Development of Microdevices for Proteome Research and Diagnosis

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki,
for public critism at Haartman Institute, Lecture Hall 2 (Haartmaninkatu 3),
on June 26th 2009 at 12 noon.

HELSINKI 2009

Cover Graphic: reprinted with permission of graphic designer Débora Rodrigo Garciá.
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ISBN 978-952-10-5620-8 (paperback)
ISBN 978-952-10-5621-5 (PDF)

http://ethesis.helsinki.fi/

Helsinki 2009
Helsinki University Print

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Come then, and let us pass a leisure hour in storytelling, and our story shall be the education of our heroes.
- PLATO (Republic, book II)

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ABSTRACT

The basic goal of a proteomic microchip is to achieve efficient and sensitive high throughput protein analyses, automatically carrying out several measurements in parallel. A protein microchip would either detect a single protein or a large set of proteins for diagnostic purposes, basic proteome, or functional analysis. Such analyses would include, for example, interactomics, general protein expression studies, detecting structural alterations, or secondary modifications. Visualization of the results may occur by simple immunoreactions, general or specific labelling, or mass spectrometry. For this purpose we have manufactured chip-based proteome analysis devices that utilize the classical polymer gel electrophoresis technology to run one and two-dimensional gel electrophoresis separations of proteins in miniaturised format. In total, we manufactured three functional prototypes of which one performed a miniaturised one-dimensional gel electrophoresis (1-DE) separation, and the second and third performed miniaturised two-dimensional gel electrophoresis (2-DE) separations.

The 1-DE polyacrylamide slab gel electrophoresis-chip (PASGE-chip) was tested against a commercial instrument, the mini-PROTEAN 3 cell from Bio-Rad. The chip separated proteins in approximately 10 minutes with a gel-to-gel repeatability of approximately 3.8%. Due to the low-diffusion of the protein bands, a 5-fold higher sensitivity could be achieved. To mimic an authentic proteome study, we excised protein bands from Coomassie stained micro-gels, digested the protein inside the gel piece with trypsin, and analyzed the released peptides by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-ToF-MS). The peptide mass fingerprinting analyses of the individual bands by MS showed improved peak intensity of the peptide fragments with considerably lower enzyme consumption. Sequence coverage of 70% could be measured. These micro-gels could also be used for subsequent protein sequence analysis by Edman degradation.

In a two-dimensional gel electrophoresis (2-DE), proteins are separated in the 1st dimension by their net charge (isoelectric focusing, IEF) and in the 2nd dimension by their mass (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE). Different designs of miniaturised 2-DE instruments in slab gel or capillary format have been previously published by other research groups. Most of the instruments are used to separate IEF and SDS-PAGE units during the focusing step to prevent the buffer cross-contamination between the gels. To obtain good electric connection between gels agarose or transfer buffer have been used to overlay the IEF gel after the focusing. Noteworthy is that all these instruments require precise handling. Drawbacks of 2-DE separation processes are the many analysis steps and the complicated automation. In the present study, we designed two slab gel-based miniaturised 2-DE instruments with a potential for automation. The electrical connection between the 1st and 2nd dimensions is achieved in the first 2-DE microchip by combining an IEF with a PAGE unit, and in the second 2-DE microchip by pressing the 2nd dimensional gel after focusing.

Our first 2-DE microchip prototype was tested by a set of naturally-coloured standard proteins, IEF standard mixture. Focused proteins were automatically transferred to the 2nd dimensional gel system by electromigration and separated according to their molecular weight in approximately 80 minutes. To measure the gel-to-gel repeatability of the two-dimensional separation, we studied changes in the isoelectric points (pI) and the masses of the proteins. The relative mobilities (Rf) and the pI values varied by approximately 4% and 4.5%, respectively, for human and bovine carbonic anhydrase spots at the 2-D map. To mimic an authentic proteome study, we excised protein spots from Coomassie stained 2-DE gels, digested them with trypsin, and

analyzed the released peptides with MALDI-ToF-MS.

The ComPress-2DE chip represents the final prototype and a novel way of combining the 1st and the 2nd dimensional separations. The basic principle of the ComPress-2DE chip is to press the 2nd dimensional gel by a micrometer head so that it slowly expands into the connecting slot between the IEF and the 2nd dimensional gel. After the gels are connected, the focused proteins are transferred to the 2nd dimensional gel to be separated according to their mass. Again, we verified the performance of the device by using the IEF standard mixture. The full 2-DE separation took approximately 80 minutes. The ComPress-2DE chip was used to separate the glycosylated haemoglobin (Hb A_{1c}) variant linked to diabetes mellitus from the other major haemoglobin variants by native 2-DE with an ultra narrow pH gradient (pH 6.7-7.7). Changes in the post-translational modifications (phosphorylation and glycosylation) of the glial fibrillary acidic protein (GFAP) isoforms between healthy and Alzheimer's diseased patients were followed by 2-DE and subsequent immunoblotting.

The third microchip prototype turned out to be especially well suited for single or multiple biomarker identification, targeted proteomics, or protein-based diagnostics with the 2-DE run repeatability of approximately 2.5% for the Rf, and approximately 4% for the pI value of spot positions of human and bovine carbonic anhydrase.

In conclusion, during this study we designed and fabricated three miniaturised slab gel electrophoresis instruments which could be used in biomarker target screening for disease prognosis and diagnosis. They all showed the advantages of miniaturization over the commercial devices: Such as fast analysis, low sample and reagent consumptions, high sensitivity, high repeatability, and inexpensive performance. All these instruments have the potential to be fully automated due to their easy-to-use set-up.

LIST OF ORIGINAL PUBLICATIONS

This doctoral dissertation is based on the following original publications, which are referred to in the text by the respective Roman numerals (I-IV). Additional material is presented in the text on the basis of unpublished results.

I **Demianova Z**, Shimmo M, Pöysä E, Franssila S, Baumann M. (2007) Toward an integrated microchip sized 2-D polyacrylamide slab gel electrophoresis device for proteomic analysis. Electrophoresis 28:422-8.

II **Demianova Z**, Pöysä E, Franssila S, Baumann M. (2007) High Efficiency Sample Transfer Between IEF and SDS-PAGE Separations on a Miniaturised Proteomics Chip. Transducers 1:1765-68.

III **Demianova Z**, Pöysä E, Ihalainen S, Aura S, Shimmo M, Franssila S, Baumann M. (2008) Development and application of a miniaturised gel electrophoresis device for protein analysis. Mol.Biosyst 4:260-5.

IV **Demianova Z**, Pöysä E, Korolainen M, Baumann M. (2009) Targeted proteomics on a chip. Analytical Chemsitry, submitted.

AUTHOR CONTRIBUTIONS TO ORIGINAL PUBLICATIONS

	I	II	III	IV
Original Idea	*ZD, MS, EP, MB, SF*	*ZD, EP, MB*	*ZD, MS, EP, MB, SF*	*ZD, EP, MB*
Prototype Design	*ZD, MS, EP, MB*	*ZD, EP, MB*	*ZD, MS, EP, MB*	*ZD, EP, MB*
Prototype Fabrication	*EP*	*EP*	*EP, SA* (silicon fabrication)	*EP*
Study Design	*ZD*	*ZD*	*ZD*	*ZD*
Data Collection Analysis	*ZD, MS and EP* (partial contribution to chapter 3.2)	*ZD*	*ZD, SI* (preparation of cell culture and VSMC lysates)	*ZD, MK* (preparation of brain lysates)
Manuscript Preparation	*ZD, MB, SF*	*ZD, MB, SF*	*ZD, MB, SF*	*ZD, MB*

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ABBREVIATIONS

1-D	One-Dimensional
1-DE	One-Dimensional Gel Electrophoresis
2-D	Two-Dimensional
2-D DIGE	2-D Fluorescence Difference Gel Electrophoresis
2-DE/2DE	Two-Dimensional Gel Electrophoresis
APS	Ammonium PerSulfate
CA	Carrier Ampholytes
CE	Capillary Electrophoresis
CEC	Capillary ElectroChomatography
CIEF	Capillary IsoElectric Focusing
CGE	Capillary Gel Electrophoresis
CSF	CerebroSpinal Fluid
CZE	Capillary Zone Electrophoresis
DNA	DeoxyriboNucleic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ECL	Enhanced ChemiLuminescent
ESI	ElectroSpray Ionization
ICAT	Isotope Coded Affinity Tag
IEF	IsoEelectric Focusing
IPG	Immobilized pH Gradient
ITP	IsoTachoPhoresis
iTRAQ	isobaric Tag for Relative and Absolute Quantitation
GE	Gel Electrophoresis
GFAP	Glial Fibrillary Acidic Protein
GVHD	Graft-Versus-Host Disease
Hb	Haemoglobin
Hb A_{1c}	Glycosylated Haemoglobin
LC	Liquid Chromatography
LMW	Low Molecular Weight protein standard
MALDI	Matrix-Assisted Laser Desorption/Ionization
MEKC	Micellar Electrokinetic Capillary Chromatography
MD	Multi-Dimensional
MS	Mass Spectrometry
MudPIT	Multidimensional Protein Identification Technology
PAA	PolyAcrylAmide
PAGE	PolyAcrylamide Gel Electrophoresis
PASGE	PolyAcrylamide Slab Gel Electrophoresis
PDMS	PolyDiMethylSiloxane
pI	Isoelectric point
PMF	Peptide Mass Fingerprinting
PMMA	Poly(Methyl MethAcrylate)
PVDF	PolyVinylDiFluoride
PTM	Post-Translational Modification
RMW	Relative Molecular Weight
RNA	RiboNucleic Acid

RP	Reversed Phase
SDS	Sodium Dodecyl Sulphate
SELDI	Surface-Enhanced Laser Desorption/Ionization
SILAC	Stable Isotope Labelling with Amino acids in Cell culture
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
ToF	Time of Flight
UPLC	Ultra-high Pressure Liquid Chromatography
VSMC	Vascular Smooth Muscle Cell
ZE	Zone Electrophoresis

1 INTRODUCTION

Proteomics at its most basic level involves the identification, quantification, structural characterization, and localization of proteins in a proteome. This could be studied at the level of any organism, any individual organ, tissue sample, certain cell, or even in the organelle. Proteomics gives the opportunity to better understand the protein expression pattern in healthy and diseased states that can be used to discover new biomarkers for clinical diagnosis (Rifai et al., 2006a; Colantonio and Chan, 2005). Core technologies to obtain the information are one- and two-dimensional gel electrophoresis (1-DE and 2-DE) (Iwadate, 2008; Issaq and Veenstra, 2007); and one, two, and multi-dimensional liquid chromatography (1-D, 2-D and MD LC) (Kreusch et al., 2008; Baum et al., 2008). In a proteomic workflow, these are almost exclusively coupled with mass spectrometry.

There are two most frequently applied electrophoretic methods in protein separations. The first is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is a biochemical method separating denatured proteins according to the differences of their masses (relative molecular weight; RMW) (Laemmli, 1970). The other is two-dimensional gel electrophoresis (2-DE) that separates proteins by two independent parameters namely by their net charge (isolectric point) and RMW (O'Farrell, 1975; Klose, 1975). Such analyses, usually performed on slab gels, are useful in studying the protein composition of relatively complex mixtures. At least two independent separation techniques (*e.g.* isoelectric focusing, IEF, and SDS-PAGE) are necessary to separate proteins of similar physical properties. When separating proteins according to their net charge and RMW, the technique requires two analysis steps (IEF and SDS-PAGE) to keep untouched as long as the focusing is proceeding. For this reason, both separation methods are usually run separately, one after the other in a sequence. This is the most difficult step in the methodology, but crucial for 2-DE automation. Gel-free methods (2-D and MD LC) have been developed to reduce the limitation of 2-DE, but they are required when using more advanced methods to reduce sample complexity prior to liquid chromatography. The advantage of the 2-DE system over the gel-free system is the possibility to store gels and proteins in the gel, and to proceed the analyses using, for example, immunoblotting, peptide mass fingerprinting, or protein sequencing. Many complementary proteomic technologies have been developed to study expression profiling and molecular interactomics. These include protein microarrays based on affinity and ligand affinity technologies, antibody arrays, surface plasmon resonance, and surface-enhanced laser desorption and ionization techniques, etc (Spisak et al., 2007; Chaerkady and Pandey, 2008).

SDS-PAGE is a simple method to detect proteins and it is suitable for a wide range of protein. In addition, SDS-PAGE is widely used in monitoring protein purification processes. On the other hand, 2-DE offers tremendous resolving power with a single run capable of separating thousands of proteins into distinct spots (Bjellqvist et al., 1982). 2-DE is able to identify the difference in protein expression levels of healthy and diseased samples, as well as preserving post-translational modifications of proteins. This is especially important in biomarker studies because many diseases can perturb phosphorylation or other modifications. 2-DE is considered to be the most powerful protein isolation method, but it is time consuming and labour intensive. Repeatability of the method is poor between laboratories. Automation of 2-DE will help to overcome some of its drawbacks, as well as improving the repeatability of the method due to accurate handling/manipulation, efficiency, and productivity. This will make 2-DE more acces-

sible for less experienced researchers.

Miniaturisation of gel electrophoresis technology offers faster analysis times, high-throughput runs, low sample and reagents consumption, possibility of integration and automation, less chemical waste, and lower cost per analysis. Some miniaturised instruments for proteomics studies are commercially available (Agilent Technologies, Bio-Rad and GE Healthcare). Agilent's Bioanalyzer and Bio-Rad's Experion are lab-on-a-chip instruments that use capillary gel electrophoresis to analyze up to ten individual protein samples within half an hour. Both instruments are also compatible with DNA and RNA analyses. Amersham Pharmacia Biotech (GE Healthcare) developed an automated slab gel electrophoresis instrument (PhastSystem) for fast analysis of proteins using miniaturised ready-made gels. Although somewhat outdated, it is still widely used.

Nowadays, various research groups design and continuously develop new automated miniaturised electrophoresis systems for protein studies. These are mainly based on zone electrophoresis or gel electrophoresis in the channel. In order to separate proteins with similar physical properties, at least two different separation methods should be used. Successful 2-D microchips combines zone electrophoresis (ZE) with micellar electrokinetic electrophoresis (Ramsey et al., 2003), isotachophoresis with ZE (Kriikku et al. 2004), IEF with ZE (Cong et al., 2008), or IEF with a single or set of gel electrophoresis channels (Griebel et al., 2004; Liu et al., 2008), as shown previously. Miniaturisation of traditional slab gel 2-DE has been challenging due to buffer cross-contamination between the 1st and 2nd dimensional gel. For this reason, IEF and SDS-PAGE analyses are usually run separately.

In this work, we describe in detail the development of miniaturised one-dimensional (PASGE-chip, study III) and two-dimensional (2-DE microchip, study I and ComPress-2DE chip, study II and IV) slab gel electrophoresis instruments. Our goal was to develop a set of miniaturised slab gel devices for fast proteomics analysis. The instrumentation was manufactured to automate the separation process, to avoid manual handling of gels, to eliminate cross-contamination, and to make analyses faster and improve system repeatability. These devices were successfully used to separate and characterize a set of predefined standard proteins, and cell and tissue samples.

2 REVIEW OF THE LITERATURE

2.1 PROTEOMICS

The study of protein structure and function has always been an essential part of biochemistry. It is estimated that the human body may contain over two million proteins, coded for by only 20,000 - 25,000 genes. The entire protein content in a given cell, tissue, or organism is defined as the PROTEOME. Wilkins coined the word "proteome - the PROTEin complement expressed by a genOME" in 1996 (Wilkins et al., 1996a; Wilkins et al., 1996b). Proteome depict the protein complement of a genome and represent the end product of the genome. Proteomics covers the protein identification and quantification, the study of their structure and function, and also their interactions within a complex biological system at a given time or under certain environmental conditions. As stated by Ian Humphery-Smith, one of the founders of the human proteome project (HUPO), "Proteins are central to our understanding of cellular function and disease processes, and without a concerted effort in proteomics, the fruits of genomics will go unrealized".

Proteomics (the analysis of a proteome) has become one of the constantly improving areas in life science and medicine, it offers a relatively new approach to studying protein expression profiling and cellular or tissue protein identification (Lee, 2001; Phizicky et al., 2003). Branches of proteomics include 1| protein separations, identification and sequence analysis; 2| quantitative, structural, cellular and interaction proteomics; and 3| bioinformatics (Lambert et al., 2005). In its broader sense, proteomics involves protein activities, modifications, interactions, and their location in body fluids, cell, organelle, tissue and organ, or even in an organism. Most importantly, proteomics aims to work out the differences in protein action between healthy and diseased tissues or body fluids (Rifai et al., 2006a; Colantonio and Chan, 2005). Clinical research is poised to benefit enormously from these studies, with the potential to develop better diagnostic and prognostic tests, to identify new therapeutic targets, and ultimately to allow patient-individualised therapy (Petricoin et al., 2004).

Moreover, proteomics is an essential part of systems biology that aims to solve the relationships between different areas of a biological system with the goal of understanding and predicting the behaviour of the system as a whole (Smith and Figeys, 2006). Systems biology brings together researchers of different disciplines (computer scientists, mathematicians, physicians, chemists, and biologists) and combines information from biochemistry, proteomics, genomics, metabolomics, and bioinformatics to ascertain the contribution of each element to the whole system. Thereafter, it strives to arrange information into integrated interaction networks of genes, proteins, and biochemical reactions, and to create predictive mathematical models of complex biological processes or organ systems (Aggarwal and Lee, 2003). It is likely to become a dominant force in the medical sciences that gives us the opportunity to better understand living systems from single cells to whole organisms, and also to prevent and cure disease.

2.2 CLINICAL PROTEOMICS

As molecular medicine moves beyond genomics to proteomics, the goal becomes to characterize the cellular circuitry and to understand the impact of a disease and therapy on cellular networks. Clinical proteomics strives to successfully translate basic scientific knowledge into

clinical applications for the benefit of the patient. Major interests include the early detection and diagnosis of disease, prediction of a disease outcome over time, as well as how a specific patient will respond to the treatment, and the identification of novel protein biomarkers (Petricoin et al., 2002). Biomarker is a characteristic that is measured and evaluated as an indicator of normal biological, pathogenic processes, or a pharmacological response to a therapeutic intervention. Biomarker discovery includes biomarker identification, prioritization, verification, and clinical validation. Only an inconsiderable number of protein biomarkers have achieved approval by the Food and Drug Administration (FDA) (Ludwig and Weinstein, 2005). A good biomarker predicts the extent and duration of organ damage, anticipates clinical outcome, and evaluates the usefulness of therapeutic strategies. Typically, there are two strategies in biomarker discovery: 1| biomarkers identified in tissue and validated in serum, or 2| biomarkers identified and validated in serum or plasma.

A wide variety of methods and a combination thereof are applied to find novel protein biomarkers. These include two-dimensional gel electrophoresis (2-DE) and two-dimensional fluorescence difference gel electrophoresis (2-D DIGE) (Iwadate, 2008; Issaq and Veenstra, 2007); one-, two- and multi-dimensional liquid chromatography (1-D, 2-D and MD LC) (Kreusch et al., 2008; Baum et al., 2008); mass spectrometry (MS) and protein microarrays (Spisak et al., 2007). Although gel-based methods are widely used in proteomics they have several drawbacks, such as difficulties in detecting membrane proteins and low-abundant proteins, and such methods are laborious and not applicable for high-throughput screening or automation. The combination of 2-D LC or MD LC with tandem MS has automated and improved the separation and identification of proteins especially in complex protein samples, *e.g.* shotgun proteomics (Motoyama and Yates, 2008) and Multidimensional Protein Identification Technology (MudPIT) (Liu et al., 2002). During the verification of protein biomarker candidates, an extended number of samples (broader range of cases and controls) is used to capture the environmental, genetic, biological, and stochastic variation in the population (Rifai et al., 2006b). Thus, in verification, biomarker candidate sensitivity is affirmed and biomarker specificity is taken into consideration. This process narrows down the large number of candidates to just a few that are then further validated. Validation of protein biomarker candidates can be performed by several independent methods, such as immunodetection, enzyme-linked immunosorbet assays (ELISA), and immunohistochemistry. However, such proteomic studies have to be planned very critically from the beginning to get statistically significant results. A summary of some published studies on biomarker discoveries using proteomic tools is shown in Table 1.

2.3 ANALYTICAL TECHNIQUES APPLIED IN PROTEOMICS

Every organism possesses one genome and multiple proteomes. Cells express genes, which encode proteins with unique cell-specific function. More than 200 different post-translational modifications of proteins (PTMs) exist, involving a wide variety of reversible or irreversible chemical reactions that influence the diversity, affinity, function, cellular abundance, and transport of proteins. This makes proteome analysis incredibly complex.

Proteomics is rapidly developing towards new applications and also improvements of basic technologies. It generally seeks for proteome identification of any cell, protein isoforms, PTMs, protein interacting partners, and higher-order complexes. These present significant analytical

Table 1 *Proteomic technologies used in laboratory (adopted from (Chaerkady and Pandey. 2008) with a few modifications)*

Proteomic method	Clinical application	Disease	Ref.
Isotopic labelling methods	13C-isotope-labeled synthetic peptides as internal standards were used to quantify C-reactive protein from prefractionated serum	Rheumatoid arthritis	(Kuhn *et al*., 2004)
	Protein cleavage-isotope dilution MS was used to quantify prostate-specific antigen directly from serum	Prostate disease	(Barnidge *et al*., 2004)
	SILAC was used for the identification of secreted proteins as biomarkers	Pancreatic cancer	(Gronborg *et al*., 2006)
	iTRAQ was used to identify unique biomarker proteins in CSF	Alzheimer's disease	(Abdi *et al*., 2006)
	ICAT was used for qualitative and quantitative profiling of laser capture microdissection tissues for biomarker discoveries	Hepatocellular carcinoma	(Li *et al*., 2004)
	18O-isotope labelling was used on LMW proteins as biomarkers from the serum of mice	Lung cancer	(Hood *et al*., 2005)
2-DE coupled with MALDI-ToF-MS	From 70 protein spots a total of 27 proteins and their isoforms were identified and significantly up- or down-regulated	Tonsillar cancer	(Roblick *et al*., 2008)
	Chloride intracellular channel protein 1 and tumour protein D52 were identified as potential biomarkers	Colorectal cancer	(Petrova *et al*., 2008)
	Extracellular glutathione peroxidase and apolipoprotein were identified as potential biomarkers	Diabetic nephropathy	(Kim *et al*., 2007a)
	Potential biomarkers (apolipoprotein E, serum amyloid P-component) studied in maternal plasma	Down's syndrome	(Kolialexi *et al*., 2008)
Imaging MS	Direct imaging of cancer tissue with the photocleavable mass-tagged secondary antibody by MALDI-ToF-MS	Melanoma	(Thiery *et al*., 2007)
	Direct imaging of fine-needle aspirates by MALDI-ToF-MS	Lung cancer	(Amann *et al*., 2006)
SELDI-ToF-MS	Serum proteomic pattern of 8 m/z species were shown to discriminate GVHD from non-GVHD	Acute GCVHD	(Srinivasan *et al*., 2006)

SELDI-ToF-MS	Fine-needle aspiration and collection of intact sample from breast tissue for protein pattern expression of breast neoplasms	Breast cancer	(Fowler *et al*., 2004)
	Pattern in sera of patients pre- and post-surgery; serum amyloid alpha was identified as a potential biomarker	Renal cell carcinoma	(Engwegen *et al*., 2007)
Antibody array	Validation of thrombospondin as a potential biomarker	Prostate cancer	(Shafer *et al*., 2007)
	Novel cytokines, chemokines, and growth factors were detected in CSF	Infectious meningitis	(Kastenbauer *et al*., 2005)
	Leptin, prolactin, osteopontin, and insulin-like growth factor II antibodies were analyzed in serum	Ovarian cancer	(Mor *et al*., 2005)
Autoantigen arrays	The prevalence and clinical significance of a spectrum of autoantibodies in systemic lupus erythematosus and incomplete lupus syndromes were investigated	Systemic lupus erythematosus	(Li *et al*., 2007)
	Identification of autoantibodies to citrullinated epitopes using synovial arrays as early markers of disease	Rheumatoid arthritis	(Hueber *et al*., 2005)
Reverse-phase protein arrays	Study of specific molecular pathways in cell survival and progression from normal epithelium to invasive carcinoma directly from tissue	Prostate cancer	(Grubb *et al*., 2003)
	Quantification of an active extracellular signal-regulated kinase in tissue lysates using a phosphopeptide reference	Ovarian cancer	(Sheehan *et al*., 2005)
Bead-based arrays	Multiplexed analysis of serum for human papilloma viral proteins	Cervical cancer	(Waterboer *et al*., 2005)
	Quantification of circulating interleukin-8 and anti-interleukin-8 autoantibodies	Ovarian cancer	(Lokshin *et al*., 2006)
Magnetic beads biosensor	Detection of human antidengue virus Immunoglobulin G in serum	Dengue virus infection	(Aytur *et al*., 2006)

Key: *CSF*, Cerebrospinal fluid; *GVHD*, graft-versus-host disease; *ICAT*, isotope-coded affinity tag; *iTRAQ*, isotope tagging for absolute and relative quantification; *LMW*, low molecular weight; *MALDI*, matrix-assisted laser desorption/ionization; *SELDI*, surface-enhanced laser desorption/ionization; *SILAC*, stable isotope labelling with amino acids in cell culture; *ToF*, Time of Flight.

challenges owing to high proteome complexity and low abundance of a large number of proteins, this requires high sensitivity and resolving power.

Proteomics uses a combination of separation methods (polyacrylamide gel electrophoresis (PAGE) or 2-DE; 1-D, 2-D, or MD LC) together with MS or tandem MS (MS/MS), amino acid sequencing, and bioinformatics. This is due to the fact that currently there is not a single proteomics technology that has sufficient analytical power to detect and identify an entire proteome of body fluids, cells, or tissue samples. An analytical strategy of a proteomic workflow is illustrated in Figure 1. Basically it includes 1| sample collection, preparation and storage; 2| protein enrichment, fractionation and separation; 3| protein digestion and peptide separation; and 4| sample analysis by MS and data interpretation. Single cell analysis (Diks and Peppelenbosch, 2004) represents a new challenge in proteomics that greatly calls for technical innovations in current analytical chemistry, *e.g.* microfluidics devices (Huang et al., 2007), smart droplet tools for nanodigestion (Lanigan et al., 2008; Lanigan et al., 2009), and ultra high sensitivity MS and MS/MS.

2.3.1 Liquid Chromatography

Protein separation by 1-D, 2-D, and MD LC has been extensively used in proteomics. LC can separate proteins or their peptide fragments according to differences in size, charge (pI), or hydrophobicity, *i.e.* the physical characteristics that define any protein. In general, chromatography involves a mobile phase with a sample to be separated over a stationary phase. Proteins in the mobile phase will have different interactions with the stationary phase, this leads to separation of proteins. Proteins with tighter interactions will tend to move more slowly through the phase than those proteins with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the stationary phase. Typically, each mode of separation requires a specifically tailored stationary phase. The most commonly used chromatographic techniques are reverse phase-liquid chromatography (RP LC) (Neverova and Van Eyk, 2005; Ducret *et al*., 1998), ion exchange chromatography, and affinity chromatography (Lee and Lee, 2004; Pieper *et al*., 2003). 1-D LC is generally applied for peptide separations after enzymatic protein digestion or for separating intact proteins prior to MS (McDonald *et al*., 2006; C.T. Mant and R.S. Hodges, 1991). Liquid chromatography is widely used for sample concentration, clean up, or desalting.

A variety of multi-dimensional chromatographic combinations that increase total separation power have been reported (Link *et al*., 1999; Anderegg *et al*., 1997; Mawuenyega *et al*., 2003). These methods may use various chromatographic and electrophoretic techniques (off-line or on-line) that are coupled with electrospray ionization (ESI) MS/MS. MS limits the number of dimensions used. MD LC analysis combines orthogonally together strong cation exchange, strong anion exchange, size exclusion, affinity chromatography, and reverse-phase chromatography. In the first step of MD LC, a protein sample of interest is digested into constituent peptides. MD LC then separates the resulting peptides that are finally introduced into MS and/or MS/MS. The MS/MS spectra are searched against protein databases using computer algorithms such as SEQUEST or Mascot (Perkins *et al*., 1999). This technology is also known as shotgun proteomics (McDonald and Yates, 2002; Wolters *et al*., 2001; Fournier *et al*., 2007). The most

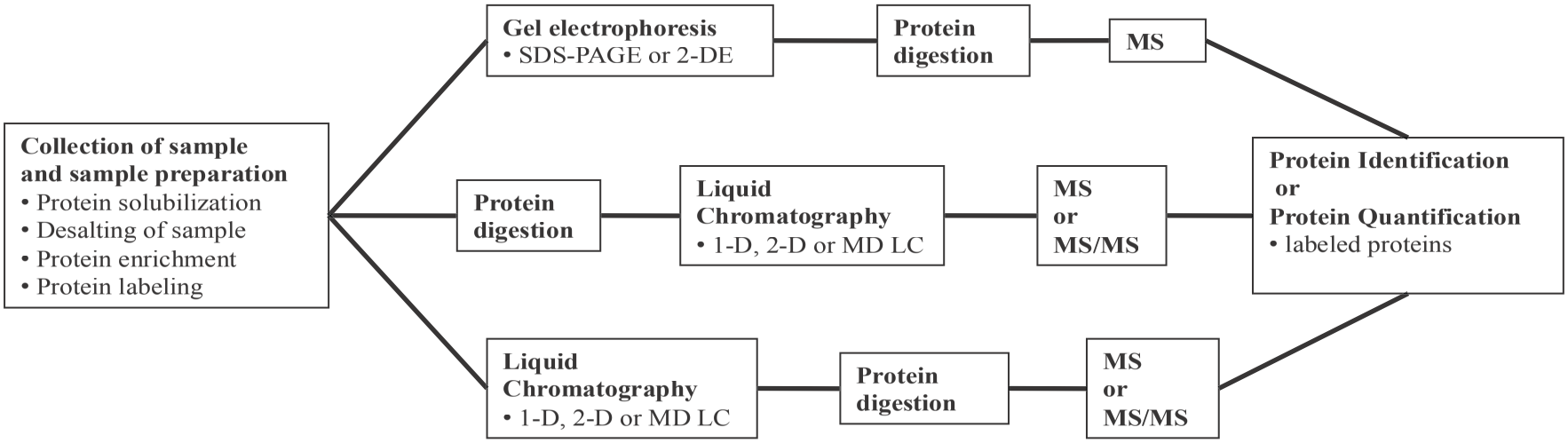

Figure 1 *A typical proteomic workflow. 1-D, one-dimensional; 2-D, two-dimensional; 2-DE, two-dimensional gel electrophoresis; LC, liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MD, multi-dimensional; MS, mass spectrometry.*

widely used shotgun approach is to combine strong cation exchange chromatography with RP LC followed by MS/MS, which is also referred to as MudPIT (Wall *et al*., 2000; Sheng *et al*., 2006; Kislinger and Emili, 2005). Thus, shotgun proteomics increases the possibility to facilitate the analysis of a large dynamic protein spectrum, including their specific isoforms, PTMs, and low abundant proteins.

Recently, liquid chromatography has undergone significant improvements in protein and peptide separation. Efforts to improve RP LC separation have resulted in the development of Ultra-high Pressure LC (UPLC) and the use of less than 2 μm porous silica particles as the stationary phase (Mazzeo *et al*., 2005). The smaller particle diameter improves resolution due to the minimized diffusion of analytes across the particles. UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity. Recently, UPLC was applied to MS-based shotgun proteomics (Motoyama and Yates, 2008; Shen *et al*., 2004).

2.3.2 Electrophoresis

Electrophoresis has become increasingly important in laboratories for fundamental biomedical research and for clinical applications to diagnose a disease or its progression. The technique was pioneered in 1937 by the Swedish chemist Arne Tiselius for protein separation based on their movement due to the influence of a direct electrical current (Kyle and Shampo, 2005). Electrophoresis has now been extended to separate many different classes of analytes; from small organic and inorganic molecules to macromolecules, such as peptides, proteins, RNA, and DNA that can be separated according to their physical properties (RMW, pI and hydrophobicity).

Electrophoresis can be performed in narrow bore fused silica capillaries (capillary electrophoresis; CE) or in slab gels (SDS-PAGE or 2-DE). CE can be segmented into several separation techniques; such as capillary zone electrophoresis (CZE), isotachophoresis (ITP), capillary isolectric focusing (CIEF), capillary sieving/gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), and capillary electrochromatography (CEC) (Landers, 1996). CE is characterized by its selectivity, high separation efficiency, high sensitivity, and high speed of analysis. Due to its simplicity, CE was the first method integrated into the planar microchip format (Manz *et al*., 1992; Harrison *et al*., 1992). However, CE is not often applied to separate intact proteins.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Gel electrophoresis (GE) is widely used for the analysis of macromolecules, and suitable equipment for GE can be found in almost every biochemical/biological laboratory. The popularity of GE is based on its high resolving power; its ability to analyze many samples simultaneously; its high sensitivity for protein, RNA, and DNA samples; its possibility to recover proteins, RNA, and DNA molecules after electrophoresis; the possibility for several subsequent analysis, *e.g*. immunodetection, peptide mass fingerprinting (PMF), and protein sequencing by Edman degradation; and the modest cost of the method. The main advantage of slab gel electrophoresis, compared to LC or CE techniques, is its suitability for the analysis of multiple samples

simultaneously. GE separates charged molecules according to their physical properties as they are forced to move through a gel in an electrical field. The gel is composed of either agarose (mainly for RNA and DNA separation) or polyacrylamide (PAA) (mainly for protein separation). The pore size of the gel is defined by the concentration of agarose or PAA. GE can be performed in slab format or in a capillary.

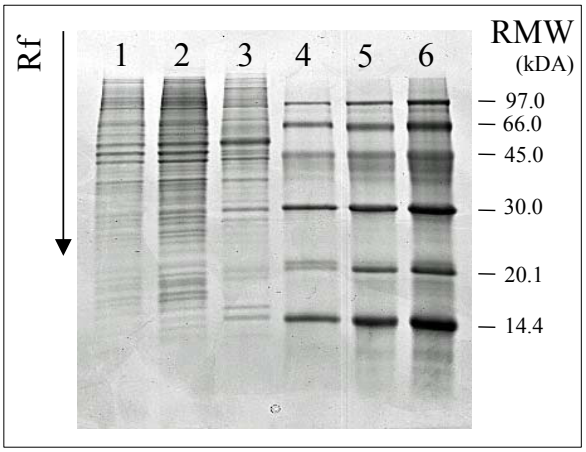

Figure 2 *Demonstration of protein separation by SDS-PAGE. Line 1| 10 µg and line2| 15 µg total protein amount of HEK 293 cell lysate, line 3| 10 µg total protein amount of K99B+/+ cell lysate, and line 4| 30 µg, line 5| 15 µg and line 6| 7 µg total protein amount of LMW standard. The proteins were separated within 90 minutes on a 12% SDS-PAA gel at 150 V.*

GE performed in a polyacrylamide slab gel (Figure 2) is used to characterize an individual protein in a complex sample, to follow the purification of protein sample, to determine the expression of a protein, or to study protein-protein interaction. PAGE can also be used as a preparative tool to obtain a pure protein sample, or as an analytical tool to provide information on the mass and charge. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein. Several forms of PAGE exist and can provide various information about the protein(s). Native PAGE separates proteins according to their mass to charge ratio, m/z (Wittig and Schagger, 2008), SDS-PAGE separates proteins primarily by mass (RMW) (Laemmli, 1970), and IEF separates proteins based on their net charge (pI) (Righetti *et al*., 1997). 2-DE separates proteins by net charge in the 1st dimension (IEF) and by mass in the 2nd dimension (SDS-PAGE) with a resolution power of thousands of proteins in a single gel (O'Farrell, 1975; Klose, 1975).

Proteins can be visualized in gels using various detection methods. The most common protein staining methods include Coomassie brilliant blue (Merril, 1990a), silver (Merril, 1990b), and fluorescence staining (Cyanine or SYPRO dyes) (Unlu *et al*., 1997; Steinberg *et al*., 1996a; Steinberg *et al*., 1996b). The chemiluminescent detection (enhanced chemiluminescent, ECL) (Durrant, 1994) is used for the immunodetection of proteins, which are transferred to a polyvinyldifluoride (PVDF) or a nitrocellulose membrane from a gel. Gel images are evaluated and analyzed manually or with commercially available software packages. Such software is used to compare gel patterns using complex algorithms, highlight differences between gel images (protein spots that are qualitatively different, or up- or down-regulated), and store the relevant information in a database.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

Currently the most effective and widely used method for proteomics is 2-DE with an almost unrivalled capacity for the separation of complex protein mixtures (O'Farrell, 1975; Klose, 1975). 2-DE can routinely separate up to 2 000 proteins and with larger format gels up to 10 000 proteins. 2-DE is primarily used as an analytical tool for the characterization of proteins by their net charge (pI) and mass (RMW). As the charge and mass properties of proteins are essentially independent parameters, this orthogonal combination of charge (pI) and size (RMW) results in the sample proteins being distributed across the whole 2nd dimensional gel profile building a 2-D map (Figure 3). Achieving optimal separation of protein mixtures relies greatly on how the sample is handled and processed prior to 2-DE.

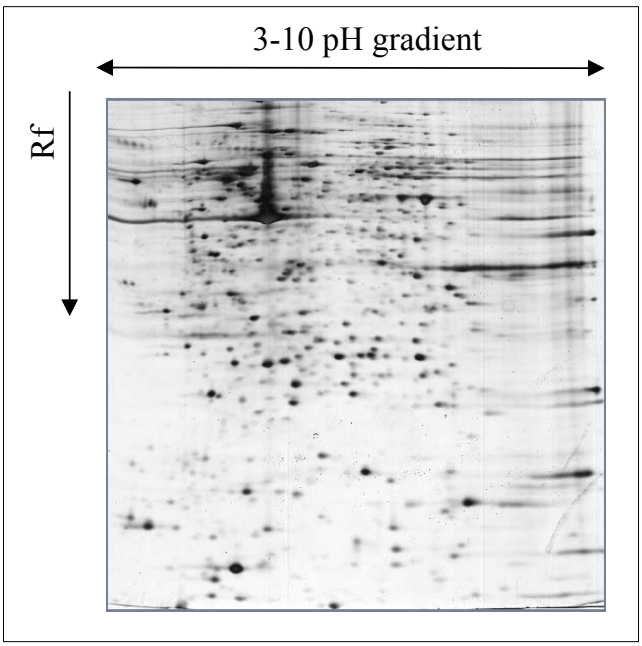

Figure 3 *A 2-DE separation of approximately 60 µg of a vascular smooth muscle cell lysate (18 cm IPG strip pH gradient 3-10, 12% 2nd dimensional gel).*

Sample preparation is perhaps the most important step in a 2-DE experiment as impurities introduced at this stage can often be magnified with the potential to impair the validity of the obtained results. No single method for sample preparation can be universally applied owing to the diverse nature of samples that are analyzed by 2-DE. Disturbing substances, such as salts, lipids, polysaccharides, and nucleic acids have to be removed during the sample preparation to avoid any interference in the 2-DE separation. A crucial factor of a successful 2-DE separation is the protein solubilisation, since the proteins need to remain soluble during the whole 2-DE process. To keep proteins soluble, high concentrations of urea and other detergents are used in sample preparation as well as in the IEF step (Rabilloud *et al*., 2007).

Generally, 2-DE consists of two separate analysis steps, *i.e*. IEF and SDS-PAGE, which are manually combined together. One analysis run may take several hours and on larger sized gels it is even longer. During this procedure the focusing gel is rehydrated, focused, equilibrated, and finally placed on top of the 2nd dimensional gel. SDS-PAGE separates focused proteins ac-

cording to their masses, subsequently the 2nd dimensional gel is stained to visualize separated protein spots. The 2-DE methodology has improved in sample pre-treatment, instrumentation, and technology, as well as commercial softwares packages used to analyze 2-D maps.

The critical point in the development of 2-DE was the introduction of an immobilized pH gradient (IPG) for the 1st dimension IEF step to allow precise tailoring of narrow or wide range of pH gradients (Neverova and Van Eyk, 2005; Molloy, 2000; Ong and Pandey, 2001; Bjellqvist *et al*., 1982). The most common IPG strips are at the pH range of 3–10, that can be in linear or nonlinear pH gradient. However, intermediate (*e.g*. pH 4–7, 6–9) and ultra narrow (*e.g*. pH 4.0–5.0, 4.5–5.5) ranges of IPG strips are also available. Furthermore, immobilized ampholytes greatly enhance the stability of a pH gradient. IPG strips overcome problems with the cathodic drift of the pH gradient made of carrier ampholytes and the possible degradation of carrier ampholytes due to long runs (Righetti *et al*., 1983; Baumann and Chrambach, 1975).

2-D fluorescent difference gel electrophoresis (2-D DIGE) is a mode of 2-DE where two or more protein samples are differentially labelled and electrophoretically separated on the same gel (Timms and Cramer. 2008). This technology overcomes the problem of 2-DE gel-to-gel variation of healthy and diseased patient samples due to low inter-gel variation. Differentially labelled samples are subjected to the same handling procedures and raise the confidence with which protein changes can be detected and quantified, thus reducing the number of gels that need to be run. Prior to 2-D DIGE separation, the different protein samples (*e.g*. healthy/diseased) are labelled with CyDye DIGE fluor Cy minimal dye (Cyanine Cy2, Indocyanine Cy3 and Indodicarbocyanine Cy5) with different excitation/emission maxima (λex/em Cy2 482/501 nm, λex/em Cy3 552/570 nm and λex/em Cy5 640/661 nm) and then separated in a single gel (Unlu *et al*., 1997; Chakravarti *et al*., 2005). 2-D DIGE is extremely sensitive with a detection limit of less than 1 fmol (Lilley *et al*., 2002). One major reason for 2-DE's success is its high resolving power with the possibility for fast protein identification by MS (Karas and Hillenkamp, 1988; Hillenkamp and Karas, 1990; Hillenkamp *et al*., 1991).

2.3.3 Mass Spectrometry

MS has revolutionized proteomics and protein-based biomarker searches especially with the development of methods for ionizing large biomolecules (Guerrera and Kleiner, 2005). These include ESI (Fenn *et al*., 1989) and matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988; Tanaka *et al*., 1988). These ultra sensitive methods have substantially improved protein identification, MS speed, and sensitivity. The most common MS strategy is to analyse peptides rather than full-length proteins. To analyse proteins or peptides effectively by mass spectrometry they must be ionized in the gas phase. ESI is the most widely used atmospheric-pressure ionization technique, where a strong electric field is applied to the liquid sample stream, *e.g*. from the LC column, which is then nebulised and desolvated with the assistance of a high-temperature gas flow to produce gas-phase ions. MALDI is a soft ionization technique used in MS where laser pulses strike the sample in the solid or viscous phase. When the sample takes up the energy from the laser pulse it is "blasted" into small fragments. The fragments fly one after another, and are released as intact hovering molecule ions with low charge then as accelerated molecules ions in an electrical field, such are detected by recording their time of flight (ToF). The advantage of MALDI-ToF-MS over ESI-MS relies on its relatively high tolerance

for contaminants, the higher sensitivity and speed. MALDI is able to analyse more than 30 000 samples/week in its automated configuration. Consequently, MALDI-ToF-MS peptide mapping is used for the initial screening of a large set of protein digests, and the other mass spectrometric techniques often for in-depth analysis.

The combination of 2-DE and MS has become an important analytical technique for the characterization of complex protein populations extracted from cells, organelles, and tissue fractions. The spots of interest in 2-D gels are excised, the proteins subjected to in-gel digestion (Patterson *et al*., 1996; Shevchenko *et al*., 1996; Clauser *et al*., 1995) and the resulting peptide fragments eluted from the gel are identified by MS (Cottrell, 1994). The peptide masses are then in silico compared against protein databases that contain known protein sequences to identify the unknown/original proteins. The whole process from protein in-gel digestion to its identification is called PMF (Mann *et al*., 1993; Pappin *et al*., 1993). Various protein databases with different focuses (*e.g*. extracellular protein-protein and protein-carbohydrate interactions, plasma protein database, nuclear protein database, *etc*.) were developed. Major publicly available databases are SwissProt (http://au.expasy.org/sprot/), Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), NCBI Entrez Protein (http://www.ncbi. nlm.nih.gov), UniProt (http://www.uniprot.org), and Protein Information Resource (http://pir.georgetown.edu/) (Gasteiger *et al*., 2001; Sussman *et al*., 1999; Apweiler *et al*. 2004).

If information on protein isoforms, PTMs, or "absolute" identification of proteins in complex mixtures by 1-D and 2-D LC is needed, then tandem MS is required. MS/MS spectra are usually generated by an ion trap, MALDI ToF-ToF, or quadrupole-ToF mass spectrometer, which allows the generation of de novo sequences and exact localization of PTMs (Guerrera and Kleiner, 2005). Several stable isotopic labelling (*e.g*. SILAC, iTRAQ, *etc*.) (Ong and Mann, 2005) and label-free (Wang *et al*., 2008) techniques have been developed for proteins and peptides.

Proteome fractionation of a biological fluid sample by ClinProt (Bruker Daltonics, Bremen, Germany) is based on different bond affinities with an activated surface of magnetic beads into groups of proteins before MALDI analysis (Ketterlinus *et al*., 2005). ClinProt methodology is dedicated to peptide and protein profiling, and biomarker discovery. The newest MS application in proteomics is imaging mass spectrometry. This technology is used to profile and image peptides and proteins directly from a thin tissue section (Goodwin *et al*., 2008).

2.4 MINIATURISED ELECTROPHORESIS SYSTEMS

Currently, there is growing interest in miniaturizing analytical systems especially for biological/biochemical applications, mainly because of the advent of large-scale proteomics (Lion *et al*. 2004). The motivation for turning from macro scale analytical procedures to micro-analytical systems is defined in the literature as listed in Table 2.

Various researchers have developed different designs of miniaturised systems, as well as integrating one or more techniques onto the same microchip. Miniaturised instruments are commonly fabricated using standard photolithographic, etching, and bonding techniques (Franssila, 2004). They are typically made of glass, silicon, or polymers with an overall size from millimetres to centimetres, and with micro volume channel networks (Dittrich *et al*., 2006).

Table 2 *Advantages of miniaturised systems*

faster analysis time - increase productivity
reduce sample and reagent consumption
minimize the risk of losing valuable samples
reduce cost
improve reproducibility, accuracy and reliability
integration and parallelisation
possibility for portability and disposability
automation
environmentally friendly • low power consumption • less material - less waste • elimination of rare or aggressive material polluting the environment

Polymers have gained wide acceptance due to their low cost, availability, and ease to produce (Becker and Locascio, 2002). In recent years, remarkable progress has been made in simplifying conventional techniques such as CE for miniaturisation (Kutter and Fintschenko, 2006a; Janasek *et al*., 2006; Tudos *et al*., 2001; Guttman and Ronai, 2000; Han and Singh, 2004; Tsai *et al*., 2004). Electrophoresis became the primary target for miniaturisation. Miniaturised electrophoresis consists basically of two channels that are crossed: the sample loading and the separation channels. A schematic diagram of an electrophoresis microchip with classical and double T sample injection crossing is illustrated in Figure 4. The sample injection is done in a cross section of a channel network by switching the voltage. The injected sample is then electrokinetically driven through the separation channel by electroosmotic flow generated inside the channel. Hence, this overcomes the need for mechanical pumps or valves to drive fluid movement. Furthermore, its application can range from small ion separations to proteins, RNA, DNA, and cell analyses (Tuomikoski *et al*., 2005; Huang *et al*., 2008; Peng *et al*., 2008; Dolnik and Liu, 2005; Wang *et al*., 2003).

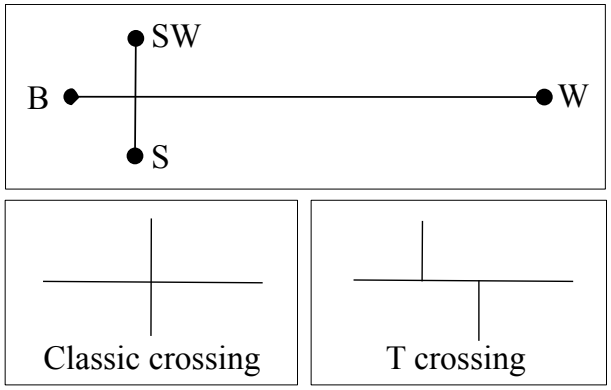

Figure 4 *Schematic diagram of the electrophoresis microchip with different sample injection types. B, buffer reservoir; S, sample reservoir; SW, sample waste; W, waste.*

Separation of macromolecules (protein, RNA and DNA) using the microchip has become very popular due to fast analysis time and the low amount of analytes required. Nevertheless, there

are a number of challenges to using chip-based systems such as tight bonding of microchip wafers, evaporation of already small amounts of liquids in reservoirs, and the formation of H^2 and O^2 gasses in electrodes that interfere with the separation and decrease the sensitivity of detection (Kutter and Fintschenko. 2006b).

Currently, considerable attention is being paid to the development of microchip-based analytical systems suitable for the separation and/or analysis of proteins. Among different available electrokinetic modes, ZE and GE remain the most widely adopted ones to achieve protein separation on a microchip. Slab gel electrophoresis is rarely applied in miniaturised systems, but is daily used in proteomics research. Various groups have presented miniaturised 1-D and 2-D slab gel electrophoresis instruments based on polyacrylamide gels that allow faster analysis with low protein loads (Usui *et al*., 2006; Samuel, 1995; Manabe, 2000; Sasaki *et al*., 1999; Sluszny and Yeung, 2004; Hiratsuka *et al*., 2007; Neuhoff, 2000; I; III).

The integration of two or more methods of different analysis parameters is necessary to distinguish between proteins that behave similarly or to concentrate low protein amounts (Cong *et al*., 2008; Liu *et al*., 2008; Olvecka *et al*., 2004; Liu and Sweedler, 1996; Michels *et al*., 2004; Hu *et al*., 2004; Sheng and Pawliszyn, 2002). Recently, Ramsey *et al*. showed that peptide mixtures can also be separated by 2-D electrophoresis microchips: They combined MEKC (1st dimension) and ZE (2nd dimension) for peptide separation on a microchip (Ramsey *et al*., 2003; Rocklin *et al*. 2000). A combination of open channel electrochromatography (a C18-coated spiral channel) and ZE on a microchip was also demonstrated (Gottschlich *et al*., 2001). Electrophoresis microchips have been coupled with 1| MS by ESI-nozzle, or with 2| MALDI technology to perform sensitive and fast separations (Sung *et al*., 2005; Mellors *et al*., 2008; DeVoe and Lee, 2006). The combination of electrophoresis microchips (fast analysis time, low sample loads, high sensitivity) with MS require precise manual handling that may result in incomplete protein transfer, low reproducibility, or cross-contamination.

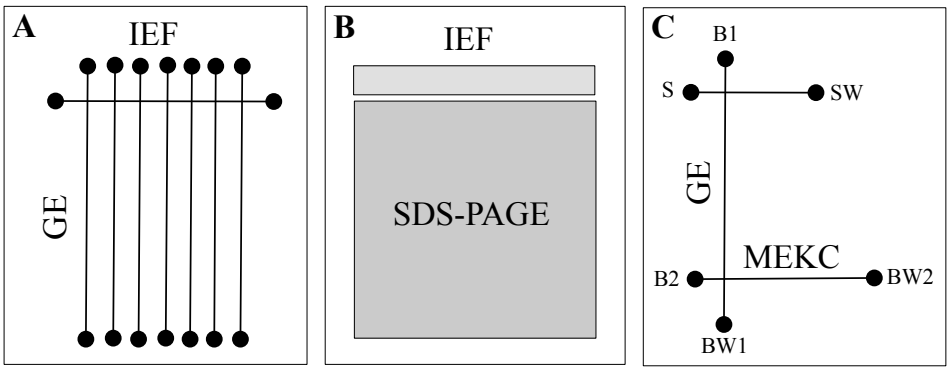

Figure 5 *Schematic diagrams of different electrophoresis microchips. A| IEF channel perpendicular to parallel GE channels, and B| scaling down the physical size of a conventional apparatus, and C| coupling two orthogonal separation channels (modified from Herr et al. 2003). IEF, isolectric focusing; GE, gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MEKC, micellar electrokinetic chromatography; S, sample reservoir; SW, sample waste, B1, GE buffer reservoir, B2, MEKC buffer reservoir; BW1, GE buffer waste; BW2, MEKC buffer waste.*

Table 3 *Recent development of 2-D electrophoresis instrumentation for protein separation and analysis on a microchip.*

Method		Material	Performance	Interface	Detection	Ref
Channel structure	GE-MEKC	PMMA	Protein mixture (10 standard proteins) were separated < 12 min	The sample was electrokinetically controlled for its transfer from 1-D to the 2-D so-called rapid sampling effluent	LIF	(Shadpour and Soper, 2006)
	IEF-ZE or GE	PDMS	IEF standard sample was pre-labelled using fluorescent dye. The separation took 20 min	The IEF and ZE were isolated by 2 sets of valves. By the opening and closing of the valves the targeted protein peaks are isolated. Interdiffusion and band dispersion during transfer to the 2nd dimension is prevented	Inverted epi-fluorescence microscope	(Wang *et al.*, 2004)
	IEF-GE	COC	Network of 29 GE channels orthogonal to the IEF separated 4 similar (pI or RMW) proteins in < 10 min	An array of pseudo-valves (in-situ polymerized acryalmide solution) was created for introducing different separation media	LIF	(Das *et al.*, 2007)
		Glass	Network of 20 GE channels orthogonal to the IEF was used to analyze the expression of MBP of E.Coli in 1 h	Passive valves of structure of narrower and shallower channels were used as an interface between IEF and GE channels	Confocal fluorescent scanner	(Emrich *et al.*, 2007)
		PDMS	Network of 100 GE channels in one layer and an IEF in another layer separated the protein mixture within a few min	The PDMS membranes that enclose the gel were used in a capillary during the first separation and provide passages for the proteins to migrate into the array of orthogonal channels	LIF	(Griebel *et al.*, 2004; Chen *et al.*, 2002)

Slab gel format	IEF-PAGE	Glass/ other material	Separation of E.Coli protein extract in less than 2.5 h. The gel size was ~1 cm2 with resolving power of ~200 spot	The gap separates an IEF from PAA gel and fills it with poly(ethylene oxide) solution during focusing	Fluorescence	(Sluszny and Yeung, 2004; Xu *et al*., 2005; Zhang and Yeung, 2006)
		PMMA	Separation of 9 standard proteins within 1 h on gel area ~12 cm2	An 0.5 mm air space separates IEF from PAGE during focusing	Coomassie blue staining	(I)
			Mouse brain tissue lysate was separated in 1 h with on-line detection. The gel size was ~24 cm2 with resolving power of ~100 spot	The IEF gel was rehydrated and expanded downwards; liquid agarose was poured into the junction structure between 1-D and 2-D regions	Fluorescence	(Usui *et al*., 2006)
			Fully automated and computer controlled 2-DE device. The gel size was ~24 cm2. Mouse liver tissue lysate separated in 1.5 h	Conveyer based on 2-DE workflow moves the IEF strip to different PMMA parts and places the strip on top of 2D gel prior to PAGE	Fluorescence	(Hiratsuka *et al*., 2007)

Key: *1-D*, one-dimensional; *2-D*, two-dimensional; *COC*, cyclic olefin copolymer; *GE*, gel electrophoresis; *IEF,* isoelectric focusing; *LIF*, laser induced fluorescence; *MBP*, maltose binding protein; *MEKC*, micellar electrochromatography; *PAA*, polyacrylamide; *PAGE*, polyacrylamide gel electrophoresis; *PDMS*, polydimethyl siloxane; pI, isoelectric point; *PMMA*, poly(methyl methacrylate); *RMW*, relative molecular weight; *ZE*, zone electrophoresis.

Certainly, the most fundamental task in proteomics is to detect and identify proteins from biological samples. One of the methods used is 2-DE due to its high resolving power and potential to store 2-D protein gels. Miniaturisation of this technique and its further automation is challenging. Until now, the IEF and SDS-PAGE separations are run separately on different instruments because of buffer incompatibility (high concentration of salts and SDS). Denaturation of proteins in the presence of salt or SDS and IEF separation is precluded. However, various microchips (channel or slab gel format) with the potential to replace the conventional 2-DE instruments have been published. These include models to combine the IEF channel perpendicular to parallel GE channels (Griebel *et al*., 2004; Li *et al*., 2004), scaling down the physical size of a conventional apparatus (Usui *et al*., 2006; Samuel, 1995; Manabe, 2000; Sasaki *et al*., 1999), or coupling two orthogonal separation channels (Ramsey *et al*., 2003; Cong *et al*., 2008; Gottschlich *et al*., 2001; Herr *et al*., 2003; Kriikku *et al*., 2004). A schematic diagram of different types of electrophoresis microchips with two different separation mechanisms is illustrated in Figure 5. A list of recent developments of 2-D electrophoresis instrumentation for protein separations and analyses on microchips is listed in Table 3.

2.5 MICROCHIPS FOR DISEASE DIAGNOSTICS

Miniaturisation, robustness, and simplicity of the operation are key factors in making microchips feasible for routine diagnostic tests. Sophisticated diagnostic tools such as versatile microchips that are cheaper, portable, specific, and sensitive would bolster the nascent transformation of clinical medicine towards personalized medicine. There are basically three approaches: miniaturised analytical systems (affinity-based electrophoresis and electrochromatography) (Hou and Herr, 2008), different separation techniques integrated into the same platform (lab-on-a-chip or micro total analysis systems) (Schmut *et al*., 2002), and microarray instruments (protein microarrays) (Spisak *et al*., 2007). The affinity separations face key technical challenges in the analysis of clinically relevant analytes. One of these challenges is analytical sensitivity, because the proteins of interest are often present only in low abundance. The high sensitivity, low patient sample volume, and analysis simplicity are the benefits of a miniaturised analytical system. Due to this, many laboratories have concentrated on developing microfluidic immunoassay instruments for clinical diagnostics (Lee *et al*., 2006; Bai *et al*., 2006; Henares *et al*., 2008c; Kartalov *et al*., 2006). These instruments have great potential to replace the widely used ELISA method in clinical laboratories. However, their operation is still challenging and requires precise handling. Only a few cases of protein analysis by microfluidic instruments can be found in literature (Hou and Herr, 2008; Derveaux *et al*., 2008; Henares *et al*., 2008a; Henares *et al*., 2008b; Dupuy *et al*., 2005).

On the other hand, simple, rapid, quantitative, and sensitive lab on a chip electrophoresis instruments (Agilent's Bioanalyzer and Bio-Rad's Experion) have become more accessible to the end-user. Various analyses have been done using lab on chip instruments, such as checking the kinetics of protein adsorption from serum onto nanoparticles complementary to 2-DE (Kim *et al*., 2007b), quantification of Immunoglobulin G produced in hybridoma cell cultures (Ohashi *et al*., 2002), and determination of RMW of intact, partly, and completely de-N-glycosylated human serum glycoproteins (Muller *et al*., 2007). Various efforts have been made to provide new compact and fast diagnostic tools for hospital and personal use.

Different protein microarrays have been widely used in clinical proteomics due to their applicability for the fast screening of biomarkers. They play an important role in disease diagnosis. Protein microarrays have been developed for binding selected proteins prior to MS. Protein microarrays are analogous to DNA microarrays. These microarrays aim to bind individual proteins from biological samples, such as serum or urine, and to allow high-throughput screening for disease-associated proteins (Petricoin *et al*., 2004). Protein microarrays combined with advanced bioinformatics of genomic microarrays (Quackenbush, 2001) are currently being used to identify the molecular signatures of individual tumours based on protein pathways and signalling cascades (Kricka and Master, 2008; Calvo *et al*., 2005; Gulmann *et al*. 2006).

Protein microarray formats can be divided into two major classes: 1| an analyte-specific ligand (*e.g*. antibody, antigen, recombinant protein) is applied in solution, or 2| the sample analyte is immobilized (*e.g*. lysate from laser capture microdissected cells) on the solid phase. Detection of the array is achieved by probing with a tagged antibody, ligand, or serum/cell lysate. The signal-generating tagging molecule generates a pattern of positive and negative spots. The signal intensity of each spot is proportional to the quantity of applied tagged molecules bound to the target molecule.

Depending on the type of microarray, the array surface is loaded with antibodies, antigens, ions, recombinant proteins, peptides, or various biological samples. The most commonly used protein microarrays are antibody and reverse-phase protein microarrays, such as planar glass-slide antibody microarrays with accurately characterized antibodies which are immobilized on an array surface to capture the protein of interest (Kopf and Zharhary, 2007; Knezevic *et al*., 2001; Gao *et al*., 2005; Haab, 2005; Chen *et al*., 2007) and reverse-phase protein microarrays, where known proteins are immobilized on an array surface to capture the specific antibodies or the protein of interest from body fluids, cell, or tissue lysates (Grubb *et al*., 2003; Tibes *et al*., 2006; Speer *et al*., 2008). Reverse-phase protein microarrays are designed for quantitative, multiplexed analysis of specific phosphorylated, cleaved, total forms (with and without the PTM) of cellular proteins from a limited amount of sample, or to study protein-protein interactions (Espina *et al*., 2007; VanMeter *et al*., 2007).

Other microarray technologies include bead-based, cellular, peptide, aptamer, affinity (surface-enhanced laser desorption/ionization, SELDI), and tissue microarrays (Spisak *et al*., 2007; Chaerkady and Pandey, 2008). In SELDI the protein sample is spotted on a surface (ProteinChip Arrays) modified with a chemical functionality, such as weak or strong ion exchange, hydrophobic surface, and metal-binding interactions. Surfaces can also be functionalized with antibodies, other proteins, or DNA. Only proteins of interest are bound on the surface, while the others are washed to waste. After washing, the matrix is applied to the surface and bound proteins are analyzed by MS (Vorderwülbecke *et al*., 2005). Protein microarrays are applied for screening of many diseases, *e.g*. aging-associated diseases, cardiovascular diseases, cancer, type 2 diabetes, and Alzheimer's disease, etc. (Table 1).

3 AIMS OF THE STUDY

When this work began, there was an urgent need for fast and simple instruments that would successfully and reliably separate proteins in polyacrylamide gels. The primary aim of the research was both to design and to develop miniaturised one- and two-dimensional slab gel electrophoresis instruments for protein studies, especially focusing on clinical proteomics. One of the main goals concerning the miniaturisation of two-dimensional gel electrophoresis instrument was to make it simpler and easier to be automated. The aim was to find a novel way of combining 1st dimension (IEF) with 2nd dimension (SDS-PAGE), without them interfering with each other during the first separation step. In addition to the design and fabrication of microchips, the aim was to use common biochemical/biological methods to test the performance of such microchips. The great hope was to develop microchips that would be accessible to untrained end-users, and would provide fast and reliable protein separation.

The more detailed aims of the work were as follows:

- to study the suitability of various polymers for gel electrophoresis
- to manufacture prototypes for automated GE in microchip format
- to investigate the functionality of the prototype
- to optimize the performance of these miniaturised instruments
- to perform faster and cheaper analyses with lower sample consumption
- to apply biochemical/biological methods on these miniaturised instruments
- to compare the reliability, repeatability, and detection limits of miniaturised and commercially available gel electrophoresis instruments
- to apply microchips for the determination of clinically relevant protein samples

4 EXPERIMENTAL SECTION

Protein samples, methods, and the commercial instrumentation used in this study (I-IV) are listed in Tables 4-6, and described in detail in the original publications. In addition to the methods presented in Table 5 and the two experimental descriptions presented herein (4.1 and 4.2), standard molecular biology methods such as potentiometry, spectrophotometry, and various gel staining methods were used. Total protein concentration of cell/tissue lysates (III and IV) and of the cytosolic protein fraction (study IV) were measured by the 2D Quant kit (GE Healthcare).

Table 4 *Protein samples used in studies I-IV*

Protein Sample	**Study**
ß-lactoglobulin	I, III
IEF standard	I, II, IV
Precision Plus Protein All Blue	III
Low Molecular Weight Calibration Kit	III
Peptide Calibration Standard	III
Vascular smooth muscle cell (VSMC) lysate	III
Cytosolic protein fraction of red blood cells	IV
Lysate of post-mortem brain tissue	IV

Table 5 *Methods used in studies I-IV*

Method	**Study**
2-DE in a microchip	I, II, IV
Slab gel electrophoresis in a microchip and mini-PROTEAN 3 cell	III
Peptide mass fingerprinting	III
Protein Sequencing by Edman degradation	III
1-D Immunoblotting	III
2-D Immunoblotting	IV

Table 6 *Commercial instrumentation used in experimental work of studies I-IV*

Instrument (manufacturer)	**Study**
mini-PROTEAN 3 cell (Bio-Rad)	I, III
Autoflex MALDI-ToF-MS (Bruker)	I, III
Applied Biosystems 477A/120A fully automated protein/peptide sequencer (Applied Biosystems)	III

4.1 CASTING OF MICRO-GELS FOR THE PASGE-chip

(STUDY III)

The pump-controlled set-up for the preparation and casting of miniaturised polyacrylamide (PAA) slab gels was designed to repeatably cast PAA gels with ultra narrow dimensions. The casting set-up consisted of a LC 414 pump (Kontron Analytic; Zürich, Switzerland), polymer

tubes and the PASGE-chip casting module. The micro-gels (15 x 11 x 0.37 mm) were prepared according to Laemmli (Laemmli, 1970). The micro-gels consisted of a stacking PAA gel (~ 17 μl of 4% polymer solution) and a resolving PAA gel (~68 μl of various percentage of polymer solution depending on the application). Both polymer solutions were consecutively pumped into the casting module with a piston pump at a flow rate of 200 µlmin^{-1}. This flow rate was chosen to prevent mixing of the stacking and resolving PAA polymeric solutions during gel casting. Figure 6 schematically illustrates the casting of the PAA polymer solutions into the PASGE-chip casting module.

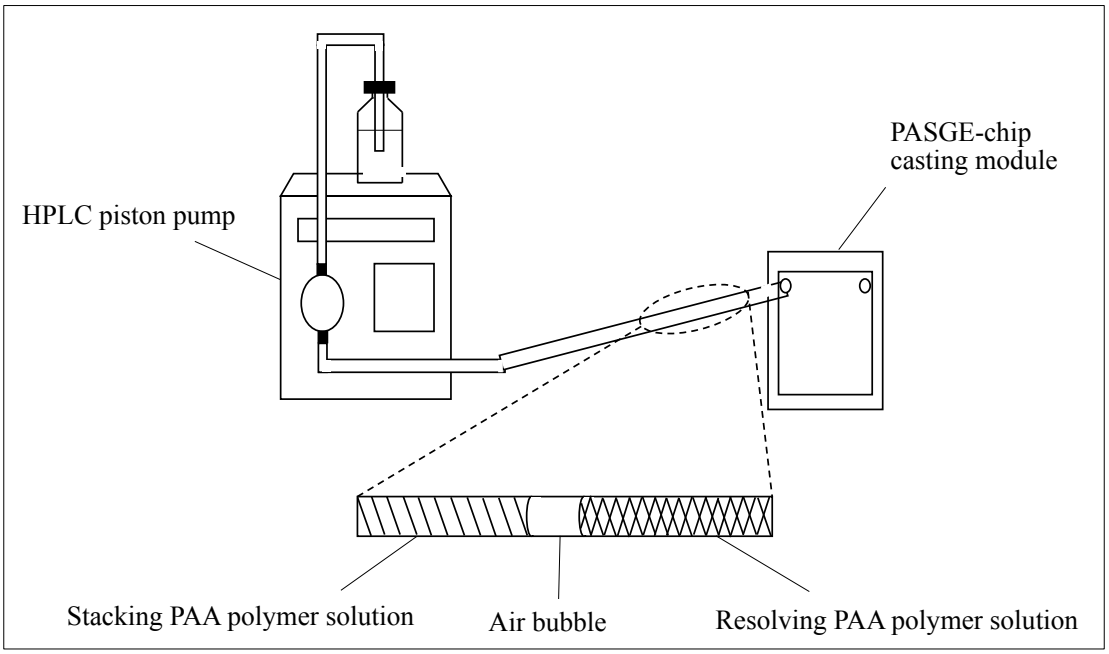

Figure 6 *Schematic illustration of casting the micro-gel (III).*

1.7 OPTIMISATION OF THE pH GRADIENT MADE BY CA

(ADDITIONAL TO STUDY IV, DATA NOT PUBLISHED)

Optimisation of the IEF separations was performed to ensure the stability of the pH gradient formed by carrier ampholytes (CA). IEF was performed without a sample using different running conditions by the ComPress-2DE chip. Three separation parameters were tested; 1| how long does it take to form a stable pH gradient and how long is the pH gradient stable? 2| what is the best CA mixture to keep a stable pH gradient for an hour, and 3| how to prevent water evaporation from the IEF gel during focusing?

The denaturing IEF gel was prepared as described in study IV. Various solutions of CA were mixed to achieve a 1% concentration of CA in the IEF gel. The mixture consisted of the following CA solutions: 1| pH 4-6; 2| pH 3-5 and pH 4-6 (1:1, v/v); 3| pH 3-10, pH 5-8 and pH 4-6 (1:1:8, v/v/v). The assembled IEF unit was stored at 4°C in a moist chamber until used.

To slow down water evaporation during focusing from the IEF gel, the pre-wetted filter pads were placed onto cathodic and anodic ends of the IEF gel. Filter pads were soaked in two dif-

ferent electrolytes (see Table 7).

Table 7 *Different combinations of electrolytes used to pre-wet the filter pads*

	Cathode	**Anode**
1	20 mM NaOH	10 mM H_3PO_4
2	20 mM tris base	10 mM Citric citric acid
3	0.5% CA pH 5-8	0.5% CA pH 3-5
4	H_2O	10 mM Citric citric acid
5	H_2O	10 mM Formic formic acid
6	H_2O	10 mM H_3PO_4
7	H_2O	H2O

Key: *CA*, carrier ampholyte

Focusing was performed under a constant current (100 μA). During focusing, the voltage was gradually increased from approximately 20 V to 200-2 000 V, depending on the focusing time. After focusing, the IEF gel was repositioned on the glass plate and evenly cut into approximately 15 gel pieces (approximately 1 mm-thick gel pieces). Each gel piece was moved to a 500 μl eppendorf tube, covered with water (approximately 50 μl), and vortexed for 5 minutes three times every 10 minutes. The pH of the solution was measured by a digital pH meter (JENCO; San Diego, CA, US).

4 RESULTS AND DISCUSSION

The main results with some additional studies are described. Further details on the experimental conditions and results are to be found in the original publications I-IV.

4.1 ON THE WAY TOWARDS THE FINAL DESIGN

(DATA NOT PUBLISHED)

Our 1st prototype consisted of three parts, which resembled a CD format, and contained: the bottom plate, the gel chamber unit, and buffer reservoir unit with integrated electrodes. These parts were pressed together with four clamps to prevent PAA gel solution leakage prior to polymerisation (Figure 7 A). The instrument was fabricated from PMMA material and was operated in two steps: 1| the gel formation step, and 2| the running position step. Step 1| is changed to step 2| by a 90º turn of the buffer reservoir unit. The first successful separation of low molecular weight (LMW) proteins on an 8% PAA gel (2 mm-thick 5.2 x 4 cm (length x width)) is shown in Figure 7 B. The buffer solution (1xTris-glycine-SDS buffer) was continuously pumped through the buffer reservoirs with a syringe pump. The sample was loaded into a filter pad and placed on top of the gel. The main disadvantages of the 1st generation of 1-DE prototype were: 1| the leakage of the PAA solution during gel polymerisation, 2| PAA gels cracked when parts were turned, 3| challenging sample loading, and 4| the set-up was complicated in use.

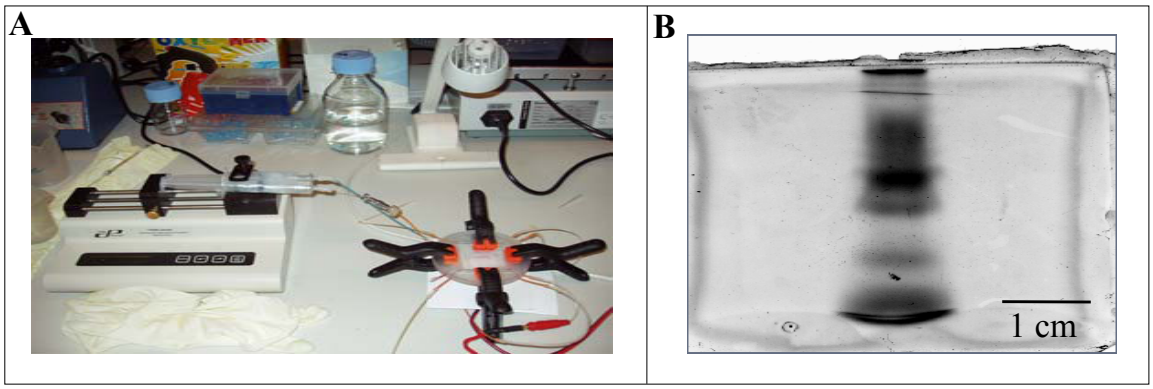

Figure 7 *The 1st generation 1-DE prototype. A| the whole assembly of the prototype, and B| separation of LMW standard on an 8% PAA gel.*

After testing the 1st prototype, the whole concept of the miniaturised gel electrophoresis device was changed from CD format to rectangular format microchip. The moving of the buffer reservoir unit from step 1| to step 2| was excluded from the design of the 2nd prototype. Different materials were tested for compatibility with PAA (I). PMMA and silicon dioxide coated silicon showed the best performance. PMMA was the material of choice due to easy fabrication and low cost. PMMA was subsequently used to fabricate further prototypes, except when the gel chamber height was below 0.5 mm. In this case, silicon dioxide coated silicon material was used. PAA gels were prepared in a separate casting module (gel chamber unit and cover plate). After the gel polymerized, the cover plate was removed and the buffer reservoir/electrode unit placed on top of the gel chamber unit. The top unit was made of an elastic polymer (polydimethyl siloxane, PDMS). The PDMS prevented buffer leakages during analysis due to its adhesion to the gel chamber unit. As PDMS interfered with the PAA gel polymerisation, it was not used

for the gel chamber fabrication. After the running buffer was pipetted into the buffer reservoir, the sample was loaded into the sample well. With the 2nd generation 1-DE prototype it was possible to separate three samples of a protein mixture (0.5 µg bovine albumin (66 kDa), 1 µg ovalbumin (45 kDa) and 1 µg ß-lactoglobulin (14.4 kDa)) within half an hour (Figure 8). The gel size was 52 x 40 x 0.75 mm (length x width x height) with an actual separation area of 27 x 22 x 0.75 mm.

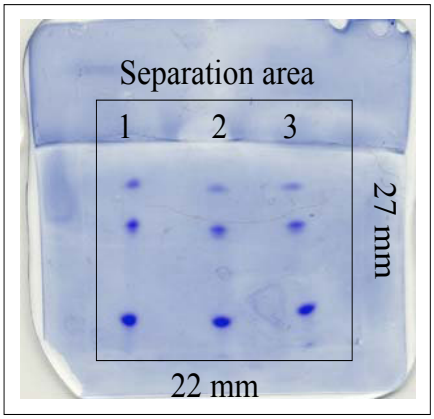

Figure 8 *Testing the performance of the 2nd generation 1-DE prototype. Separation of 0.5 µg bovine albumin, 1 µg ovalbumin and 1 µg ß-lactoglobulin on a 12% PAA gel stained with Coomassie brilliant blue.*

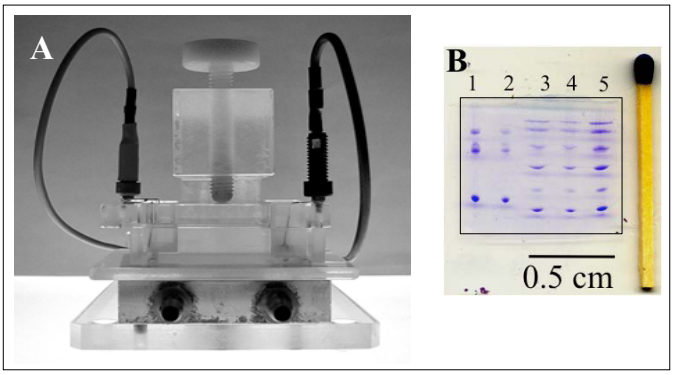

Figure 9 *The 3rd generation 1-DE prototype. A| the whole prototype assembly in a holder with the integrated water-cooling unit, and B| 1-DE protein separation of line 1| 0.25 µg bovine albumin, 0.5 µg ovalbumin and 0.5 µg ß-lactoglobulin, line 2| 0.125 µg bovine albumin, 0.25 µg ovalbumin and 0.25 µg ß-lactoglobulin, line 3 and line 4| 0.72 µg, and line 5| 1.4 µg total protein amount of LMW sample standard on a 12% PAA gel.*

A 3rd prototype (miniaturised polyacrylamide slab gel electrophoresis instrument, 1-DE microchip) was designed. The 1-DE microchip was assembled into the external chip holder with an integrated water-cooled plate, as shown in Figure 9 A. 1-DE microchip could simultaneously separate proteins in five samples in less than 10 minutes (Figure 9 B). This prototype contained three parts: A PMMA coverlid with integrated electrodes, a buffer reservoir unit made of PDMS with five sample loading wells, and a plate with gel chamber (37 x 25 x 0.3/0.5 mm). Difficulties were encountered after the miniaturisation of the slab gel electrophoresis device, such as 1| overheating of the gel during the separation creating "smiley" bands, 2| handling ultra thin gels, 3| producing thinner gels (below 0.5 mm), and 4| high gel-to-gel variation. By instrumentation

some disadvantages were overcome, integrating a water-cooled aluminium plate into the external holder solved the heating problem. Silicon dioxide coated silicon was used to fabricate the gel chamber where gel thickness was below 0.5 mm.

4.2 MINIATURISED 1-D ELECTROPHORESIS (PASGE-chip)

(STUDY III)

The 1-DE microchip was further miniaturised to study the influence of gel-size on protein separation. The 4th prototype (PASGE-chip) could rapidly separate a set of predefined standard mixture, as well as protein lysates.

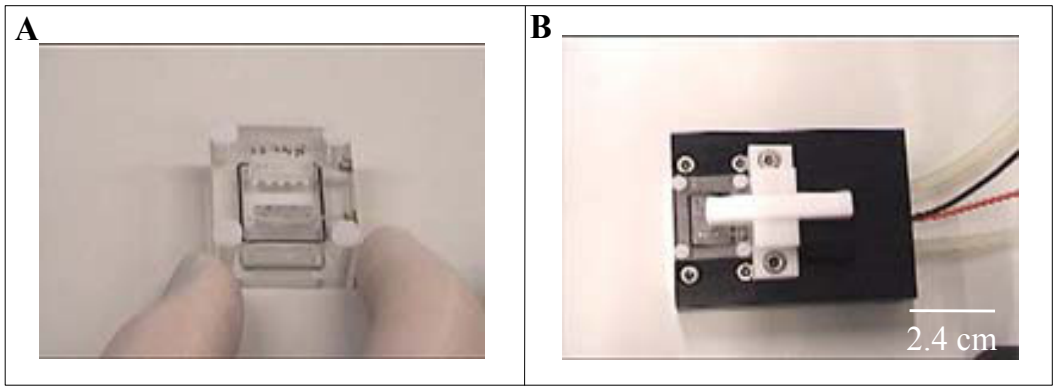

Figure 10 *Photo of A| 4th generation 1-DE prototype (PASGE-chip), and B| assembled PASGE-chip in the holder with integrated pin electrodes and cooling unit (III).*

PASGE-chip assembly is shown in Figure 10 A. It consists of a loading compartment (3 x 2.5 x 1 cm, width x length x depth) with integrated buffer reservoirs, running electrodes and five sample inlets, and a plate with gel chamber (11 x 15 x 0.5 mm). The microchip was inserted into a holder containing pin connectors for electrode wires and an aluminium water-cooling unit (Figure 10B).

Separated proteins were detected by Coomassie brilliant blue (I). Several materials were tested for their suitability for micro-gel preparation, *e.g.* polyester, polycarbonate, silicon dioxide, and PMMA, as published elsewhere (I). According to our results, PMMA turned out to be one of the few materials which did not interfere with the electrical field of the gel electrophoresis device. Furthermore, it also allows a homogenous PAA polymerisation of gels thicker than 0.5 mm. Although PMMA does not react with PAA polymerisation of gel thicker than 0.5 mm, it failed to allow a proper polymerisation of gels below 0.5 mm in thickness (Figure 11 A and B). For this reason, silicon dioxide coated silicon became the support material for gels with thickness below 0.5 mm. The effect of a micro-sized stacking gel was investigated. The shape of protein bands was slightly improved by using stacking gel above the resolving gel (Figure 11 C).

The final version of the PASGE-chip separated proteins from 12 kDa to 150 kDa within 10 minutes with a gel-to-gel repeatability of approximately 3.8%. The detection limit for ß-lactoglobulin was approximately 2-3 ng with silver staining and 10 ng with Coomassie brilliant blue staining, corresponding to a 10-fold increase in sensitivity compared to the separation us-

ing the conventional mini-PROTEAN 3 cell instrument (Bio-Rad).

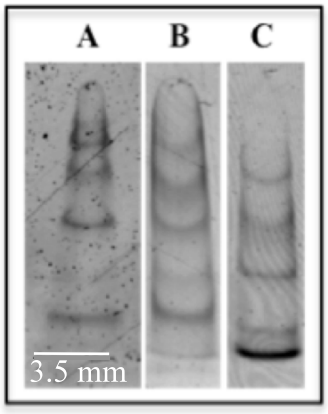

Figure 11 *1-DE separation of LMW standard mixture. The PAA was polymerized in A| PMMA and B| silicon dioxide coated silicon without stacking gel, and C| silicon dioxide with the PAA stacking gel. The gel thickness was approximately 300 μm. Protein bands were visualised with Coomassie brilliant blue. The 1-DE separation was carried at constant voltage (40 V).*

Micro-gels were also used for immunodetection, PMF analysis, and protein sequencing. The sensitivity of an immunodetection was measured by staining α-actin from a VSMC lysate sample (540 ng of total protein). Due to the low-diffusion of the protein bands separated in the micro-gel, a 5-fold higher sensitivity could be achieved compared to protein bands in the 10 x 7 x 0.75 cm gels. PMF analysis showed a 2-fold increase in peak intensity of the peptide fragmentation with considerably lower enzyme consumption due to the smaller area of protein bands in the micro-gel. MASCOT identification of β-lactoglobulin revealed a high probability score of 98 ($p < 0.05$) and sequence coverage of 70%. Edman degradation was performed for protein sequence identification of ß-lactoglobulin after micro-gel blotting to a PVDF membrane. The repetitive yield through ten cycles was 93.9%. All these results were compared to results obtained from a mini-PROTEAN 3 cell.

4.3 MINIATURISED 2-D ELECTROPHORESIS

The goal was to develop a miniaturised device which would be able to run a full 2-DE in a single analysis, thus avoiding the laborious workflow of conventional 2-DE instruments. Two different microchips were fabricated; the 2-DE microchip and ComPress-2DE chip.

4.3.1 2-DE Microchip

(STUDY I)

The 2-DE microchip included five components made of PMMA; an IEF and a PAGE coverlid both with integrated electrodes, a buffer reservoir unit with five sample loading wells, a plate with a gel chamber, and a IEF gel lid with loading channels for the IEF gel solution (Figure 12 A). However, the actual analysis device hosted only three separate components, the IEF or PAGE coverlid, the buffer reservoir unit, and the gel plate, all of which could be assembled

into one single unit (Figure 12 B). Figure 13 represents an IEF separation of naturally-coloured standard proteins, IEF standard mixture. Proteins were separated on an IEF gel (4% PAA) with 1% carrier ampholytes pH 3-10. For protein identification see Table 8.

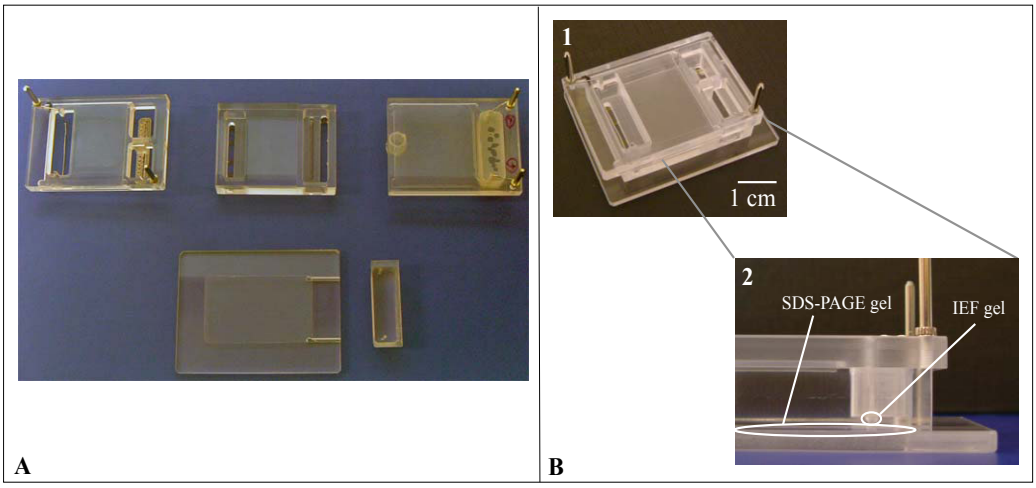

Figure 12 *The 2-DE microchip: A| all five components, and B| actual running assembly; 1| top view of the SDS-PAGE unit, and 2| side view of IEF unit.*

The performance of the microchip was tested with a set of naturally-coloured standard proteins, IEF standard mixture (Bio-Rad). An IEF separation of the protein mixture is shown in Figure 13. The protein focusing was completed in approximately 60 minutes. Focused proteins were then electrophoretically transferred horizontally in a 90°C angle to the PAA gel, and separated according to their molecular weights in approximately 20 minutes. Total 2-DE separation of 6.6 μg of an IEF standard mixture was completed in approximately 80 minutes (Figure 14). To measure the repeatability of the 2-DE performance changes in the pI and RMW, the position of the proteins were measured. On average, the gel-to-gel retardation factor (Rf) variation for human and bovine carbonic anhydrase was approximately 4%, and pI variation approximately 4.5%.

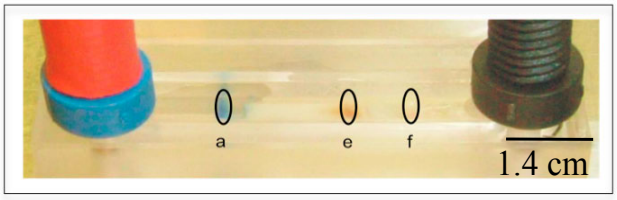

Figure 13 *IEF separation of naturally-coloured standard proteins (IEF standard mixture). Proteins were separated on an IEF gel (4% PAA) with 1% carrier ampholytes pH 3-10. For protein identification see Table 8.*

To mimic an authentic proteome study, we excised all protein spots from Coomassie-stained 2-D gels (Figure 14), in-gel digested them with trypsin and released peptide fragments were analyzed by MALDI-ToF-MS. The MS/MS spectra from peptide fragments were searched against protein databases using Mascot computer algorithms. The identified proteins are listed in Table 8.

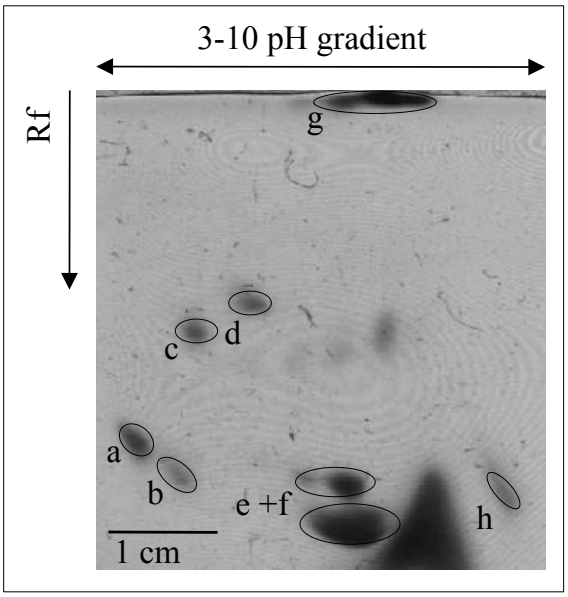

Figure 14 *2-D map of an IEF standard mixture on a 15% SDS-PAA gel. Protein spots a-h identification see also Table 8 (I).*

Table 8 *Proteins identified by MALDI-ToF-MS. Table was modified from IEF standard instruction manual (Bio-Rad).*

	Colour	**pI***	**Protein name**
a	blue	4.45, 4.65, 4.75	phycocyanin
b		5.1	ß-lactoglobulin
c		6.0	bovine carbonic anhydrase
d		6.5	human carbonic anhydrase
e	brown	6.8, 7.0	myoglobin
f	red	7.1, 7.5	haemoglobin
g		7.8, 8.0, 8.2	lentil lectin
h	red	9.6	cytochrome c

* Theoretical pI values of identified proteins

5.3.2 Testing the Stability of the pH Gradient

(ADDITIONAL TO STUDY IV, DATA NOT PUBLISHED)

The 2-DE microchip and ComPress-2DE chip separated successfully a predefined set of sample proteins (IEF standard mixture, Table 8). Difficulties were discovered when analysing biological samples (see Study IV). Biological samples required urea, thiourea, and other detergents to be added into the sample and rehydration buffer as well as into the IEF gel. These are needed to keep the proteins soluble during the 2-DE separation. Identical conditions to those used in a traditional 2-DE separation were applied. The use of high urea concentration (8 M) and the strong inhibition of the PAA gel formation by thiourea complicated the IEF gel preparation. Thiourea inhibits the PAA polymerisation chemically initiated with ammonium presulfate

(APS) and TEMED (Rabilloud, 1998). The urea/IEF gel solution was heated to 37ºC prior to polymerisation. The IEF gel had to be used within two hours after polymerisation because of urea crystallization when stored at 4ºC. The stability of the pH gradient, formed by the CA in the IEF gel, was tested due to possible urea degradation. The pH gradient formed by SEVALYT 4-6 was stabilised within 15 minutes of focusing, as shown in Figure 15. Cathodic drift of the pH gradient is a typical phenomenon when using CA, being caused by electrolysis at the cathode (Baumann and Chrambach, 1975). A 15-minute pre-focusing prior to sample loading was used for the formation and stabilization of the pH gradient made by CA.

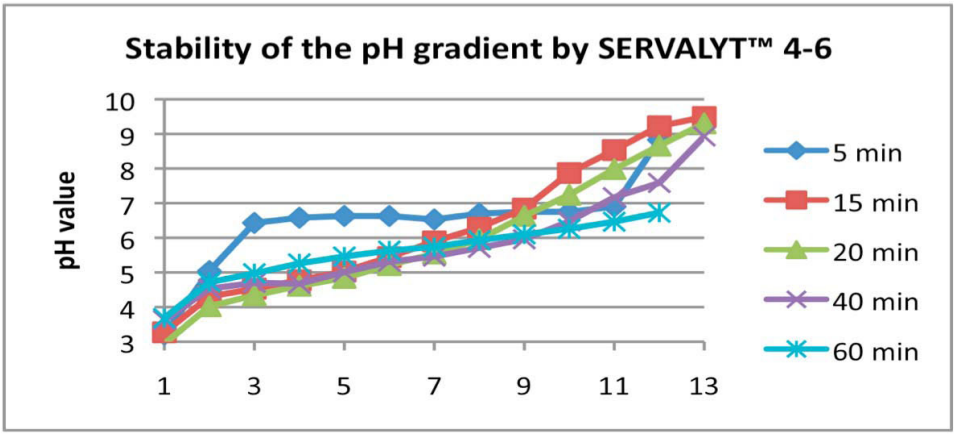

Figure 15 *Chart representing pH stability during focusing. The x-axis represents the number of pieces from the IEF gel (1 gel piece was approximately 1 mm thick).*

High evaporation of water during IEF was observed in the ComPress-2DE chip. However, this was not observed in the 2-DE microchip where the IEF unit was closed by the IEF lid. To minimize evaporation during focusing, filter pads were used at both ends of the IEF gel. Filter pads were pre-soaked in different electrode buffers (for the composition, see Table 7). No difference between the filter pads pre-wetted in water or in the electrode buffers was observed (data not shown). Thus, the water pre-wetted filter pads and a 15-minute pre-focusing were chosen for all IEF separations on the ComPres-2DE chip.

5.3.3 ComPress 2DE-chip

(STUDY IV)

Our final prototype for 2-DE (ComPress-2DE chip) included a new way to move the IEF and PAGE together. ComPress technology was able to control the movement of proteins between the 1st and 2nd dimensions. An empty connecting slot and a blocking membrane separated the IEF and the 2nd dimensional gel contact during IEF. This avoided any possible ionic contamination and prevented the disturbance of the pH gradient during the focusing step. An adjustable micrometer head was used to press the 2nd dimensional gel, to slowly expand the PAA polymer, and to fill the connecting slot for smooth protein transfer from the 1st to the 2nd dimensional gel by electromigration. By simply pressing and expanding the 2nd dimensional gel, a steady linkage between the IEF and the 2nd dimensional gel was kept. The ComPress-2DE chip was made of PMMA by micro-milling and micro-drilling, with a gel size of 26 x 33 x 0.5 mm (Figure 16).

Various samples were successfully separated and detected by the ComPress-2DE chip under native and denatured conditions.

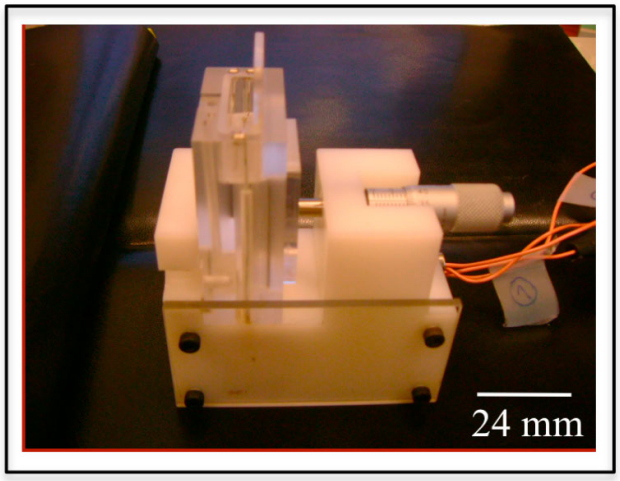

Figure 16 *Complete assembly of the ComPress-2DE chip. The microchip is placed in the chip holder with the integrated electrode pin contacts and the micrometer screw.*

A wide pH range, 3-10, was used for the separation of IEF standard mixture (Figure 17 A). The protein spots on the 2-D protein map were not as diffused as the 2-D protein map for the 1st 2-DE prototype (I). In this first prototype, the 2-DE separation was done with different lids, which were changed between the IEF and PAGE separations. The 2-DE microchip was designed to transfer the focused proteins at a 90° angle from IEF to PAGE gel (Study I). The ComPress-2DE chip moved the focused protein directly into the 2nd dimensional gel without a 90o angle. A 2-DE analysis on the ComPress-2DE chip took approximately 80 minutes. Figure 17 A shows the 2-D protein map of the IEF standard containing nine proteins. The gel-to-gel variation for human and bovine carbonic anhydrase for pI and for Rf with respect to 2-D coordinates and the origin in the centre of Phycocyanin, was approximately 2.5% and 4%, respectively.

The ComPress-2DE chip was used to separate proteins from clinical samples (cytosolic extraction of red blood cell and tissue lysate of the frontal cortex of human brain). Our main interest was to separate and detect PTM proteins using stringent running conditions. An ultra narrow pH gradient (pH 6.7-7.7) was chosen to separate the glycosylated haemoglobin (Hb A_{1c}) from other haemoglobin variants. Native 2-DE preserved the natural conformation of the haemoglobin structure and the denaturation of haemoglobin molecules to smaller subunits. A 2-D map of cytosolic proteins of red blood cells is illustrated in Figure 17 B.

As a proof-of-principle, the ComPress-2DE chip was used to run the control and AD brain samples, followed by immunoblotting to show expression differences of glial fibrillary acidic protein (GFAP) isoforms between healthy and AD patient brain samples (Figure 17 C). The micro-sized device could separate and identify each of the AD samples (n = 4) from their controls (n = 2) based on GFAP isoform distribution differences (Korolainen et al. 2005).

These experiments illustrated the possibility to use the ComPress-2DE chip for the fast screening of diseases where the target is known and the separation conditions are tailored. The Com-

Press technology made the 2-DE method easier to be automated. Nevertheless, it is important to understand that micro-separations are more sensitive to precise handling, reagents purity, accurate buffers preparation, and pre-treatment of biological samples than separations using the conventional 7 or 18 cm wide gels.

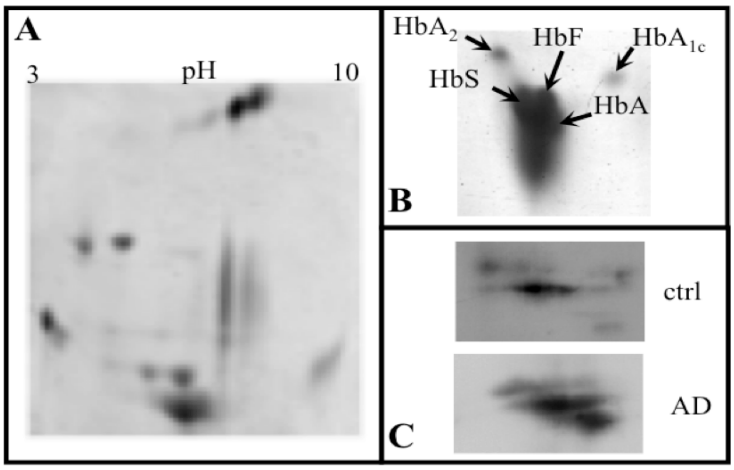

Figure 17 *Testing the performance of the ComPress-2DE chip. 2-D maps of A) the IEF standard; B) haemoglobin variants from human blood; C) post-translation modification differences of glial fibrillary acidic protein isoforms between healthy and Alzheimer's disease (AD) patient from the frontal cortex of human brain (Immunodetection), are shown. Hb, haemoglobin; ctrl, healthy patient sample (IV).*

6 CONCLUSION AND FUTURE PROSPECTIVES

Proteomics is a rapidly developing and changing scientific discipline, which has seen some major advances in fundamental techniques and its expansion into new applications. Developing sophisticated diagnostic tools such as microchips would bolster the nascent transformation of clinical medicine towards personalised medicine. The advantages of microchips are their low cost and portability, short analysis time, high sensitivity, and automation due to their simple design. In addition, many are customised for a particular purpose. Analytical microchips provide a good alternative to macro-scale procedures, generally applied for the detection and identification of proteins from complex biological samples.

In the present thesis, the designs of three different miniaturised GE instruments for microchip analyses of proteins are presented. One such instrument was made to reduce the regular 1-DE system, and the others two were instrumented to miniaturise 2-DE. The aim was to develop microchips that are easy-to-use, can be made from low cost materials, and have the possibility of automation. Our microchips were used in immunodetection, PMF, Edman degradation, and for analysing two clinically relevant protein modifications. The main goal was to overcome the challenge of combining two different separation methods (IEF and PAGE) and to enable automatisation of 2-DE.

Two different miniaturised 2-DE instruments were designed. The first 2-DE prototype (2-DE microchip) had two different cover lids (IEF and PAGE cover lid). Lids were changed after the IEF run was completed, and the IEF and PAGE separation units were joined together. Focused proteins were then transferred from the 1st to the 2nd dimension by electromigration. The second 2-DE prototype (ComPress-2DE chip) had IEF and PAGE separation units integrated onto the same microchip. To achieve an electrical contact between them, the 2nd dimensional gel was pressed by a micrometer head so that the gel slowly filled the gap between these two contacts. The ComPress-2DE system allowed automated 2-DE. Both 2-DE microchips successfully separated a pre-defined IEF standard sample within 80 minutes. The second design was additionally used to separate cytosolic red cell lysates, which distinguished the glycosylated haemoglobin (Hb A_{1c}) from other haemoglobin variants. The ComPress-2DE chip was also used to detecte different post-translational stages of GFAP in healthy and AD patient brain samples.

A conventional-size automated 2-DE device with a gel size of 10 x 7 cm (length x height), was fabricated in parallel with the ComPress-2DE chip. The principle of the ComPress-system was also utilized in this device. This prototype could adopt immobilized ampholytes (commercial IPG strips) in the IEF run and perform a 2-DE analysis comparable to the original ComPress-2DE chip. We could show that this device was running an automated 2-DE with the immobilized IPG strips. Due to the universality of the ComPress method, it can be adapted for any commercial instrument with a small modification to the current instrumentation.

Furthermore, we have also initiated a project to incorporate a 3rd dimension into the ComPress-2DE chip. This would allow either an automated protein digest in the microchip for subsequent PMF MS analysis, or alternatively, to perform immunodetection. For the preliminary experiments we designed a membrane with immobilized enzyme, directly applicable for MS. In addition, we tested new nanoporous filters that would capture the digested proteins after they pass

through the trypsin membrane. We were able to obtain MS data using MALDI-ToF-MS from β-lactoglobulin that was bound to a diethylaminoethyl cellulose or nitrocellulose coated PVDF membrane, as a possible capture membrane.

In conclusion, during this study we designed and fabricated three functional miniaturised slab gel electrophoresis instruments, which showed the advantages of miniaturisation over the commercial devices: Such as fast analyses, low reagent consumption, high sensitivity, high repeatability, and inexpensive performance. All these instruments have the potential to be automated due to their easy-to-use set-up.

ACKNOWLEDGEMENTS

This thesis is based on research which commenced in summer 2003 and was completed in spring 2009 at the Protein Chemistry - Proteomics - Peptide Synthesis - Array Unit, Institute of Biomedicine - Anatomy, Biomedicum, University of Helsinki. It was here that most of the experimental work was performed. Microfabrication of silicon wafers was collaborated with the Department of Micro and Nanosciences, Helsinki University of Technology. Brain tissue lysates were prepared at the Department of Neuroscience and Neurology, Clinical Research Centre/Meditekmia, University of Kuopio by Minna A. Korolainen. This project was carried out in cooperation with the BioFunc group.

I owe my deepest gratitude to Doc. Marc Baumann for giving me the opportunity to be part of his dream to make the life of biochemists both easier and faster. For his encouragement, support, helpful advice, hours of discussion, and clear-cut scientific thinking. His enthusiasm for the application of proteomics into the miniaturised world, especially the development of new devices and their improvement, has made a deep impression on me. Thank you for being not only the boss but also a friend.

I wish to thank all the co-authors for their contribution to this work, especially Eemeli for fabricating the microchips which without your accurate hands would have been so less effective. Also big thanks go to Masahiko and Janne. All of us gave something remarkable to this project and in so doing made the impossible possible. A special thanks goes to Doc. Sami Franssila for his excellent co-operation and valuable comments on my work. It has been my great pleasure and honour to collaborate with you.

I am grateful to the reviewers of this thesis, Prof. Heli Sirén and Prof. Jörg Kutter for their availability and feedback. Heli, it was my pleasure to meet you once again on my road to becoming a scientist. Thank you also for your support and help with my previous project at the Laboratory of Analytical Chemistry, University of Helsinki. I thank Prof. András Guttman for accepting the role of opponent and to challenge my knowledge in public defence.

My time in the lab has been nicer thanks to the people who have and currently populate it, and for the knowledge about protein chemistry which they shared with me. Thanks also go to my scientific friends at the Biomedicum, Haartman Institute, Kumpula and Viiki, for long discussions on work-related and unrelated topics, and particularly to Ale and Nuno for the all-you-can-eat lunch-breaks.

Warm thanks go to all my friends, you kept my days shining even on the coldest winter days and made my stay in Finland unforgettable. Also to all my flatmates, especially the ones at Hämeentie squat 55 that survived my cleaning terror ;) and became amazing friends. It was a great experience to live with you! The Axé goes to mestres, teachers, and members of the groups Capoira Nativos and FICA-helsinki, who taught me how to play capoeira, introduced me to amazing Brazilian culture which I now love, and many times challenged me at rodas. Capoeira will always be part of my life! The following are only a few of the many friends I had the pleasure to meet and share one of too many beers: André, Anna P., Anna V., Birgit, Daiva, Daniel, Danielle, Emilie, Janka, Jaska, Joni, Juha, Jukka, Katri, Kpadja, Maria, Marja-Liisa, Michael, Salla, Suvi,

Ondrej, Paula, Petra, Rocio, Tania, Teppo, Ville, and Yan Peng.

Unforgettable thanks go to my Slovak friends Dano, Evička, Fón, Janka, Kundy, Magduška, Maja, Maroš, Matuško, Michal, Monika, Palino, Peta, Zuzka, and many others. Ďakujem, za prijemné chvíle strávene pri pivke, za nezabudnutelné akcošky, a za pripomínanie, že moja slovenština je už trosku zastarala a poangličtená.

The biggest thanks go to my family. Also teta Vlasta who told me that the only way to get out of Bratislava is to do electro-migration methods. This changed my scientific direction, and subsequently I moved to Helsinki to continue this methodology. Poďakovanie určite patrí aj mojej babke, ktorá my stale pripomínala, že sa chce dožiť mojej promócie a neskôr PhD obhajoby. Tvoj hlas stále zniel v mojej hlave a posúval ma ďalej. Starému ocovi, ktorí ma vždycky povzbudzoval pri získaní nových skúseností a objavovania sveta. Teta, ďakujem že ste my dali občas vedieť čo sa deje doma a vydržali všetky tie moje argumenty počas celých tých rokov. Finally, heartfelt thanks goes to my sister and father for their love and always being there when I needed them. Oci ďakujem, za to že si aký si. Veľa si ma naučil, ukázal a máš veľke dočinenie na tom aká som!

The work was financially supported by TEKES and the Academy of Finland.

All of you, each and everyone who I met in Finland helped me to bring this project to a successful end. Thank you once again.

Zuzana Demianová

Helsinki 2nd of June 2009

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APPENDIX: ORIGINAL PUBLICATIONS I-IV