of acetonitrile to denaturate the protein and, more importantly, to counteract adsorption to the carrier particles of protein and peptides produced during proteolysis. Unfortunately, the addition of acetonitrile to the digestion buffer makes sample dilution necessary prior to reversed-phase LC separation in order to prevent loss of peptides. Implementation of such a step in an on-line SPR-LC-MS/MS system would require the addition of two pumps and mixing units flanking the reactor, which makes such a set-up more complicated. Taking this into account and the fact that NSA would be detrimental to the result of the analysis, a dextran hydrogel was used as a coating for the fused-silica capillaries. As discussed above, this material is very efficient in the prevention of NSA and allows the immobilisation of considerable amounts of enzyme.

Various materials for protein digestion are commercially available such as Poroszyme and trypsin-modified agarose. Unfortunately, preliminary tests using home-made micro-columns containing these commercially available

materials or trypsin-modified porous glass did not perform as expected and showed incomplete digestion of the model protein cytochrome C. In contrast, both types of open-tubular reactors proved to be capable of efficient proteolysis of proteins at concentrations varying from 1 nM to 20 µM in buffer. Protein carry over was not observed in these experiments. This demonstrates that both reactors are a useful tool for the characterisation of unknown proteins. Use of the developed IMERs is not necessarily limited to SPR-LC-MS/MS. As these open-tubular reactors essentially consist of coated fused-silica, they can be produced easily, reproducibly and inexpensively. The IMERs show hardly any backpressure and can easily be implemented in both LC-MS and CE-MS methods and miniaturised multi-dimensional analysis systems. Other applications may include enzyme-inhibitor studies.

8.4. On-line SPR-nanoLC-MS/MS system with on-line digestion

Although the advantages of combining SPR with MS are clear, the possibilities for direct coupling and the creation of an on-line system are limited due to the fact that most SPR instruments are equipped with a flow-through cell that tolerates limited backpressure. Consequently, to bring SPR on-line with MS, a low-pressure sample-transfer method is necessary. In the described experiments the ligand, desorbed from the SPR sensor surface, is collected in a loop and immediately directed to the developed on-line digestion reactor after which the formed peptides are accumulated and desalted on a trapping column prior to nanoLC separation and MS/MS analysis. The system is automated and programmed in a sequence of timed events that can easily be adapted to meet particular requirements. Although the protein sensitivity is comparable with other systems combining SPR and LC-MS/MS, the total run time is less due to efficient sample collection combined with the on-line digestion. Additionally, a new analysis can be started while the running cycle is not finished. In contrast to the existing SPR LC-MS methods that employ on-chip digestion, and consequently a replacement of the sensor-chip after each analysis, this allows multiple analyses and thus a more economical use of materials and equipment.

In reported SPR-MS approaches quantification generally implies the determination of the absolute *amount* of protein isolated on the sensor surface. Due to the fact that the sensor chip can be reused for a new sample, which is not possible when either digestion or MS analysis is performed on-chip, it is possible to calibrate the SPR analysis and to use it repeatedly for protein *concentration* determination. It is demonstrated that by means of the developed SPR-MS method, the concentration of β_2-microglobulin present in a urine sample can readily be determined. Additionally, the identity of the protein can be established in a biologically relevant concentration range. The proposed method allows a rapid protein quantification and confirmation of identity taking only 55 min for a full analysis cycle. The results show that proteins present in a diluted sample can be isolated on an antibody-modified sensor surface and efficiently removed from the surface using a glycine-regeneration solution, allowing for subsequent acidic proteolysis using pepsin and MS identification of the fragments.

8.5. General conclusions and perspectives

The aim of the research project was to develop an on-line SPR-MS system for protein concentration determination and identification. The current methods are either variations of the on-chip SPR-MS setup or elution-based procedures, the characteristics of which are discussed in **Chapter 2**. In the developed system, manual intervention was omitted. The efficiency of the developed sensor surfaces and protease micro-reactors proved sufficient for system operations and the results show that the automated, on-line system for SPR-digestion-nanoLC-MS/MS is able to isolate, quantify and identify proteins present in a complex sample. The developed system could be a relevant tool in e.g. proteomics, residue analysis, biomarker identification and (orphan) receptor screening. Future studies using the SPR-digestion-nanoLC-MS/MS system may focus on the analysis of biological samples using other antibody or receptor molecules, e.g. for interaction proteomics purposes, the investigation of signalling pathways and metabolomics.

The characteristics of the available MS instrument determine what the possibilities of the developed system will be. Taking into consideration that the sensitivity of SPR sensors is approximately 5 pg mm^{-2}, which equals 5 x 10^{-16} mol mm^{-2} for a 10 kDa protein, the MS should have a sensitivity that allows the detection of such amounts of protein. Several papers in which SPR instruments are coupled with MALDI-ToF MS, show that the sensitivity of both methods is of the same order of magnitude allowing protein determination and identification at a fmol mm^{-2} level. MALDI, like SPR, is dependent of the surface density of the molecules being studied, allowing the study of surface areas of only a few square micrometer. The ToF MS is capable of an accurate mass determination, allowing protein identification after digestion on the basis of a Peptide Mass Fingerprint (PMF). Therefore, the SPR-MALDI-ToF approach is a good combination that is also suited for use in array systems. A disadvantage of PMF confirmation of identity is that the protein sequence has to be known. Additionally, mixtures containing multiple proteins are difficult to process. For these latter samples MS/MS analysis is necessary to obtain sufficient specificity for protein identification based on peptide fragmentation and sequencing.

Various MS instruments are suitable for use in on-line SPR-LC-MS/MS, including Q-ToF instruments, combining the accuracy of the ToF-MS with the possibilities offered by two quadropoles for both selection and fragmentation of the ions of interest. Alternatively, iontrap (IT) MS can be used. An IT is capable of selecting and capturing ions in an electric field formed between the electrodes from which it consists. Two variations of IT instruments exist, i.e. linear and 3D. The mass spectrometer used in this thesis is of the latter type which, compared to the linear IT MS, possesses a limited ion storage capacity. This means that higher sensitivity can be obtained using a linear IT, which would be preferable for an on-line SPR-MS such as presented here. To improve relatively low mass accuracy of the IT a hybrid MS such as the LTQ-Orbitrap might be employed, combining a high capacity linear IT with an accurate Orbitrap MS.

To increase the throughput, several approaches are possible. First, when using an antibody that displays no or limited cross reactivity, verification may be possible based on the mass of the protein and digestion may not be necessary. This is also true when the interacting molecule is a peptide or other small-molecule interactant. In these cases trapping and washing of the desorbed ligand on a trapping column prior to LC-MS analysis is sufficient for confirmation based on the accurate determination of the molecular mass. Second, by adopting shorter columns with high separation efficiency, such as polymeric monolithic stationary phases and/or sub-2 µm particle LC columns, much shorter separation times and improved detection limits caused by higher efficiency might be obtained. Lastly, in the near future developments in imaging SPR (SPRi) sensors may allow the analysis of multiple ligands in a sample. Compared to conventional SPR, SPRi allows the visualisation of an entire sensor chip surface and the possibility to continuously monitor multiple interactions at the same time. In this way an array with possibilities for creating all kinds of controls to correct for e.g. non-specific adsorption can be created. Several SPRi array platforms are commercially available nowadays, allowing 16 to 400 interactions to be monitored simultaneously in a flow-cell volume ranging from 6 to 46 µL. Although the use of SPRi does not increase throughput perse as the analysis time is still the same, the number of interactions per SPR-experiment is higher. Consequently, the information-density for a sample is increased in a time-efficient way.

Unfortunately, the possibilities for coupling the current generation of SPRi sensor arrays with MS are limited to direct on-chip MS detection due to the high risks of cross contamination when processes such as sample recovery or proteolysis are executed on the sensor surface. Further drawbacks of these systems are the flow-cell volume that, in spite of sample recovery, leads to large material consumption and the fact that the whole chip is contacted with the sample meaning that parallel analysis are not possible. However, as described in Section 2.5, recent developments in the field of SPRi show that it is possible to monitor multiple ligands against different receptor molecules using a

parallel 264-microarray chamber. The fluidics system is designed for small volumes (700 pL) and able to recover samples after SPR measurement with minimal cross contamination, thus allowing MS identification. These developments show that SPR quantification in combination with MS identification is still ongoing and that the advance of lithographic technologies, when used in biosensor design, opens new fields of research and application of SPR-MS.

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Nederlandse samenvatting

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Curriculum Vitae

Publicatielijst

Immunosensoren gebaseerd op het principe van Surface Plasmon Resonance (SPR) worden, sinds zij commercieel verkrijgbaar zijn, toegepast in diverse onderzoeksgebieden. Deze lopen uiteen van concentratiebepaling en screening van eiwitten en toxines tot de bepaling van affiniteitconstanten. De kracht van SPR is gelegen in het feit dat enerzijds de interactie tussen receptor en ligand real-time kan worden gevolgd zonder (één van) de bindingspartners chemisch te labelen en, anderzijds, dat de massa die gebonden is aan het sensoroppervlak gevoelig en nauwkeurig kan worden gemeten. De selectiviteit van de SPR-metingen wordt bepaald door de selectiviteit/specificiteit van het gebruikte, geïmmobiliseerde antilichaam. De SPR-systemen zijn robuust en, dankzij een groot aantal verkrijgbare sensoroppervlakken, zeer flexibel inzetbaar.

Ondanks genoemde gunstige eigenschappen zijn er bij het gebruik van SPR-immunosensoren ook beperkingen. Aangezien de respons wordt bepaald door de totale hoeveelheid massa die gebonden is, kan er naast het - *gewenste* - specifiek-gebonden ligand ook - *ongewenst* - materiaal, b.v. uit een biologische matrix, niet-specifiek worden geadsorbeerd door het sensor oppervlak, waardoor de juistheid van de meting nadelig wordt beïnvloed. Een andere beperking, inherent aan het gebruik van antilichamen, is de selectiviteit. Antilichamen worden opgewekt tegen (een epitoop van) een eiwit. Het antilichaam herkent de epitoop van het ligand, maar eiwit-"metabolieten" of andere eiwitten kunnen dezelfde of een vergelijkbare epitoop hebben en dus ook worden gebonden door de sensor.

Het is van belang een tweede technologie te gebruiken om de identiteit van het specifiek-gebonden materiaal een-eenduidig te vast te stellen. Een techniek die hiervoor bij uitstek geschikt is, is massaspectrometrie (MS). De combinatie van SPR en MS stelt de gebruiker in staat de interactie tussen de geïmmobiliseerde receptor en het (eiwit-)ligand te volgen en de identiteit en hoeveelheid van het aanwezige ligand te bepalen.

De eerste publicatie die een combinatie tussen SPR en MS beschrijft, dateert van 1997. Sindsdien zijn diverse verschillende SPR-MS systemen

beschreven, zoals beschreven in **Hoofdstuk 2** en grafisch weergegeven in figuur 2. De mogelijkheden zijn samen te vatten in een tweetal benaderingen, namelijk:

a) *On-chip SPR-MS* waarbij zowel de SPR-bepaling van de interactie tussen receptor en ligand als de MS identificatie plaatsvindt op het sensoroppervlak.

b) *Elutie SPR-MS* waarbij het door de receptor gebonden ligand wordt gedesorbeerd en met MS kan worden geanalyseerd.

Bovengenoemde benaderingen hebben een aantal belangrijke voor- en nadelen. Bij *on-chip SPR-MS* systemen wordt de MS-analyse uitgevoerd met MALDI-ToF MS. De belangrijkste voordelen van deze strategie zijn het gebruiksgemak en de gevoeligheid van de methode. Nadelen zijn het gebrek aan automatiseringsmogelijkheden met de doorgaans gebruikte apparatuur en het feit dat de sensor slechts eenmalig kan worden gebruikt.

Een belangrijk voordeel van *elutie SPR-MS* is de mogelijkheid het sensoroppervlak te gebruiken voor een serie analyses. De gangbare methoden in elutie SPR-MS zijn off-line wat inhoudt dat manuele handelingen nodig zijn om het geïsoleerde ligand nader te analyseren. Een probleem hierbij kan zijn dat onder invloed van de oplossing gebruikt voor desorptie van de ligand, bijvoorbeeld een zuur, denaturatie en daardoor verlies van het geïsoleerde eiwit kan plaatsvinden. De gebruikte oplossing moet daarom zo snel mogelijk worden geneutraliseerd. Daarnaast kan het geïsoleerde ligand door (pipetteer-) handelingen ook deels verloren gaan. Deze nadelen kunnen worden voorkomen als alle benodigde stappen worden geautomatiseerd. De ontwikkeling van een systeem, waarbij SPR en LC-MS/MS worden geïntegreerd, wordt beschreven in dit proefschrift.

Om te komen tot een geautomatiseerd, on-line SPR-MS systeem moeten onderdelen worden ontwikkeld c.q. verbeterd. De minimalisering van de niet-specifieke adsorptie en optimalisatie van de mogelijkheden voor eiwit (receptor) koppeling zijn belangrijke punten. Een ander aspect is het realiseren

van een efficiënte manier van (on-line) digestie. Dit is noodzakelijk om tandem-MS identificatie van het geïsoleerde eiwit mogelijk te maken. Tenslotte moet een verbinding tussen SPR enerzijds en de LC-MS anderzijds worden gerealiseerd.

Succesvolle toepassing van SPR interactie-monitoring is afhankelijk van de mogelijkheid om een receptor te immobiliseren en gelijktijdig niet-specifieke adsorptie te voorkomen. In **Hoofdstuk 3** zijn de eigenschappen van een aantal oppervlakte modificaties bestudeerd met (verdund) bloedplasma als biologische matrix. De beste resultaten v.w.b. de niet-specifieke adsorptie werden behaald met dextraan-coatings. Minimale niet-specifieke adsorptie gecombineerd met goede mogelijkheden voor covalente koppeling van antilichamen werd bereikt met ongemodificeerd dextraan. Een grotere immobilisatiegraad werd gerealiseerd met carboxyl-gemodificeerd dextraan, dat echter enige niet-specifieke adsorptie vertoont, vermoedelijk door de grote concentratie carboxyl-groepen die na antilichaam immobilisatie nog in de hydrogel aanwezig zijn. Langdurige inactivatie met een hoge concentratie van bijvoorbeeld ethanolamine neemt veel van deze groepen weg waardoor de (niet-specifieke) adsorptie-eigenschappen verbeteren. De kennis, opgedaan bij de bestudering van deze hydrogels, is gebruikt bij de ontwikkeling van protease reactoren beschreven in de volgende hoofdstukken.

Voor de identificatie van een eiwit met de massaspectrometer is doorgaans tandem-MS informatie nodig van peptiden die worden verkregen door proteolyse. Om dit mogelijk te maken in een geautomatiseerd SPR-LC-MS systeem is een on-line digestie systeem nodig dat wordt geplaatst tussen de SPR en de nanoLC. De **Hoofdstukken 4 en 5** beschrijven de ontwikkeling van een open-tubular reactor zowel van trypsine als pepsine. De eisen die aan deze reactoren worden gesteld zijn een minimale niet-specifieke adsorptie en een hoge enzymatische activiteit. Om de digestie zo efficiënt mogelijk te doen verlopen wordt de diameter van de reactor zo klein mogelijk gehouden. Om die reden wordt gebruik gemaakt van fused-silica capillair. De daarin aan te

brengen oppervlakte modificaties werden eerst bestudeerd met SPR. Het blijkt dat de beste resultaten v.w.b. de te immobiliseren hoeveelheid enzym worden bereikt met een dubbele dextraanlaag. De werking van het in deze hydrogel geïmmobiliseerde trypsine is met een activiteitsbepaling vastgesteld. De eiwitten cytochroom C en myoglobine worden bij 37°C, pH 8.5 en een verblijftijd van 165 s in een reactor met een interne diameter van 50 µm volledig gedigesteerd. Deze eiwitten kunnen worden geïdentificeerd met tandem-MS op basis van de geproduceerde peptiden.

Verbreking van de niet-covalente binding tussen een ligand en een antilichaam gebeurt veelal met een zure buffer. Helaas is een dergelijke oplossing niet compatibel met het enzym trypsine, waardoor vooraf een neutralisatiestap nodig is. In **Hoofdstuk 6** is aangetoond dat zo'n stap mogelijk is. Het is echter eenvoudiger om de digestie uit te voeren bij een lage pH. Dit is de hoofdreden voor de ontwikkeling van een pepsine reactor. Een tweede argument is dat het gebruik van verschillende proteases / specificiteit een meerwaarde kan bieden voor de identificatie van een onbekend eiwit. Bij het maken van deze reactor is de kennis opgedaan bij de ontwikkeling van de trypsine reactor gebruikt. Bij de karakterisering van de pepsine reactor bleek dat deze, evenals de trypsine reactor, zowel bij opslag als bij gebruik stabiel is. Identificatie van een reeks eiwitten en eiwitmengsels was mogelijk op basis van de verkregen tandem-MS informatie.

Hoewel de voordelen van de integratie van SPR en (LC-)MS duidelijk zijn, is directe koppeling van SPR en LC-MS moeilijk. De reden hiervoor is dat SPR systemen doorgaans zijn uitgerust met een doorstroomcel die slechts een zeer beperkte tegendruk tolereert. Daarom wordt in het on-line systeem zoals is beschreven in **Hoofdstuk 6**, het gedesorbeerde ligand tijdelijk bewaard in een loop, waarna het via het ontwikkelde digestiecapillair wordt geleid naar een trapping kolom, waar de gevormde peptiden geconcentreerd en ontzout worden alvorens te worden geanalyseerd met nanoLC-MS/MS. Het beschreven systeem is volledig geautomatiseerd en de opeenvolgende serie stappen is eenvoudig aan te passen aan de eisen die worden gesteld aan de betreffende toepassing.

Een analyse met het ontwikkelde platform is sneller voltooid dan met andere beschreven systemen die SPR combineren met LC-MS. Een nieuwe SPR-analyse kan worden gestart terwijl de voorgaande LC-MS bepaling wordt afgerond. Een volledige SPR-LC-MS/MS analyse kan worden uitgevoerd in 60 minuten..

In de meeste SPR-MS systemen wordt niet de *concentratie* van het in het monster aanwezige eiwit bepaald. Reden is het eenmalig gebruik van het sensoroppervlak vanwege on-chip MS of on-chip digestie. In deze gevallen is kwantificering meestal beperkt tot bepaling van de *hoeveelheid* gebonden ligand. De sensorchip in het beschreven on-line SPR-LC-MS systeem kan echter na calibratie worden hergebruikt voor een gehaltebepaling. Dit is beschreven in **Hoofdstuk 7**. Er wordt aangetoond dat gebruikmakend van de SPR en MS het gehalte β_2-microglobuline in urine kan worden bepaald in biologisch relevante concentraties van 0.2 tot 4 µg mL^{-1}. Tevens kan de identiteit worden bevestigd op basis van de verkregen MS-data.

Een geautomatiseerd systeem voor on-line SPR-LC-MS is ontwikkeld en de toepasbaarheid hiervan is aangetoond. Het platform biedt de mogelijkheid zowel de identiteit als het gehalte van een ligand te bepalen. De bindingscapaciteit van de ontwikkelde coating in combinatie met het grotere sensoroppervlak en de efficientie van de protease reactoren zijn compatibel met de gevoeligheid van de LC-MS. De resultaten tonen aan dat het SPR-digestie-LC-MS/MS systeem in staat is een eiwit-ligand te isoleren, de concentratie daarvan te bepalen en de identiteit vast te stellen op basis van tandem-MS data. Het ontwikkelde systeem kan een belangrijke bijdrage leveren in interactie-proteomics onderzoek, receptor screening en residue analyse. Toekomstig onderzoek kan zich richten op de analyse van diverse liganden in biologische monsters gebruikmakend van antilichaam-antigeen combinaties.

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ES

Edwin Stigter werd geboren op 28 november 1968 te Dordrecht. Het VWO werd in 1987 voltooid aan het Develstein College te Zwijndrecht. Vervolgens is hij biotechnologie gaan studeren aan de Hogeschool Rotterdam & Omstreken te Delft. Na afronding van een afstudeeropdracht bij TNO - Voeding te Zeist kon hij het onderzoek op het gebied van geïmmobiliseerde biomoleculen voor katalyse en bioanalyse daar voortzetten bij de groep "Biochemie en Enzymologie" en later bij de groep "Bioengeneering en Biomonitoring". In 2001 volgde een overstap naar de Universiteit Utrecht, groep Biomedische Analyse, waar onder leiding van dr. W.P. van Bennekom en drs. M. Bart onderzoek werd verricht naar (de combinatie van) Surface Plasmon Resonance en Electrochemical Impedance Spectroscopie sensoren. In 2003 is hij gestart met het in dit proefschift omschreven promotieonderzoek. Na afloop van deze onderzoeksperiode is hij in 2009 begonnen aan een door het Nederlands Metabolomics Centrum gefinancierd postdoconderzoek aan het UMC Utrecht, afdeling Metabole en Endocriene Ziekten.

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