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MICROFLUIDIC BIOSENSING SYSTEMS BASED ON ENZYMES, ANTIBODIES AND CELLS

Richard Davidsson

Akademisk avhandling som med vederbörligt tillstånd av Naturvetenskapliga fakulteten vid Lunds universitet för avläggande av filosofie doktorsexamen kommer att offentligt försvaras å Kemicentrum, Sölvegatan 39, hörsal B, lördagen den 28 februari 2004, kl 10.15.

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Abstract

The rapid developments within the life science and biotechnology areas put up ever-new demands on more sophisticated techniques and methods for chemical and biological analysis to reach deeper insight into the events at molecular level. Miniaturisation of assays and systems on microchips is one way to increase throughput and advance through parallel analysis lines, multiplexing and integration. This thesis shows some aspects on the use of silicon microchips as a platform for immobilisation of enzymes, antibodies and cells for chemiluminometric biosensing assays.

For antibody- and enzyme-based systems, special attention was paid to the immobilisation chemistry due to its influence on stability and activity of the attached biomolecules. In general it was found that surface coatings with a layer of polymer, e.g., polyethylenimine or dextran, increased both stability and signal intensity of the systems, compared to ordinary silanisation-based attachment chemistry.

Furthermore, two cell-based monitoring systems, using either yeast cells or a reporter gene modified human (HeLa) cell line, were developed. These systems demonstrated the usefulness and advantages of microfluidics, e.g. in continuous monitoring of cellular events in real time, which stands in great contrast to the common end-point microtiter plate assays. The demands on yeast compared to human cells are however quite different, where the latter has highly specialised requirements on the physical and chemical environment. Special attention was thus paid on investigating different factors that can lead to unspecific and stress-related expression of the reporter gene, hence affecting the quality of the data and overall performance of the system.

In conclusion, the main focus of this thesis has been to develop and apply analytical techniques and methods on microchips on flow-format. During the period the which this research was conducted, many interesting and elegant microfluidic analytical systems have been reported in the literature, however, the attention is often drawn mainly to the hardware set-up and construction. The systems developed in this thesis (paper I to V) are technically simple but show real applications in which biological elements, such as living cells, enzymes and antibodies, are handled on silicon microchips.

Key words: Microfluidic, microchip, miniaturisation, enzyme, cell, immobilisation, chemiluminescence.

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Vigten af kemisk kunskap.

Om vi icke kunna undgå att häraf finna, huru en outgrundelig gudomlig allmakt skapade de kemiska processerna till tjenare, för att medelst dem frambringa den eviga omvexling, som vi alldagligen skåda omkring oss i naturen, och att genom dem ur döden ständigt åter framkalla nytt lif, så måste äfven af sig sjelft inses, huru vigtig och nödvändig för hvarje tänkande menniska den vetenskap är, som erbjuder henne förklaring öfver denna omvexling och en noggrannare insigt i skapelsens under.

Ur *Kemi-skola* av J. A. Stöckhardt, 1855.

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II. **Microfluidic enzyme immunosensors with immobilised protein A and G using chemiluminescence detection.** J. Yakovleva, R. Davidsson, M. Bengtsson, T. Laurell, J. Emnéus, *Biosensors and Bioelectronics 2003, 19, 21-34.*

III. **Microfluidic biosensing systems part I: Development and optimisation of enzymatic chemiluminescent µ-biosensors based on silicon microchips.** R. Davidsson, F. Genin, M. Bengtsson, T. Laurell, J. Emnéus, *submitted to Lab on a Chip.*

IV. **Microfluidic biosensing systems part II: Monitoring the dynamic production of glucose and ethanol from microchip-immobilised yeast cells using enzymatic chemiluminescent µ-biosensors.** R. Davidsson, B. Johansson, V. Passoth, M. Bengtsson, T. Laurell, J. Emnéus, *submitted to Lab on a Chip.*

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A-II. **A Chemiluminescence flow immunosensor based on a porous monolithic metacrylate and polyethylene composite disc modified with protein G.** S. Rani Jain, E. Borowska, R. Davidsson, M. Tudorache, E. Pontén, J Emnéus, *Biosensors and Bioelectronics 2003, in press.*

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Harmful azo colorants in leather - Determination based on their cleavage and extraction of corresponding carcinogenic aromatic amines using modern extraction techniques. C. Sparr Eskilsson, R. Davidsson, L. Mathiasson, *Journal of Chromatrography A 2002, 955, 215-227.*

Monitoring of human chemiluminescent reporter cell lines expressing G-protein coupled receptors in a microfluidic system. R Davidsson, J. Bristulf, K. Kotarsky, B. Olde, C. Owman, M. Bengtsson, T. Laurell, J. Emnéus, *Micro Total Analysis Systems 2002* (Eds. Y. Baba, S. Shoji, A. van den Berg), Kluwer Academic Publishers 2002, vol. 2, pp. 811-813.

A micro total analysis system (μTAS) for monitoring immobilized yeast cells using an enzymatic chemiluminescent detection system. R. Davidsson, M. Bengtsson, V. Passoth, T. Laurell, J. Emnéus, *Micro Total Analysis Systems 2001* (Eds. J. M. Ramsey, A. van den Berg), Kluwer Academic Publishers 2001, pp. 287-288.

Highly sensitive silicon microchip based flow immuno biosensor using immobilized affinity proteins and chemiluminescence detection. J. Emnéus, J. Yakovleva, R. Davidsson, A. Lobanova, S. Eremin, T. Laurell, M. Bengtsson, *Micro Total Analysis Systems 2001* (Eds. J. M. Ramsey, A. van den Berg), Kluwer Academic Publishers 2001, pp. 432-434.

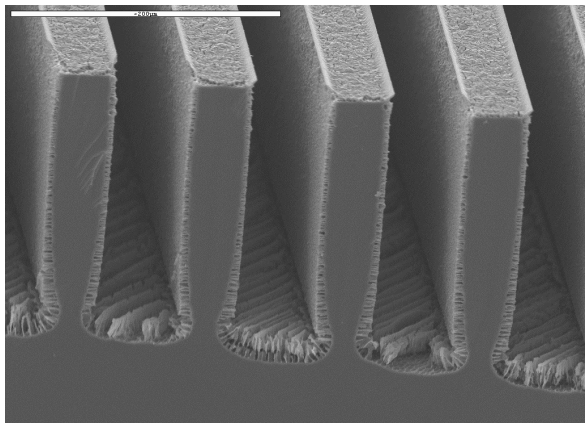
POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Kemister kallas de, som förstå att utreda hwad hwarje sak består utaf, och huru man af beståndsdelarne må kunna sammansätta nya ämnen. Kunskapen härom kallas Kemi. Den största Kemisten war wår landsman Jacob Berzelius, som föddes 1779 i Wäfwersunda i Östergötland och dog i Stockholm 1848.

Så skriver N. J. Berlin i sin *Läsebok i Naturläran för Sweriges Allmoge* utgiven år 1852. Denna beskrivning är fortfarande sann, men i något modernare ordalag uttryckt så kan *kemi* beskrivas som vetenskapen om ämnens sammansättning, egenskaper och omvandlingar. Vetenskapen kemi är ett mycket omfattande område där det idag finns många olika delområden. Forskningen, som ligger till grund för denna avhandling, är lokaliserad inom delområdet *analytisk kemi*, som omfattar tekniker och metoder för att karaktärisera (analysera) kemiska föreningar, och blandningar av kemiska föreningar. Några enkla applikationer på vad man kan utföra med analytisk kemi är t.ex., att bestämma koncentrationen nitrit/nitrat i dricksvatten (intressant ur vatten-kvalitetsperspektiv) eller glukos koncentrationen i blod (viktigt att känna till för diabetiker där glukoskoncentrationen i blodet är avgörande för hälsotillståndet). Även analytisk kemi kan delas in i olika delområden, antingen på basis av vad för typ av material som analyseras eller med vilken teknik som analysen utföres. Forskning inom analytisk kemi syftar till att utveckla nya tekniker samt att innovativt kombinera olika tekniker till att utgöra system för bättre problemlösning och kemisk analys.

Under de sista tio-tolv åren ser man en tydlig trend mot att utveckla miniatyriserade system för kemisk analys på små mikrochips, tillverkade i kisel, glas eller plast. Tillverkningsteknikerna är ursprungligen hämtade från mikroelektronik-industrin där de används för att tillverka elektroniska mikrochips för t.ex. datorer. På mikrochipsen för kemisk analys finns det istället för koppartrådar (som leder elektrisk ström), kanaler och reaktionskamrar, i vilka vätska transporterar prover och reagens för att utföra kemisk analys. Kanalerna är vanligen mellan 10 och 100 µm (1 µm är 0,000001 m) i tvärsnitt och dimensionerna på mikrochipen är ofta några, upp till ett tiotal kvadratcentimeter stora. Jämfört med ordinär kemisk analysutrustning och

teknik vill man med mikrochipminiatyriseringen reducera de fysiska dimensionerna i de steg där vätska, reagens och prov hanteras, transporteras och reagerar. Orsaken till detta intresse är tvåfaldig: analytiskt-kemiskt-tekniskt intressanta effekter från miniatyrisering (däribland förbättrad kvalitet och snabbare analys) samt ekonomiska fördelar. Dessa två är dock intimt relaterade till varandra, t.ex. kan förbättrad och snabbare analys via miniatyrisering ge högre genomströmning av prov per tidsenhet, vilket kan vara ekonomiskt gynnsamt. Vidare, så är en praktisk konsekvens av miniatyrisering att kemikalie- och energikonsumtion minskar, vilket är både ekonomiskt och miljömässigt fördelaktigt.

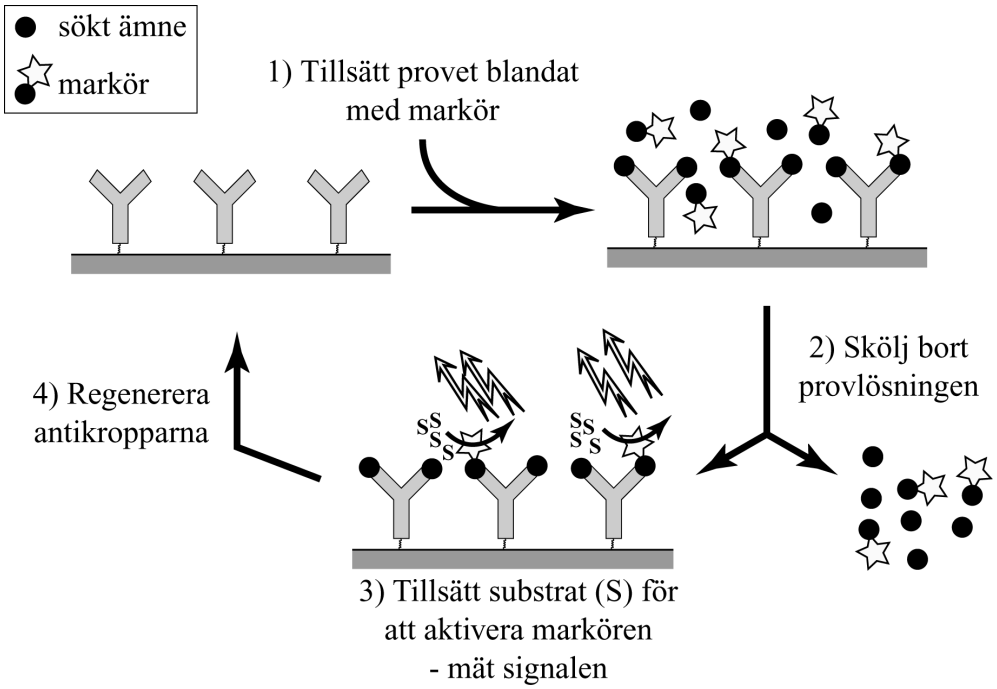


Figur 1. Här visas strukturen på en typ av mikrochip som använts i det här arbetet. Det består av 42 parallella kanaler, 235 µm djupa och 25 µm breda. Kanalerna mynnar i var ände i en bassäng. På mikrochipens yta immobiliserades (d.v.s. bands fast) enzym eller antikroppar. Reagens och prov tillsattes i ett vätskeflöde som kontinuerligt pumpades genom chipet, vanligen 10 till 50 µl/min (1 µl är 0,000001 liter).

Syftet med den forskning, som ligger till grund för denna avhandling, har varit att utveckla och undersöka potentialen med miniatyriserade system, där nyckel-komponenten för mätningen är en biologiskt aktiv komponent, t.ex. en antikropp (artikel I och II), ett enzym (artikel III och IV) eller en levande celler (artikel IV och V). En typ av mikrochips som använts för detta visas i figur 1, tillverkad i kisel med ett parallellt kanalverk i vilket vätska med reagens och prov pumpas. På mikrochipens yta binds de biologiska komponenterna fast på kemisk väg (processen kallas immobilisering) och innebär att dessa kan återanvändas; detta är kostnadseffektivt

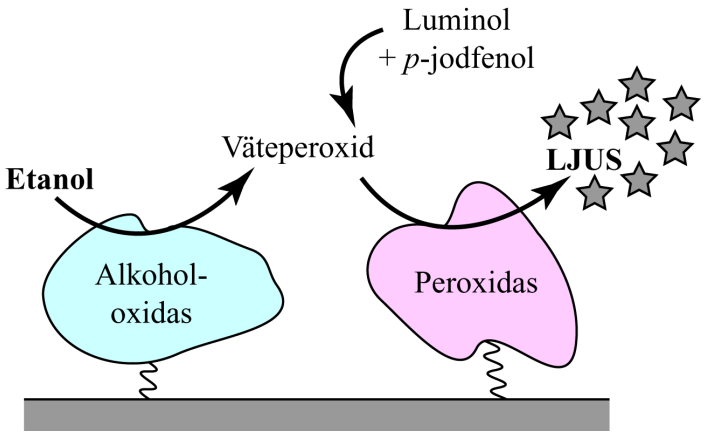
eftersom både enzym och antikroppar är dyra kemikalier. Enzym och antikroppar är mycket stora molekyler som finns i alla levande organismer där de är involverade i olika kemiska processer; antikroppar deltar i immunförsvaret och enzymer katalyserar kemiska reaktioner (d.v.s. gör så att de sker snabbare). Båda dessa grupper av biologiska molekyler har en förmåga att kunna binda speciellt med vissa ämnen eller grupper av ämnen. Detta kan utnyttjas för kemisk analys genom att t.ex. låta antikroppars specifika bindning ”fiska ut” det man är intresserad av ur en blandning (se figur 2). En stor fördel med denna ”biologiska specificitet” är att provet ofta inte behöver renas upp före analys, vilket annars skulle kräva både kostsamma och tidsödande procedurer. På liknande sätt kan enzym utnyttjas där detta endast katalyserar reaktionen med de ämnen som kan binda specifikt till det (se figur 3). De enzym- och antikroppschip som utvecklats i det här arbetet har först karaktäriserats genom att analysera prov med känd sammansättning, och därefter har prov med okänd koncentration analyserats för att bestämma deras innehåll. För antikropp- och enzymchipsen ägnades mycket stor uppmärksamhet åt att bestämma hur immobiliseringskemin (det sätt på vilket molekylerna var fastbundna till ytan) påverkade de biologiska molekylernas stabilitet och aktivitet. Dessa egenskaper är viktiga att känna till för att kunna erhålla tillförlitliga data. Miljön på mikrochipsen är väldigt olik den som antikroppar och enzym härstammar ifrån (d.v.s. inuti levande organismer) och blir därigenom lätt förstörda om de inte hanteras på rätt sätt. Genom att använda olika tillvägagångssätt och reagens vid immobiliseringsproceduren kan ett mikroklimat skapas runt de biologiska molekylerna på mikrochipsen som gör att de blir stabilare under en längre tid. Vilken immobiliseringsmetod som leder till en, för biomolekylerna, gynnsam miljö är inte lätt att i detalj förutse utan måste utvärderas experimentellt.

Levande celler kan också användas som nyckelkomponent i ett system för kemisk analys. Systemet blir då mer komplext och informationen som erhålls är oftast inte koncentrationen av ett sökt ämne utan istället t.ex. giftighet (toxicitet), biologisk tillgänglighet och/eller biologisk aktivitet. Beroende på vilken typ av celler som systemet är baserat på kommer informationen att variera. I det här arbetet utvecklades två modellsystem, först med jästceller (artikel IV) och sedan med en human cancercellstam kallad HFF11 (artikel V).



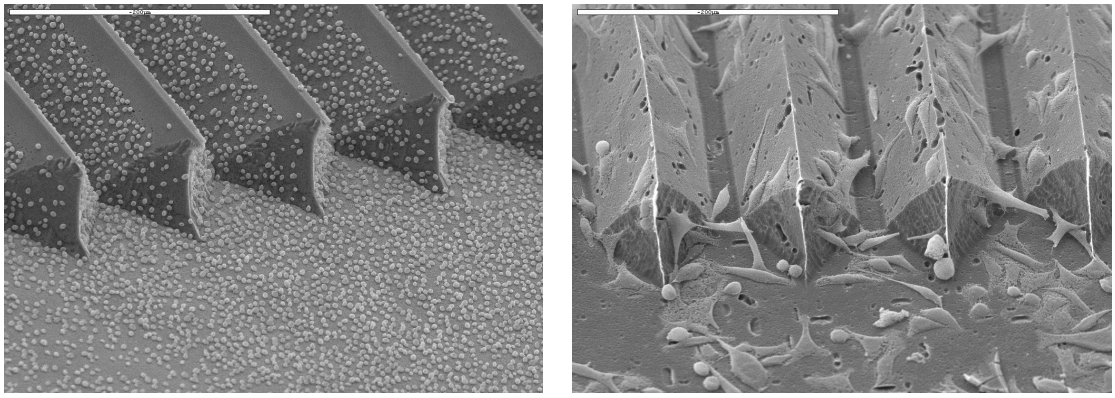
Figur 2. I figuren visas principen för analys baserad på antikroppar immobiliserade på mikrochipen (se figur 1) som utvecklades i artikel I. De svarta prickarna i figuren representerar det sökta ämnet och i det aktuella exemplet var det bekämpningsmedlet atrazin. För att kunna mäta hur mycket atrazin som binder in till antikropparna tillsättes en liten mängd markör till varje prov som skall analyseras. Markören är av samma typ som det sökta ämnet men till denna har man dessutom kopplat en grupp som kan generera en signal. I exemplet här användes atrazin som kopplats till ett enzym som kallas peroxidas. Antikropparna visas som Y:n vilka sitter fasthäftade på ytan. I det första steget tillsätts prov som innehåller atrazin med tillsats av markör (svart prick kopplad till stjärna), vilka kommer att tävla om att få binda in till antikropparna. Ju högre koncentration av atrazin som finns i provet desto färre platser blir över för markören att binda till. Efter några minuter sköljs provet bort och kvar på ytan sitter antikropparna bundna med atrazin samt markör och då är det dags att tillsätta substrat (S). Detta är ett ämne som bryts ner av enzymet på markören samtidigt som ljus skickas ut (s.k. kemiluminescens). Ljussignalen registreras och dess styrka står i direkt proportion till hur många markörer som fanns bunda till antikropparna. Om koncentrationen atrazin var hög i provet kommer endast ett fåtal markörer vara bundna till antikropparna och därav genereras endast en liten mängd ljus och vice versa gäller om

koncentrationen av atrazin var låg i provet. Genom att analysera prover med olika kända koncentrationer av atrazin kan en matematisk relation erhållas mellan koncentration och ljussignal vilken sedan kan användas för att analysera prover med okänt innehåll. Efter att signalen registrerats regenereras antikropparna genom att i några minuter tillsätta en sur (lågt pH-värde) lösning vilket gör att atrazin och markör trillar av antikrpparna. Antikropparna är sedan redo för analysera ett nytt prov.



Figur 3. Ovan visas principen för enzymbaserad analys. I artikel III och IV användes denna detektionsprincip för att bestämma etanol. På mikrochipets yta sitter två slags enzymer immobiliserade, nämligen alkoholoxidas och peroxidas. Till varje prov tillsätts från början en portion av reagensen luminol och p-jod-fenol, vilka krävs för att analysen skall vara möjlig. Då ett prov som innehåller etanol kommer i kontakt med mikrochipet katalyserar alkoholoxidas en reaktion där bland annat väteperoxid bildas. Det senare kommer i sin tur att reagera med luminol och p-jodfenol som katalyseras av peroxidas samtidigt som ljus skickas ut (kemiluminescens), vilket registreras med en ljusdetektor. Desto mer etanol det finns i provet desto mer ljus kommer att produceras. Genom att analysera prover med känd etanolkoncentration kan en en matematisk relation sätt upp mellan koncentration och mängden producerat ljus. Därefter kan man analysera prover med okänt innehåll och med hjälp av den uppmätta signalen samt den matematiska relationen räkna ut koncentrationen etanol i provet.

Målet var här att visa möjligheterna, men också svårigheterna, med att använda mikrochips som bas för ett cellbaserat analytiska system. Figur 4 visar två mikrochips med immobiliserade jästceller och HFF11-celler. Den initiala frågeställningen var om mikrochipsen kunde användas som en plattform för analys av levande celler och hur dessa skulle må i miljön på mikrochipen. Detta undersöktes först för jästceller eftersom dessa är robusta mikroorganismer (se artikel IV), och resulterade i positiva resultat. Nästa steg blev ett något mer komplext system och för detta valdes cellstammen HFF11 (se artikel V), vilken är genetiskt modifierad för att kunna användas för utveckling av nya läkemedel. Frågeställningen var liknande den för det föregående jästsystemet, men i jämförelse ställer HFF11-cellerna avsevärt högre krav på den fysiska och kemiska miljön de befinner sig i. Felaktigheter eller dramatiska förändringar i cellernas miljö kan leda till cellstress och ge upphov till icke-tillförlitlig data. Vidare är analystiden mycket längre, mellan 10 och 30 timmar, vilket skall jämföras med ca en till två timmar för jästcellerna. En stor del av arbetet ägnades därför åt att studera hur dessa celler påverkades av olika hanteringssätt, vilket slutligen resulterade i ett mikrosystem som kunde användas för att studera och följa cellernas aktivitet under ca 30 timmar.



Figur 4. Denna figur visar mikrochips på vilka celler immobiliserats; till vänster jästceller och till höger humana cancerceller.

Under den tid som det här arbetet har utförts har andra forskargrupper rapporterat om eleganta mikrochipssystem, men fokus har ofta legat på den tekniska konstruktionen och dess utförande. I denna avhandling har tekniskt sett relativt enkla system

utvecklats, där tyngdpunkten lagts på utvärdering av hur olika biologiska komponenter beter sig i mikroformatet, vilket mer sällan har beskrivits i litteraturen.

Vad gäller framtidsutsikterna generellt så finns visioner om att ett helt laboratorium skall kunna rymmas på ett mikrochips, därav har begrepp som ”laboratorie-på-ett-chip” eller ”laboratoriechip” myntats. Detta innebär att analyser som det annars krävs ett helt laboratorium för att utföra skall kunna rymmas på ett litet mikrochip. På ett sådant laboratoriechips skall man kunna sätta en droppe prov (t.ex. blod, saliv, dricksvatten) som sedan portioneras ut via kanaler på chipet till små stationer där olika analyser utförs. Några minuter senare skall ett komplett resultat från analysen erhållas. Vidare är tanken att det inte skall behövas någon bred kemisk kunskap för att kunna hantera chipset, ungefär som persondatorernas ”plug-and-play” system, som bara kräver att man trycker på startknappen. Laboratoriechipsen skall kunna ersätta tidsödande rutinmässiga analyser som idag utförs med manuell hantering, involverar flera personer och transport mellan olika enheter där separata analyser sker. Ett exempel är alla de kliniska analyser som idag sker där prov tas från patienter vid olika vårdcentraler och sjukhus och som sedan transporteras till ett centralt laboratorium där analyserna sedan sker. Resultatet skickas sedan tillbaks till den lokala vårdpersonalen och slutligen görs bedömningen av patientens hälsotillstånd. Här skulle ett laboratoriechip direkt kunna hanteras av sköterskan eller läkaren ute på det lokala sjukhuset/vårdcentralen och inom en avsevärt kortare tid ge provresultaten för patienten ifråga. Man kan t.o.m. tänka tanken att framtidens patienter själv kontrollerar sitt hälsotillstånd, ungefär som en diabetiker idag kan kontrollera sin glukoskoncentration och därmed sitt behov av insulin.

Abbreviations

7TM	Seven-transmembrane
AOX	Alcohol oxidase
APTS	3-Aminopropyltriethoxysilane
ATP	Adenosine 5'-triphosphate
BPEI	“Branched” polyethylenimine (only in paper I)
CE	Capillary electrophoresis
CL	Chemiluminescence
CPG	Controlled pore glass
EOF	Electroosmotic flow
FI	Flow injection
FIA	Flow injection analysis
µFIA	Micro flow injection analysis
GA	Glutaraldehyde
GOPS	3-Glycidoxypropyltrimethoxysilane
GOX	Glucose oxidase
HRP	Horseradish peroxidase
HTS	High-throughput screening
IC	Integrated circuit
LOD	Limit of detection
LOV	Lab-on-valve
LPEI	“Linear” polyethylenimine (only in paper I)
LUC	Luciferase
MPV	Multiposition valve
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PIP	*p*-Iodophenol
SEM	Scanning electron microscopy
SIA	Sequential injection analysis
µSIS	Micro sequential injection system
µTAS	Micro total analysis system

1. INTRODUCTION

Biological interactions (DNA/RNA, enzyme-substrate, antibody-antigen, receptor-ligand etc.) occur persistently and en masse inside cells, and are vital for cell function, cell-to-cell communication and higher-level organisations of all living matter. These interactions are part of precisely tuned communication networks and are fundamental to the molecular machinery in which they control all events at the molecular level. Malfunctions in signalling occur however, due to genetically and/or environmentally governed factors, and may be determinants of various diseases, some of which we already understand today (*e.g.* cystic fibrosis and haemophilia). The understanding of how different biological elements are involved in the complex signalling networks of life is thereby fundamental to the understanding and prevention of disease and for the development of new drugs. New high throughput screening (HTS) methodologies of substances interacting with identified biological targets (biomarkers) are important strategic positions for the pharmaceutical industry in the search for new drugs [1, 2] as well as in environmental protection for identifying, monitoring and elimination of potential toxic compounds [3]. The largest most structured attempt in this direction essentially started with the emergence of the genomics field and recently led to the sequencing of the human genome, revealing the code for individual proteins [4, 5]. In the course of these developments, the proteomics field emerged, which attempts to identify and structure determine all encoded proteins [6]. The proteome finally regulates the finely tuned levels of the metabolites, which are the final products from the information transferred from the genome. However, these three fields, genomics, proteomics and metabolomics, are highly interlaced in a huge molecular network and for better understanding how these function together and build up the cell's large-scale organisation a new discipline referred to as systems biology is rapidly emerging [7, 8]. In this new field one tries to merge all previous knowledge from literature with new surfacing knowledge from genomics, proteomics, metabolomics, cellomics etc., to generate a road map of the signalling networks significant to life [7, 8]. This is a large step towards a better biological insight and new potential drug targets and candidates are already at closer reach [9], however, the most extensive work remains, which is to identify and understand how genes, encoded proteins and protein clusters interconnect and rule the huge signalling networks, and to reveal their essential functions for life and death.

In order for the fields of systems biology, cellomics etc. to advance, there is great need for new, reliable and fast assay and screening systems, complementing already existing techniques, and this need can be met by analytical chemists. One special feature here is the increasing interest for using the whole living cell as an analytical biosensing component [10-14]. Miniaturisation of assays and systems on microchips is one way to go to increase throughput and advance through parallel analysis lines, multiplexing and integration [15-18] and will be outlined in more detail in the following chapters.

Objective

In light of the above aspects, the research in this thesis was performed with the overall objective to develop microfluidic bioanalytical platforms, making use of Nature's own macromolecular building blocks (enzymes and antibodies) as the biosensing elements, and at a higher level use the whole cell as a small analytical tool-box to assess molecular information. The biological components were reconstituted on silicon microchips and used for recognition and detection in microfluidic assays based on antibodies (paper I and II), enzymes (paper III and IV) and living cells (paper IV and V). The aim was to investigate the feasibility of these microfluidic bioanalytical systems with the specific aim to explore and solve potential problems associated with the move from a macro to a micro format.

In the following chapters, I present different aspects of importance to the work performed in this thesis: In Chapter 2, some basic principles of enzyme- antibody- and cell-based assays relevant to this work, are presented. In Chapter 3, a review of miniaturisation in general with different aspects on transfer of bioanalytical assays from a macro to a micro format is given. This is followed by a Chapter 4, which briefly describes microchip fabrication processes. In Chapter 5, the attention is turned to what has been achieved so far on chips in regards to antibodies, enzymes and cells, and is followed by Chapter 6, which is a summary of the papers included in this thesis. Finally some conclusions and future visions are given, which are followed by the individual reprints (paper I-II), submitted manuscripts (papers III-V), and an Appendix. The latter contains two published non-microchip based papers, however their content is in many ways related to the work performed in this thesis (Appendix, paper A-I and A-II), *i.e.*, a comparison of two techniques for determining *E. coli* in water (paper A-I), and a

monolithic disk-based immunoassay (paper A-II). These papers will, however, not be referred to in this summary.

2. BASIC PRINCIPLES OF ENZYME-, ANTIBODY- AND CELL-BASED ASSAYS

In this thesis the main focus is directed towards the use of enzymes, antibodies and living cells as the key component in miniaturised analytical system. These three types of biological elements all have different properties and characteristics, and will be briefly reviewed in this chapter.

The inherent ability of biomolecules (enzymes, antibodies, receptors, nucleic acids etc.) to selectively bind certain compounds can be utilised as a core for recognising and/or detecting compounds (*i.e.* analytes) in a device for chemical and/or biological analysis. A huge number of assays and systems are available in various formats, *e.g.*, biosensors, immunoassays [19, 20]. The biological (biosensing) element is reconstituted in an artificial environment, for example covalently linked or adsorbed to a solid surface, where it is used as key component in recognition and detection of the analyte. Not only separate biological molecules can be used for biosensing but also whole living cells. An overview of possible assay systems and the potential information outcome is shown in Figure 1. As can be seen the information complexity increases from using antibody- to cell-based systems. Receptor- and some enzyme-based assays are slightly intermediate since they can be used to obtain concentration values but also give a hint of biological activity, *i.e.*, if a compound binds to a receptor it is likely to have a biological effect when present in a living organism, either as agonist or antagonist (*e.g.* estrogen receptor assay for environmental estrogens [21] or AH receptor for dioxin assay [22]). Similarly, activity of specific enzymes can be used as biomarkers of disease, biological activity, and toxicity (*e.g.*, aromatase, responsible for conversion of testosterone to estradiol, is inhibited by various compounds that can be used in breast cancer and menopause therapy [23] or acetylcholine esterase, essential in neural transmission, is inhibited by various pesticides and is thus an indicator of potential toxicity of these compounds [24]. The cell-based approach results in the most complex information, which can be bioavailability, toxicity or biological activity. The advantage of using cells is that the analyte is allowed to interact with the whole cell and multiple interaction points can be discovered and dynamic data, in terms of agonist-antagonist can be revealed, which is important in drug screening [10, 11]. Through molecular genetics, the functions of cells can in

addition be modified to generate specific signals that we want and are capable of reading, and can thus be exploited as very small and specialised toolboxes that help us learn more about the complexity of life.

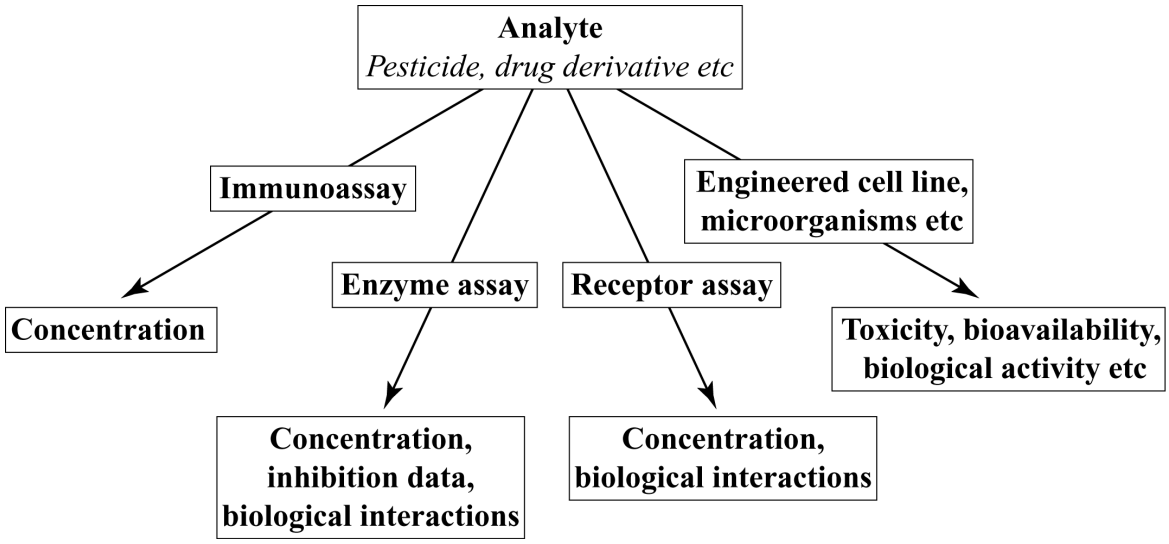


Figure 1. Categorisation of assays using biological elements for recognition and detection. The information output from immunoassays, enzyme and receptor assays is concentration. For the two latter, biological interactions can be investigated, i.e. if a compound binds to the receptor or enzyme it is highly likely to have effect in a living organism. The most complex assay system is based on living cells and depending on cell type used the output information can be for example toxicity, bioavailability or biological activity. Further on, cell systems can reveal if a receptor-ligand interaction is of agonistic or antagonistic character, which cannot be obtained in a corresponding solution-based receptor assay.

Enzymes and antibodies are both proteins but with different properties: enzymes are low affinity proteins that catalyse chemical reactions while antibodies are high affinity proteins that bind strongly to an antigen. The most important property of both is their inherent ability to selectively catalyse (enzymes) or bind (antibodies) certain compounds or groups of compounds, sometimes with absolute specificity for one analyte, which is based on the interaction between the binding site on the enzyme/antibody and the analyte. In practice this means that enzymes and antibodies can be used to "fish out" the analyte from the sample matrix and thus there may not be any need for sample clean up. The unique characteristics of

immunoassays are derived from the strength of the antibody-antigen binding force. Once the antigen has been bound it can only be removed using rather harsh treatments such as extreme pH, high salt strength and organic solvents, (often called regeneration) [20]. On the other hand, an enzyme is completely restored after the catalytic reaction has taken place and does not affect the equilibrium constant of the reaction they catalyse, but simply increase the rate at which the reaction approaches equilibrium [25].

Bioanalytical system based on enzymes, antibodies and cells can be designed in a *solution-based* or *surface-immobilised* format. The former implies that all components, analyte and biological elements react in a homogeneous liquid phase, freely moving in solution, while in the latter the biocomponent is tethered to a solid support and the reaction takes place at this interface. In solution-based systems every sample consumes a small amount of biocomponent whereas surface-immobilised assay formats allow repeated use of the same population of biological elements. The latter is usually more cost effective when using expensive reagents such as antibodies and enzymes. In the case of cells, the immobilised format potentially allows the monitoring of dynamic cellular events of the same cell population during a prolonged period, which could be of paramount importance for the emerging area of systems biology. All systems developed in this thesis are based on surface-immobilised biological components and thereby most attention will be paid to these kinds of assay formats when referring to the literature.

Enzyme-based systems

The enzyme-based biosensing involves conversion of substrates to products in which either the disappearance of a substrate or appearance of a product of the enzymatic reaction is monitored. In general, the latter is the most common principle and has also been used throughout this thesis. The possible detection principles are many (*e.g.* absorbance, fluorescence, chemiluminescence (CL) or electrochemical measurements [26]). Figure 2 shows the two-step enzyme-catalysed CL detection system used in this thesis (paper III and IV). The first enzyme, alcohol oxidase (AOX) or glucose oxidase (GOX), oxidises the analyte (ethanol or glucose) accompanied by formation of hydrogen peroxide. The latter is consumed in the second reaction where horseradish peroxidase (HRP) catalyses the CL oxidation of luminol, enhanced by addition of *p*-iodophenol (PIP) [27]. In paper I and II (microfluidic

immunoassays) the second reaction described in Figure 2 was utilised to monitor the antibody binding event via HRP-labelled analyte (see following section for more details).

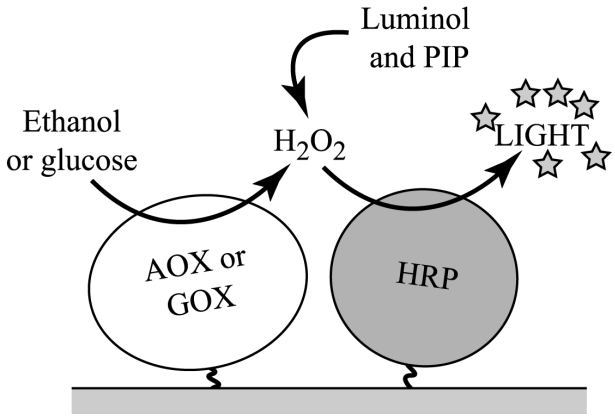


Figure 2. Schematic cartoon showing the enzyme-based assay used in paper III and IV. The system is based on a two-step reaction catalysed by alcohol oxidase (AOX) or glucose oxidase (GOX) co-immobilised with horseradish peroxidase (HRP). First, the analyte (ethanol or glucose) is oxidised by corresponding oxidase enzyme (AOX or GOX) accompanied by formation of hydrogen peroxide. The latter is consumed in the chemiluminescent (CL) oxidation of luminol catalysed by HRP, with addition of p-iodophenol (PIP) as enhancer. In paper I and II the second reaction described in the figure was utilised to monitor the antibody-binding event via HRP-labelled analyte.

Antibody-based systems

Immunoassays can be performed in very large number of formats and it is not easy to categorise, but one can distinguish two large groups, *i.e.*, the "limited reagent" methods (*competitive immunoassays*) and the "reagent excess" methods (*non-competitive immunoassays*). In competitive assays, analyte and labelled analyte (tracer) are added to a limited amount of anti-analyte antibodies. The analyte and tracer will thereby "compete" for the binding sites on the antibodies. After incubation of immuno-reagents the antibody-bound or non-bound (*i.e.* the "free") fraction of tracer is determined, which will reflect the binding ratio between analyte and tracer. In contrast, the non-competitive assays are based on adding

an excess of antibodies to the sample, which means that all analyte is bound. The immuno-complex is then determined and reflects the total concentration of analyte in the sample.

Each format described above has its own advantages and disadvantages, but briefly the sensitivity in competitive assays is determined by the affinity constant of analyte and tracer towards the antibody, while in non-competitive assays, non-specific binding of labelled immuno-reagents will be limiting [20].

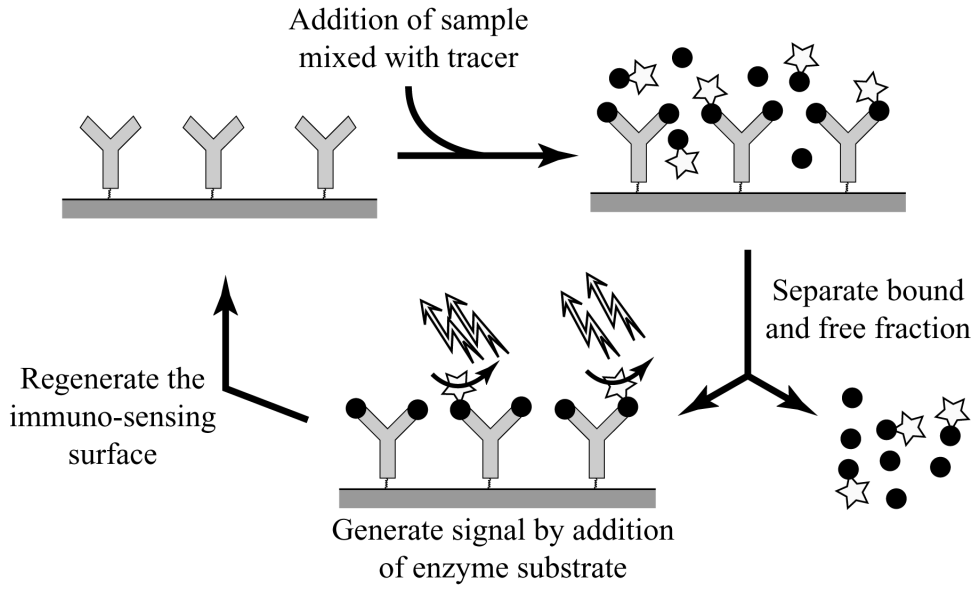


Figure 3. The figure shows the principle for the immunoassays developed in this thesis for determination of atrazine (paper I and II), based on immobilised antibodies and HRP-labelled analyte as tracer. After addition of sample the analyte (black dots) and tracer (black dot labelled with a star) compete for the binding sites on the immobilised antibodies. After incubation the non-bound fraction is removed and a signal is subsequently generated from the HRP tracer by injecting luminol-hydrogen peroxide-PIP substrate (i.e., the second reaction in the system described in Figure 2). Finally, the bound analyte and tracer is removed by changing the pH from 7.4 to 2.2, which results in an immuno-sensing surface ready for another assay cycle.

Both assay formats (competitive and non-competitive) can be further divided into *homogeneous* or *heterogeneous assays*. The former denotes that both the bound and free fraction of immuno-reagents are present when the detection is performed, while the latter

means that the bound fraction is separated from the free fraction before detection, and in this case, either of the two portions can be determined [20]. The separation needed in heterogeneous immunoassays is very often accomplished by using surface-immobilised antibodies or analytes. Thus, after formation of the immuno-complexes the free fraction can be removed while keeping the bound fraction on the support material. The immunoassays developed in this thesis (paper I and II) are heterogeneous competitive systems, using immobilised antibodies (paper I) or affinity proteins that captures the immuno-complexes (paper II). Figure 3 shows the principle for the atrazine immunoassay developed in paper I, using anti-atrazine antibodies immobilised on silicon microchips and HRP-labelled analyte as tracer (see Figure 2 for HRP reaction). The analyte is mixed with tracer and introduced in the microfluidic system. When the sample has entered the microchip the flow is stopped for an incubation period, during which the two components will compete for the binding sites on the immobilised antibodies. The carrier buffer will then remove the non-bound fraction of tracer and analyte. Next a signal is generated from the HRP tracer by injecting luminol-hydrogen peroxide-PIP substrate, which reflects the ratio of bound tracer-to-analyte. As a last step, the immuno-sensing surface is regenerated by decreasing pH to 2.2, which then is ready for another assay cycle.

The enzyme-labelled approach presented in Figure 3 and paper I and II shows a very common detection principle in immunoassays. Via the enzyme-catalysed reaction an amplification and easily detected compound can be produced and determined (*e.g.* via fluorescence, absorbance, electrochemical or CL measurements) [20]. The main disadvantage is the enzymes susceptibility to interference due to changes in assay conditions, *e.g.*, pH, ionic strength, content of organic solvents, as well as other agent, catalysts, inhibitors etc. present in the sample [25, 28]. Moreover, the enzyme-labelled immuno-reagents can adsorb non-specifically due to the high molecular weight of the conjugate, which will generate a background signal and thus affect the sensitivity of the assay [20].

Cell-based assay systems

Bioanalytical systems based on living cells usually constitute the most complex in terms of performance, data out-put and interpretation. However, these matters are highly dependent on what kind of cell type is used (mammalian cells, bacteria, yeast etc.), and is determined by the

purpose of the assay. Figure 4 shows a generic assay type for cell-based system, which for example could be used for toxicity studies [29] or drug development [10-14, 30]. A cell assay starts by stimulating the cell with the sample and depending on the cell type used it responds by different means. Often the cell is genetically manipulated to fit the purpose and can then be engineered to respond by producing a specific protein (so-called reporter genes, see for example references [31, 32]) that might be fluorescent (green fluorescent protein, GFP, is one example [33]) or catalyses a CL reaction (*e.g.* luciferase [34]). Otherwise more fundamental functions can be monitored, for instance, production of an endogenous compound or oxygen consumption that follows respiration. In a final step the signal from the cell is registered and the data collected, interpreted and correlated back to the stimulation.

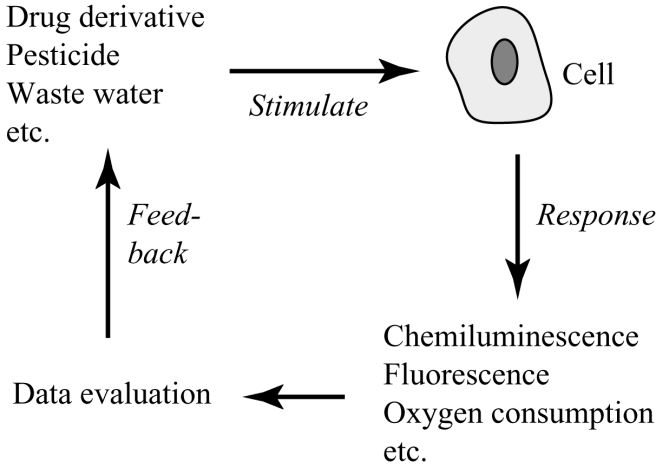


Figure 4. General principle for a cell-based assays set-up. The analyte can be drug derivative, pesticide or wastewater and depending on the cell type used it will respond by different means. The obtained read-out is then correlated back to the stimulation, and depending on the set-up, information such as toxicity, biological activity or bioavailability can be obtained.

As an example, a commonly used toxicity assay is that based on the marine bacteria *Vibrio fischeri* [29]. It is auto-luminescent, which means that it is constantly emitting light under certain conditions. The toxicity is determined by monitoring the decrease in luminescence that follows stimulation of the bacteria with the sample of interest [29]. Moreover, the human cell line used in this thesis (paper V) was constructed for HTS of ligands for seven-transmembrane (7TM) receptors [35, 36], which are potential drug targets [37]. When a ligand binds and activates the receptor it triggers a cascade of intracellular reactions, which

finally ends up with production of the two reporter proteins, enhanced green fluorescent protein and luciferase, which each can be used to monitor the ligand-receptor event [35, 36] (this is outlined in more detail in the summary of paper V in Chapter 6).

Common aspects to consider

Both enzyme- and antibody-based assays are susceptible to various interference, which may arise from cross-reacting compounds that compete with the analyte for the catalytic- or antibody-binding site [20, 25], or unspecific due to undefined compounds present in the sample matrix (including effects on enzyme labels in immunoassay) [20, 25]. Cell-based systems are obviously susceptible to similar types of interference, but are even more complex to decipher.

Modifications by immobilising enzymes or antibodies to a support have been performed extensively in this thesis and can severely influence the kinetics of the catalytic or affinity reaction, and thus the overall assay sensitivity (papers I-IV) [20, 25, 38, 39]. The immobilisation chemistry, the type of support, and the necessity of a spacer arm, are therefore important conditions to consider when developing surface-immobilised reagent assays [38, 39].

3. DOWNSIZING OF SYSTEMS FOR CHEMICAL AND BIOCHEMICAL ANALYSIS

When looking into the literature of miniaturised systems for chemical and biochemical analysis one will find a very diverse group ranging from fully on-chip integrated units with pumps, valves and detectors, to hybrid systems composed of ordinary laboratory equipment interfaced with micromachined components, such as microchip channel systems and reactors. Each system in-between the two limits has its own advantages and problems associated with the choice of construction. Thereby, at a first glance it is not easy to overview the available microanalytical systems, and above all, what is micro and what is macro, the borderline is not always clear and may depend on our background and preferences as readers. To put perspective on the research that I have performed during my Ph. D. studies, limits have to been drawn in order not to get overwhelming. The title of this thesis is ”Microfluidic biosensing systems based on enzymes, antibodies and cells” and with that phrase I mean systems based on sample and reagent handling using liquid in continuous or discontinuous flowing motion. *As a consequence, analysis based on high-density microwell or immobilised-surface reagent arrays (e.g. DNA or protein microchips) will not be included in this thesis.* In principle these latter systems rely on the same liquid handling as the test tube experiment: samples and reagents are added in discrete portions by manual or automatic pipetting. Thus, in this thesis *“microanalytical system”, “microfluidic analytical system”, “microchip-based analytical system”, and “microfluidic biosensing system” will be used interchangeable to describe a system for chemical and/or biochemical analysis based on sample/reagent handling in liquid flow* (in a few instances also gas-based handling when discussing chromatography), *having at least one microfabricated component.* The latter component(s) are thereby sometimes also referred to as the microchip(s). Finally, the applications are limited to microfluidic systems using enzymes and antibodies, and systems for handling and assaying living cells. Below follows a general overview and history of microanalytical systems, explaining basic concepts frequently appearing in the literature.

Brief retrospect on the origin and development of microchip-based analytical systems

The history of analytical microchip systems began with the fabrication of a gas chromatograph on a silicon wafer, at Stanford University in 1979 [40], using the same fabrication technology as that used by the microelectronics industry. However, the micro format did not reach much attention in the chemistry field during the 1980s, although, there were initial signs of a merging trend for miniaturisation of analytical systems. For example, in 1983 and one year later, Ruzicka and Hansen reported on the construction of a microconduits system for flow injection analysis discussing aspects on integration, miniaturisation and scaling [41, 42]. Later, in 1986 van der Linden reviewed fluid dynamics in flow injection when miniaturising [43, 44].

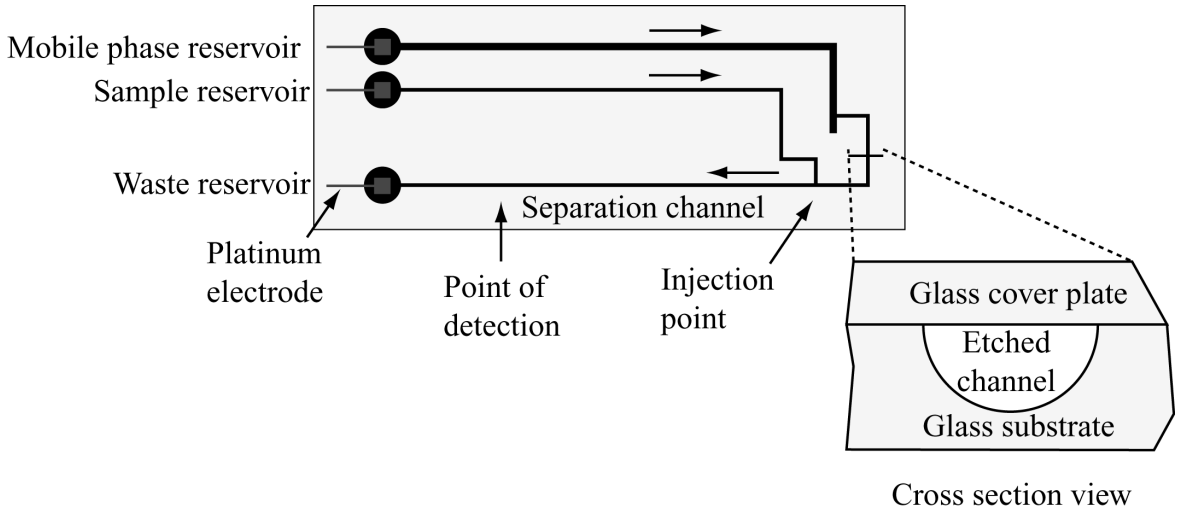


Figure 5. Example of a CE system etched in a planar glass substrate. The figure shows the construction used in the very first CE on-chip published in 1992 [45]. The substrate was 5 mm thick and the channels were 10 µm deep to which another glass substrate was attached (in a process called bonding). Access holes were drilled in the cover plate and used as buffer reservoirs and insertion of platinum electrodes.

In 1990 Manz *et al.* published their theoretical work on what they called "miniaturised total chemical analysis system" (µTAS), where the effects from downscaling were theoretically reviewed in terms of analytical performance [46]. In the same year, Manz *et al.* also reported on a microchip liquid chromatograph. The column was 6 x 2 µm and 15 cm long (1.5 nl

volume) and was etched in a silicon microchip with an integrated conductometric detector (1.2 pl volume). Eluent pumping and sample injection was performed off-chip using conventional equipment. The plate number was 8,000 and 25,000 for a 1 and 5 min separation, respectively [47]. Two years later, in 1992, Harrison *et al.* showed the first microchip capillary electrophoresis (CE) system (see Figure 5). It was fully integrated on-chip and fabricated on a single glass substrate having 10 μm deep channels. Calcein was used as model analyte and the maximum number of theoretical plates obtained was 35,000 [45]. At this time the group of Nico F. de Rooij reported on miniaturised systems using piezoelectric micropumps and ISFET sensor chips [48]. Two pumps, fluid connections and sensor chip were integrated in a stacked format measuring 22 x 22 x 11 mm^3 [49].

Thereafter, the number of reports on miniaturised systems for chemical and biochemical analysis has steadily increased and a great wealth of constructions, techniques and applications have been described (see reviews on gas and liquid chromatography [50], CE [51, 52], microchip-mass spectrometry [53, 54], genomics [55-57], proteomics [56, 58, 59] and cellomics [60, 61]). Commercial systems have also become available, for instance from Caliper Technologies Corp. (various electrophoretic separation systems and in co-development with Agilent Technologies) [62], Gyros AB (rotating disk for multiple bioassays and sample preparation) [63] or Affymetrix (array systems) [64].

Microchip-based analytical systems are fabricated using the same technology as the integrated circuit (IC) industry use to develop microchip-based electronics [65]. It is tempting to envision that analytical instrumentation will undergo a similar revolution towards microchip-based systems, as the IC industry that started making computers of room size compared to the small handheld computers and electronic calendars we have today [17].

μTAS, Lab-on-a-chip and MEMS

Among the manifold of microfluidic systems there are two concepts, which appear frequently, *i.e.*, "micro total analysis system" (μTAS) and "lab-on-a-chip". The μTAS was coined by Manz *et al.* [46], and does not contain anything new in itself, but rather defines the construction and performance of an ideal miniaturised analytical system. The μTAS should contain all necessary steps to perform the analysis, *i.e.* sampling, sample pre-treatment

(filtration, enrichment, derivatisation etc.), separation, and finally detection [46]. The lab-on-a-chip is a more undefined term and the meaning depends slightly on who is using it [66]. However, usually it is a microchip-based system that (similarly to the μTAS) contains all liquid handling steps on the chip to perform the analysis, but not necessarily all steps in the analytical procedure need be integrated on-chip [66].

Another acronym is MEMS, which reads MicroElectroMechanical Systems. This is a collection notation for mechanical components, for example various sensors, motors and pumps fabricated by micromachining [67] (recently reviewed for microfluidic systems and applications [68]). Some of the very earliest microfluidic analytical systems, appearing in the early 1990s, were based on fully integrated MEMS, having micromechanical units for pumping and valving liquids [48, 49].

Scaling effects

When decreasing the dimensions of an analytical system one will see that some properties do not scale linearly, but tend to increase or decrease due to the smaller dimensions. This includes fluid properties, diffusion time versus distance and surface-to-volume properties [18, 65, 69], and will be reviewed briefly in this section.

Fluid flow can be characterised by the Reynolds-number R_e [67, 70] and can be seen as the ratio between inertial and viscous forces of the liquid in motion. Equation 1 below describes how R_e is related to density ρ, viscosity η and velocity v of the fluid in a tubing of diameter d;

$$R_e = \rho v d/\eta \qquad (1)$$

At low flow rates the viscous forces will dominate and result in a laminar flow, while at high flow rates the inertial forces will make the fluid turbulent. The transition between laminar and turbulent flow occurs when $R_e \approx 2000$ [70]. In microchip devices laminar flow is usually the norm due to the small dimensions and low flow rates. Laminar flow can be viewed as a set of layers flowing along each other in the fluid direction. The different layers will not mix but molecules will migrate only by diffusion. In practice, laminar flow means that two fluid

streams can flow along each other without any physical barrier but without mixing (see Figure 6). However, in the interface between the two streams molecules will migrate by diffusion. As a consequence, microchip systems must often rely on mixing solely by diffusion. The relation between diffusion distance and migration time is given by Equation 2;

$$t = L^2/D \qquad (2)$$

Thus, the diffusion time t for a species having a diffusion coefficient D is proportional to the square of the distance L to travel. From this equation we see that decreasing L ten times (*e.g.* by changing tubing inner diameter from 1 to 0.1 mm) t will be shortened 100 times. The effect of decreasing the diffusion distance L upon diffusion time t is plotted in Figure 7. In practice, the diffusion distance is simply made shorter when decreasing the dimension of the channel.

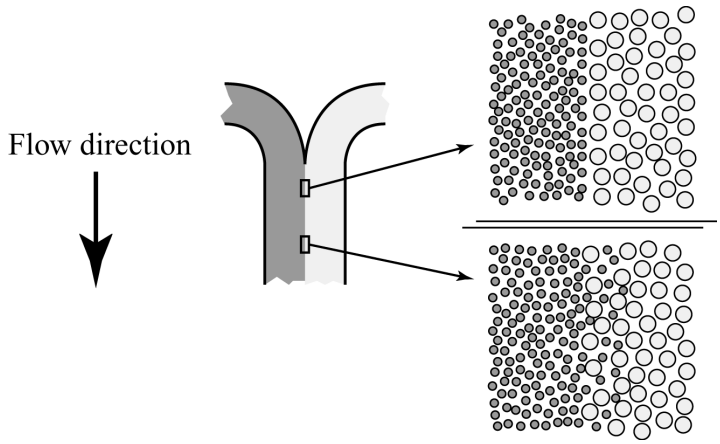


Figure 6. Schematic principle of the effect from laminar flows: Two micro-channels merge into one where the separate fluids are combined. They will not mix because of the laminar flow properties, however, at their interface molecules will migrate by diffusion. In the figure, this is exemplified by the presence of high molecular weight species in one stream (large dots) and low molecular weight in the second stream (small dots). Initially, as the streams are combined the molecules are completely separated. Further downstream, the low molecular weight molecules have moved a short distance into the neighbouring flow by diffusion. Similarly, the high molecular weight species have migrated into the other stream, but a shorter distance due to the slower diffusion. The diffusion

distance of each species into its neighbouring flow is dependent on for how long distance the two streams are allowed to flow along each other.

Another scaling effect is the increased surface-to-volume ratio and can be exemplified with a tube having a diameter d and a length L. Its volume V and area A are given by Equation 3 and 4 respectively;

$$V = \pi d^2 L/4 \quad (3)$$

$$A = \pi d L \quad (4)$$

The surface-to-volume ratio is the ratio between the two and is A/V = 4/d. Consequently as the diameter gets smaller, the surface-to-volume ratio increases proportionally to 1/d (see Figure 7). A practical implication of this is that the surface chemistry becomes a very important property to control in miniaturised systems.

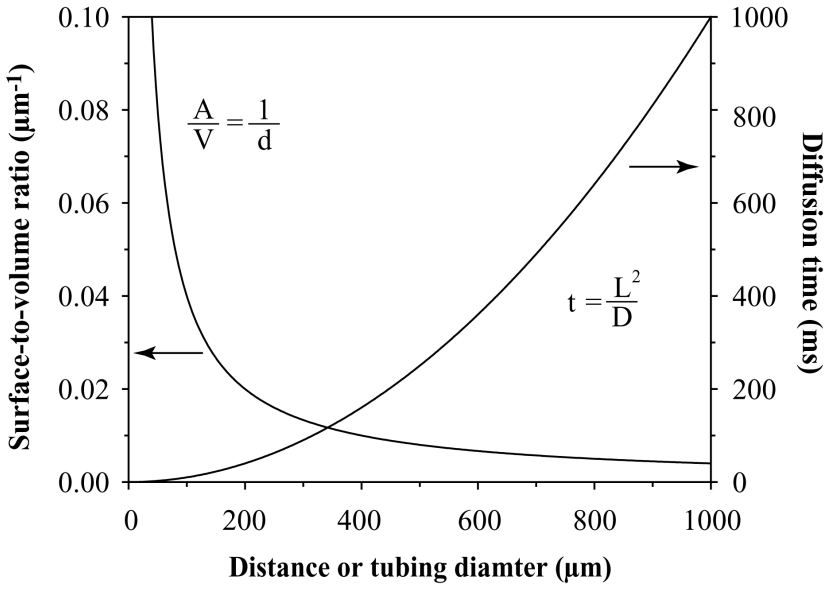


Figure 7. Plots showing the surface-to-volume ratio (combination of Equation 3 and 4) and diffusion time (Equation 2, for a species having a diffusion coefficient of 1 x 10^{-6} m^2/s) as functions of distance. The most common interior channel dimensions in microfluidic systems range approximately from 200 to 10 µm.

Why miniaturise?

The reasons for miniaturising analytical systems originate from two sources: economic benefits and increased analytical/technical performance of the analysis. However, both are interrelated; an improved analysis can have economic impact due to faster analysis, higher sample throughput, lower reagent consumption etc. Below are the most important aspects and implications listed, which normally are referred to when downscaling analytical systems [15, 46, 66, 71, 72].

- *Higher sample throughput*
- *Multiplexing*
- *Automation*
- *Integration*
- *Portable analysers (in-field/in-situ/point-of-care)*
- *Manufacturing cost*
- *Disposability*
- *Lower energy consumption*
- *Reduced chemical consumption*
- *Laminar flow*
- *Improved separation efficiency*
- *Short diffusion paths*
- *Increased surface-to-volume ratio*
- *Faster heat dissipation*

Several of the aspects listed above are intimately related to each other. Higher sample throughput can be a consequence of improved separation performance, multiplexing, and/or faster analysis due to short diffusion paths for analytes and reagents. Reactions may proceed faster because of improved thermal transport (heat dissipation), which can be exemplified with the heating-cooling cycles needed to perform in the polymerase chain reaction (PCR) [73-75]. Multiplexing can be achieved by construction of parallel analysis lines integrated on the same chip [76-79].

Compared to normal microtiter plates commonly used in all sorts of bioassays, microfluidic systems are favoured by the short diffusion paths (*cf.* Equation 2 and Figure 7) [80]. Moreover, microchips with channel systems for multiple liquid-handling steps [81-84], detection systems [85, 86] and MEMS-based liquid handling [49, 87-90] can and have been integrated, resulting in fully automated and compact units. Both integration and automation are prerequisites for efficient portable analysers [79, 89]. As a natural consequence of the small size and dimensions, both energy and chemical consumption are reduced. The latter can

be very important when working with expensive reagents and as more stringent environmental regulations is evolving.

The properties of laminar flow has already been reviewed above (see Figure 6) and this phenomenon has been used to perform liquid-liquid extraction [91, 92], immuno-affinity extraction [93] and sample fractionation [94] between two parallel flows. Thereby, the laminar flow properties can be used for performing various sample pre-treatments [95]. The increased surface-to-volume ratio (see Figure 7) can be an advantage when working with immobilised components, *e.g.* enzymes and antibodies [80, 96, 97]. However, it may, as already indicated above, also have negative side effects: unspecific adsorption of biological elements (*e.g.* proteins and cells). This problem has been reported [81, 82, 98-101] and investigated for various fabrication materials and surface modifications [102-110]. However, in the majority of these reports, electroosmotic flow (EOF) is used as the pumping technique [81, 82, 98-102, 104, 105, 107, 108, 110], which is completely dependent on the surface charge of the channel walls [111]. Surface modifications to decrease adsorption are therefore difficult to perform without loosing the important surface charges that enables the EOF in the first place (surface modification methods for microchip separation systems have recently been reviewed elsewhere [112]). McClain *et al.* [81] recently presented a possible solution to this problem by constructing a hybrid system for cell lysis with pressurised flow in channels with passivating surface coatings to avoid cell adsorption. The EOF and electrophoretic separation of cell lysis products was further performed in non-modified channels.

Even though the surface-to-volume ratio increases when miniaturising it does not necessarily mean that the total available surface area increases, which is important to note when working with immobilised reagents. One approach to overcome this problem has been to incorporate micro-beads in flow through channels or reactors fabricated on-chip [80, 86, 113-115]. Moreover, silicon can be etched into a highly porous state (reviewed further in Chapter 4), and thereby offers a very suitable coupling matrix for immobilised reagents [116]. Such microreactors have been used in all systems developed in this thesis (paper I to V).

Except that increased surface-to-volume ratio can be unfavourable, other problems associated with microfluidic systems are interfacing macro-to-micro or world-to-chip can be troublesome [17, 117-119]. Moreover, clogging of channels may occur due to non-specific adsorption, and when it comes to “real” samples, presence of particles may cause problems.

However, sample pre-treatment techniques are available (reviewed elsewhere [95]), *e.g.*, using sample extraction via laminar flow [91-94].

4. MICROMACHINING

The term micromachining (or microfabrication) is used for the manufacturing process of microchip-based systems. There are several fabrication techniques available, originally derived from the IC industry for batch production of electronic components. The properties of the resulting microstructures will be dependent on the used fabrication technique. Generally, the fabrication can be divided into two categories, namely bulk micromachining and surface micromachining [120]. The former denotes the process where the systems are made from a relatively thick substrate and the construction is made through patterning in the substrate. The latter is based on the deposition and stacking of thin films on a substrate, and results in smaller structures compared to the bulk machining. Surface micromachining is mainly used for the fabrication of sensors and will not be covered in this thesis. Bulk micromachining can be viewed as sculpturing in a piece of solid material in a process called etching. This means that material is removed by chemical reactions (induced by physical or chemical means) at desired locations and thus creating the wanted structure in the substrate. The available etching techniques can be divided into wet etching and dry etching [121]. Using the former process, the substrate is immersed in a liquid mixture of chemicals, which will react (etch) and remove material. To obtain selectivity in the etching process some areas of the substrate can be coated with a layer of another chemical composition that do not react with the etching solution, thus protecting the underlying bulk material from being etched. The protective coating is commonly called a “mask”. Dry etching is based on selective bombardment of the surface with reactive ionic species in a plasma or irradiation with for example lasers and X-ray; in either case, the exposure starts reactions on the surface that will break down the material. Both dry and wet etching can be isotropic or anisotropic. The former indicates that the material is removed at equal rate in all directions of the exposed area, while the latter means that material of the exposed area is removed at different rates depending on the direction in the material. The reason for the anisotropic behaviour is, however, different between wet and dry etching. In wet etching, anisotropy is caused by different properties among the crystal planes in the lattice of the substrate, while anisotropy in dry etching is obtained because the substrate will primary react (*i.e.* be etched) orthogonally to the trajectory line of the impinging reactive ions [121].

Several materials are being used for the construction of microsystems, and each will result in different properties, such as chemical compatibility, surface properties, transparency to UV/VIS light, thermal conductivity, electrical conductivity etc. The materials used for fabricating microfluidic systems are mono-crystalline silicon, glass or fused silica (fused quartz), and polymers (plastics). These will be reviewed in the following text, *with emphasis on wet etched mono-crystalline silicon*, which was used for fabricating the microchips used in this thesis.

Mono-crystalline silicon

Mono-crystalline silicon (referred to as just silicon in the text below) has been used since the beginning of the IC industry and occurs widely in the construction of semiconductor sensors [122]. Bulk and surface micromachining can be used to obtain very complex three-dimensional microstructures and with the existing technology, systems and sensors can easily be fabricated in large volumes. Silicon is not stable to oxygen in the atmosphere but will be oxidised. Thereby the surface layer of silicon consists of silicon dioxide (sometimes referred to as silica in this thesis), which can be chemically modified using well-known chemistries applied for silica and glass substrates [123].

The silicon crystal is diamond-like [120], which means that an atom is centred in a tetrahedron and binds four neighbouring atoms, located in the corners of the tetrahedron. However, this is an inconvenient way to describe silicon, since the bonds are not in 90° angles to each other. Another way is to use the unit cell (*i.e.*, the smallest repeating cubic unit that can describe the silicon crystal), where an atom is located in each corner, and in the centre of each face of a cube (Figure 8a). The cube is placed in a coordinate system where it is commonly described with three sets of planes (the so-called Miller indices), namely {100}, {110}, and {111} (Figure 8b). Silicon can be both dry- and wet etched, *but only the latter will be discussed here*. For some wet etching solutions the silicon crystal behaves anisotropic, and it has been found that {110} is usually the most rapidly etched planes, while {100} is intermediate and {111} the slowest. The mechanism that underlie the difference in etch rate towards certain solutions is not fully understood, but it seems to be dependent upon the density of atomic bonds on the exposed surface and the radius of the hydrated hydroxyl ions in the etching solution [120]. By adjusting the composition and temperature of the etchant,

dissolution rates of the planes can be controlled and through selective shielding (masking) of the surface, complex three-dimensional structures can be created. The etched structures will have a smooth surface, but a great feature is that silicon can be turned into a highly porous state by electrochemical etching [124, 125].

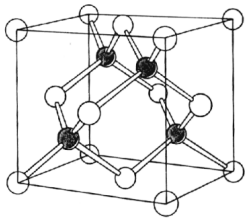


Figure 8a. The Figure shows one unit cell of mono-crystalline silicon. The white and black balls are silicon atoms at different locations in the lattice.

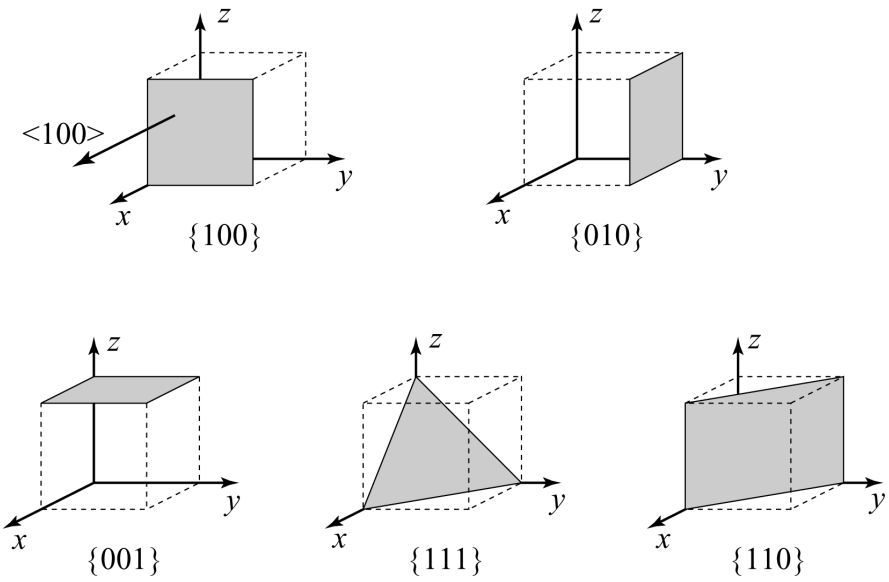


Figure 8b. The different crystal planes, which describes mono-crystalline silicon. For some wet etching solutions the silicon crystal behaves anisotropic, and it has been found that {110} is usually the most rapidly etched plane, while {100} is intermediate and {111} the slowest.

The basic fabrication process for silicon bulk micromachining, using chemical wet etching, can be divided into three parts: (i) deposition, (ii) photolithography, and (iii) etching [120] (Figure 9). This was the process used for fabricating the microchip reactors used in this thesis (paper I to V) [116, 126] and will be reviewed here.

Initially the native silicon oxide layer is made thicker by heating the substrate in a furnace with water vapour as oxygen source. The oxide layer will be used as a mask in a later etching

step and needs to be improved by this treatment. In the following deposition step (step A in Figure 9) the surface of the substrate is coated with a thin film of photoresist, which is a durable photosensitive polymer dissolved in a volatile solvent. When the solvent has evaporated a thin layer the polymer is left, which will serve as mask to prevent the silicon dioxide layer to react with the etching solution in the first etching step. The structure to be fabricated in the silicon substrate is defined by the pattern created on the surface in the photolithography step, using a glass plate (called lithographic mask) as template (step B in Figure 9). The lithographic mask is opaque, except from the areas of the pattern, which are transparent and destined to be transferred to the silicon surface. As the mask, aligned above the microchip, is irradiated with UV light the polymer breaks down in the exposed areas. Next, the polymer that was been exposed to UV light in the lithography step is removed by placing the substrate in a development solution (step C in Figure 9). The third and last part of the fabrication process is the etching. After the photolithography, the substrate has a pattern on the surface with some areas containing exposed silicon dioxide, whereas the other is still coated with photoresist. In a first etching step the silicon dioxide layer is removed in the exposed areas, using isotropic etching with a mixture of hydrofluoric acid and ammonium fluoride (step D in Figure 9). This only removes the silicon dioxide and leaves the under lying silicon, unless the acid concentration is very high, which will cause a slow etching of the silicon. Next, the remaining photoresist is removed by placing the substrate in an organic solvent (step E in Figure 9). The final structuring is done by placing the substrate in a potassium hydroxide solution that will anisotropically deep etch the silicon and create the three dimensional structure (step F in Figure 9). The silicon dioxide will serve as mask in this step, but will, however, also be etched but at a very low rate compared to that of silicon. It should be noted that the surface of the newly etched silicon is oxidised when exposed to the oxygen in air (step G in Figure 9). As mentioned above, the silicon surface can be etched into a very porous layer by electrochemical etching, which is illustrated in Figure 10. Hydrofluoric (HF) acid mixed with ethanol is used as etchant. The silicon substrate is placed in an electrochemical cell and a voltage is applied, which will create electron deficient areas, thus inducing electrochemical oxidation of silicon to obtain electrons and the formation of pores is started. Figure 11 shows an image of the final porous layer of a microchip (used for immobilising antibodies and enzymes in paper I to IV).

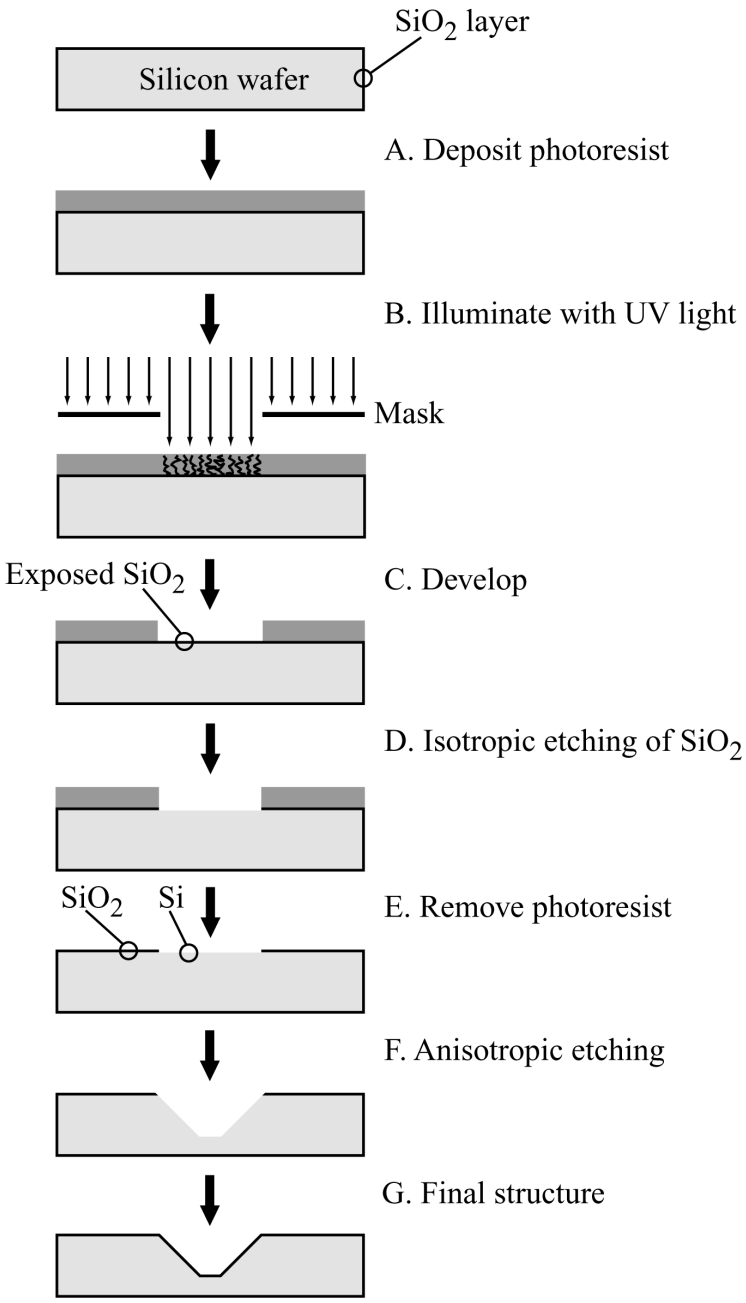


Figure 9. Schematic carton of the machining procedure performed for construction of the silicon microchips used in this thesis. See text for details.

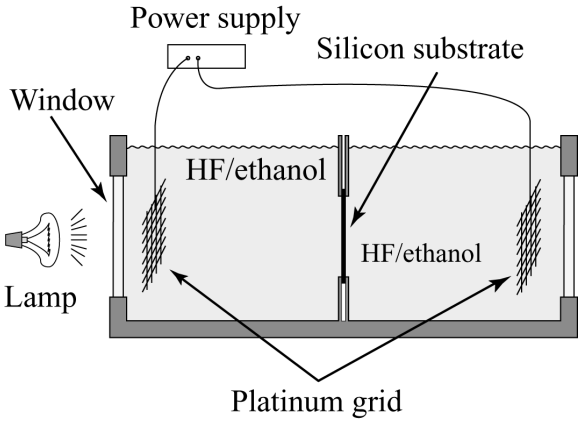


Figure 10. Electrochemical etching set-up used for making the silicon microchip surface porous. See text for details.

The disadvantage of using silicon for microfabrication is the need for high-purity silicon, which is expensive. The material is brittle and not opaque in the UV/VIS spectrum region, it is not chemically inert to basic solutions, and since it is a semiconductor it cannot withstand the voltages needed for EOF [127].

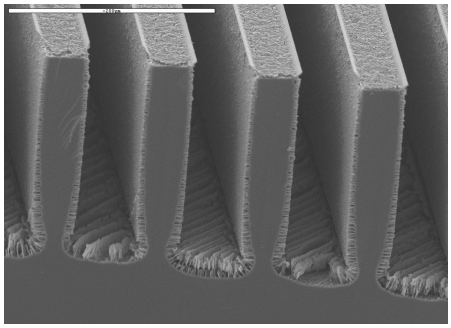


Figure 11. SEM image of a silicon microchip with porous surface: This microchip structure was used as immobilisation support for antibodies (paper I and II) and enzymes (paper III and IV). The channels were 235 µm deep, 25 µm wide and 10 mm long. Each chip had 42 channels, which in each end fall into inlet and outlet basins, with a total volume of approximately 4.7 µl.

Glass and fused silica (fused quartz)

Glass and fused silica (or fused quartz, but very often just denoted as quartz or quartz glass) are also used as substrate for microfabrication. These materials are not so expensive as

silicon, and both are transparent in the VIS spectra and fused silica also in the UV spectrum range. Glass and fused silica are extensively used for microchip-based CE since they are good insulators, which are needed to create a potential field for electrophoretic separations [51, 128]. Basically the same fabrication technique as used for silicon (*i.e.* dry and wet etching) can be used for both glass and fused silica [51, 128]. However, since both glass and fused silica are amorphous there is no regular lattice with crystal planes, thus both materials will behave isotropic to all wet etching agents and will result in curved structure because the reaction proceeds with the same rate in all directions. Still, anisotropic dry etching can be used (*e.g.* plasma etching [129]) since this process step is not dependent on crystal structure of the material.

Polymers

Synthetic polymers have gained more attention during the last years, since they are easier and less expensive to manipulate than silicon- and glass-based substrates, and thus possible to manufacture to a low cost in large volumes, which is a prerequisite for microchips to be disposable. The trend is thus moving from glass and silicon towards polymeric fabrication materials [127]. Some polymers that have been tested are poly(dimethylsiloxane) (PDMS), poly(methyl metacrylate) (PMMA) and poly(tetrafluoroethylene) (PTFE) [130]. Polymeric substrates can be structured by dry etching or by moulding against a template [127] There are several methods for performing the latter, *e.g.*, injection moulding, imprinting and hot embossing, which all rely on heating the substrate so it becomes formable and then a template (master) is applied to create the three-dimensional pattern. When the substrate has cooled the template is removed and the substrate now bears an inverse image of the structure of the template [127, 130].

The drawbacks with the use of polymers are that some are pH sensitive, not resistant to organic solvents, and may cause high background signals when using fluorescent detection, due to auto-fluorescence in the polymer substrate [127, 130].

5. MICROFLUIDIC ASSAY SYSTEMS USING ENZYMES, ANTIBODIES OR CELLS

Ruzicka and Hansen [131] introduced the concept of performing chemical analysis in a continuous flow in 1975, a technique known as flow injection analysis (FIA) or only flow injection (FI). Since then, new generations of FIA systems have evolved; sequential injection analysis (SIA) [132], and recently, sequential injection lab-on-valve (SI-LOV) [133]. All these concepts, FIA, SIA and SI-LOV, allows simple and automated liquid handling systems and a large number of applications of various set-ups have been demonstrated (see [134-138]). In this thesis, both FIA (paper I, II, IV and V) and SIA (paper III) have been used for automated sample and reagent handling in the developed analytical systems. Even though many microfluidic chip-based analytical systems look very different from traditional FIA instrumentation the underlying principle of sample handling is still the same: flow-based automatic handling of samples and reagents [139].

Pumping, valving and detection

Technically, many microfluidic systems utilise EOF for fluid propulsion and valving (the detailed operating principle is reviewed in [140]). However, when EOF pumping is used it is not unusual that the microchip also incorporates a CE separation step, which makes it into a hybrid FIA-CE [141]. If both EOF and electrophoretic movement of reagents and analytes are used, the system is often denoted as electrokinetically controlled. Another way of pumping is off-chip use of "macroscopic" liquid chromatography and flow injection pumps (most often syringe pumps) and valves, as in the case of this thesis. Except from electrokinetically controlled flow and off-chip pumping there are other techniques for fluid propulsion, but these are less common, for example MEMS-based pumps and valves [87, 142] and various non-mechanical techniques (recently reviewed by Reyes *et al.* [143]), *e.g.* electrohydrodynamic pumping [144-146], thermocapillary pumping [147, 148], and centrifugal force pumping [77].

Regarding pumps and valves on the market, there seem not be any microfabricated micropumps or microvalves commercially available yet (as far as the author has seen from browsing through the Internet and in discussion with people in the micromachining field). This probably stems from a number of reasons, *e.g.*, the field is still immature, micropumps and microvalves are complex to fabricate and thus very expensive, lack of potential buyers, lack of standardised fabrication methods, thereby making interfacing of different components difficult. However, there are commercially available microanalysis systems, *e.g.* Caliper-Agilent Technologies Bioanalyser, which can perform various assays and CE separations [62], and Gyros rotating disc system [63]. There are also several companies with interesting information available via homepages on the Internet, however, from these it is often difficult to judge what is vision and if there are any products. In contrast, manufacturer of "macroscopic" HPLC equipment can offer valves developed for microbore liquid chromatography [149, 150] and recently also valves and assemblies for micro/nano flow products and lab-on-a-chip applications [151].

Regarding detection techniques, most of the common optical detection techniques, *i.e.* absorbance, fluorescence, CL, electrochemiluminescence, have been adapted to the micro-format, as well as electrochemical detection and interfacing to mass spectrometry [72, 152]. The largest disadvantage and problem performing absorbance measurements on a chip is the very short length of the light path, which results in higher detection limits [153]. Several attempts have been made to overcome the problem by constructing a detection cell where the light is reflected by the walls in a zigzag mode to extend the light path [154, 155]. The most commonly employed detection technique on microchip is fluorescence and the very first glass chip with electrophoretic separation systems used this technique [45]. Since then a wealth of applications and systems based on fluorescence have been developed [72, 152]. The main drawbacks with fluorescence is the need for an external light source and that not all compounds exhibit fluorescence; the latter has, however, been overcome by pre- or post-column labelling of analytes [156, 157]. CL, on the other hand, does not need an external light source for detection, which results in simpler set-up and more compact systems [85, 86]. Electrochemical detection is also very suitable for microfluidics and can readily be integrated on-chip [158].

Enzyme assays and immunoassays

In regards to the reported microfluidic assays based on enzymes and antibodies one can distinguish between *solution-based* and *surface-immobilised* systems, as discussed previously in Chapter 2. In either case, the final detection does not necessarily need to take place at the same location as the molecular recognition occurred. In general, there are more examples of solution-based systems than immobilised reagent microsystems, in which the former type is predominately electrokinetically controlled. A likely reason for this is that the electrokinetic liquid control is easy to integrate on chip but has the great disadvantages of being dependent on the surface charge of the channel. Physically (adsorption) or chemically (covalently linked) immobilised reagents will alter the surface charge and thus also the EOF. Although it is not impossible to perform electrokinetically controlled surface modified assays [105, 159], associated problems with EOF and bubble formation have been reported [159]. For this reason, most assays based on immobilised enzymes or antibodies are using off-chip pumping and valving. Since the pumping technique determines to some extent what kind of assay can be performed (*e.g.* involving immobilised components), the systems described here are often referred to as to how the fluid propulsion was obtained. In the following review of microfluidic biosensing systems, *the main focus is primarily put on systems utilising surface-immobilised enzymes, antibodies or cells, because this is the approach used throughout the papers reported in this thesis (paper I to V)*.

Surface-immobilised assay system

The first application of any biological molecule as recognition element in a microfluidic system was an immobilised-enzyme assay reported in 1992 by the group of Bengt Danielsson and Ingmar Lundström [160] using silicon flow-through microreactors bearing 33 parallel V-channels, to which enzymes (catalase or penicillinase) were attached. Soon after, Isao Karube's group introduced another approach: instead of having parallel channels they used a single V-channel immobilised with enzymes (glucose oxidase and lactate oxidase) in a folded geometry on-chip, about 1000 mm in total length [161, 162]. The group of Thomas Laurell and Lars Rosengren presented a similar approach as Danielsson and Lundström, but instead of using V-shaped channels they had channels with parallel walls, which resulted in a larger surface area available for enzyme immobilisation [126]. This construction has since then been

improved further in channel dimensions and by making the surface porous (electrochemical etching, as described in Chapter 4) to increase the internal surface area [116, 163], and has resulted in the microchip structure (see Figure 11) used as the analytical platforms for the enzyme- and antibody-based systems reported in this thesis (paper I to IV).

Another approach for immobilising bioelements is based on porous microcarriers, *e.g.*, controlled pore glass (CPG). Microchambers packed with CPG material immobilised with enzymes have been performed in EOF controlled systems [164] as well as with off-chip pumps and valves [86, 114, 115]. Similarly, polystyrene beads have been used as support material for immunoassays [80, 113], having off-chip pumping.

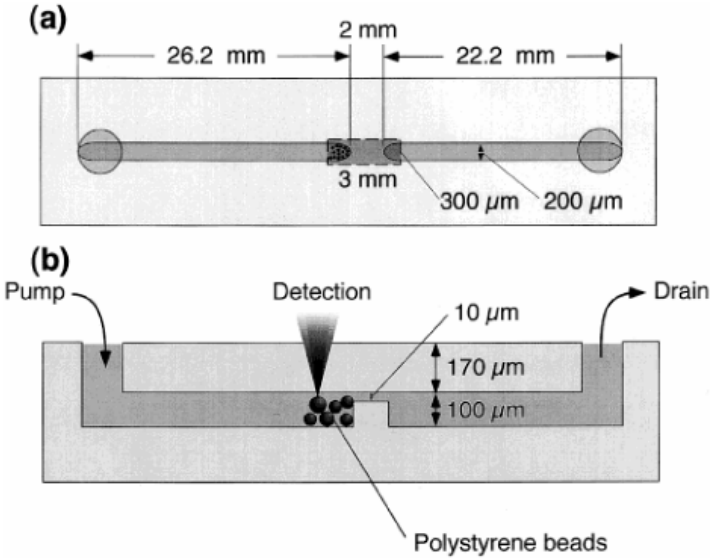


Figure 12. Quarts glass microchip for polystyrene bead-based immunoassay. The analyte (secretory human immunoglobulin A, s-IgA) was adsorbed on the beads followed by recognition with gold-labelled antibodies monitored by thermal lens microscopy (TLM) [80].

Takehiko Kitamori *et al.* [80] reported the first heterogeneous microchip immunoassay, which was a non-competitive immunoassay for human secretory immunoglobulin A (s-IgA). The assay used off-chip pumping and was based on microcarriers of polystyrene with adsorbed s-IgA and detection of the bound gold-labelled anti-s-IgA antibodies with thermal lens microscopy (see Figure 12). This type of system was later followed by similar systems [78, 113]. Moreover, immuno-reagents for heterogeneous immunoassays have also been

immobilised on the walls of microchannel sections [97, 165, 166], including systems with electrokinetic fluid control [105, 159].

Solution-based assay systems

J. Michael Ramsey's group demonstrated the first solution-based enzyme assay in 1997, which was fully integrated on-chip and used electrokinetic controlled flow of reagents and carrier buffers. The system was based on β-galactosidase and fluorescence detection [99]. The following year, Chiem and Harrison [167] presented the first solution-based immunoassay fully integrated on-chip. It was a competitive assay using theophylline as the analyte in which the antibody, antigen and labelled antigen were mixed on-chip. Since then various similar solution-based electrokinetically controlled on-chip integrated enzyme assays [168-172] and immunoassays [76, 173] have been reported.

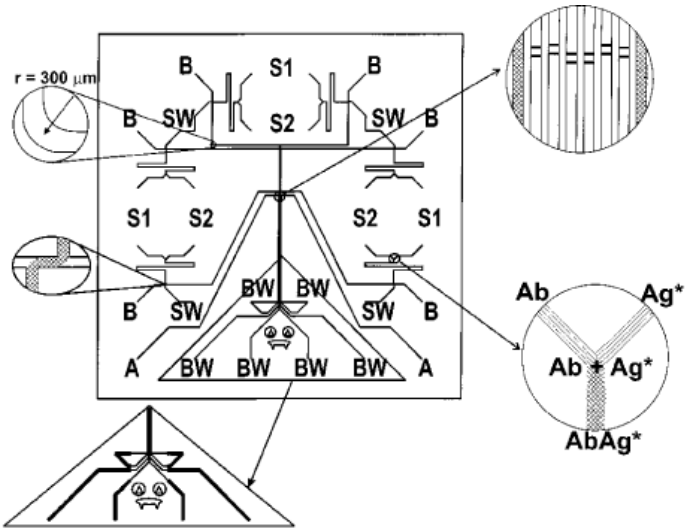


Figure 13. Multichannel CE chip fabricated on a glass substrate (B running buffer, S1 analyte, S2 antibody, SW sample waste and BW buffer waste). The channels were 50 x 10 μm and the system allowed simultaneous processing of six samples, which under optimised conditions were performed within 30 sec. The analytical system consisted of ovalalbumin and fluorescent labelled anti-ovalalbumin antibodies, or estradiol and corresponding fluorescent labelled anti-estradiol antibodies. The upper right inset shows the six parallel channels running along each other at the detection region [76].

In general, the mixing of reagents is performed in a FI manner followed by CE separation of the reaction products. Figure 13 shows an electrophoresis chip developed in D. Jed Harrison's group, which can perform six independent immunoassays simultaneously. Under optimised conditions this manifold allowed six samples to be processed within 30 sec [76].

Solution-based enzyme assays that do not utilise electrokinetic flow have also been reported, for instance using air pressurised fluid reservoirs [174] and centrifugal force on a spinning circular plastic disk for fluid propulsion [77].

Cell-based assays

Microchips for monitoring activities of living cells have gained more and more attention during the last years and the reasons are many (recently reviewed in [60, 175]). Microstructures can be fabricated comparable to the size of living cells and the chemical environment can be controlled in terms of addition/removal of reagents followed by reproducible mixing. Thus, both the physical and chemical environment can be precisely adjusted. This gives interesting features when comparing with how cell-based assays are normally carried out in batch systems, such as in microtiter plates, in which the whole solution in the well must be exchanged if a reagent needs to be removed. Furthermore, the fluidic format allows continuous (real-time) sampling from the cells, which can give information of fluctuations in cellular activities, compared to plate assays were a reaction end-point often is registered. Another large difference between microfluidic and batch systems is that waste products will be continuously removed in the former system, while accumulating in the latter.

Both transport and handling (including flow cytometry) of cells in solution [83, 84, 100, 101, 176-182] as well as assaying cell activity [81-84, 94, 183-185] have been performed in microfluidic systems. The techniques used for transporting cells have been electrophoretic and EOF [100, 101, 181, 182], dielectrophoresis [176-178] as well as pressurised flow [83, 84, 179, 180]. Electrokinetic handling has the great drawback of not being able to handle complex buffers with high ion strength [101], which very often are used as cell medium.

Furthermore, the electric field may cause membrane permeabilisation [100, 101] and adsorption of cells can be troublesome [100, 101] due to the mentioned need of charged surface on the microchannels to accomplish electrokinetic fluid motion.

The cell monitoring assays covers a broad spectrum, for example analysis of intracellular DNA [186], β-galactosidase [94] and pre-loaded cytosolic fluorescent dyes [81] by cell lysis, intracellular esterase assay in intact cells [184], membrane potential assay [183], cytochrome c distribution during apoptosis [185], calcium flux assays [83, 84] and release of insulin [82]. Figure 14 shows an interesting chip, which can separate a single cell from a suspension and transfer it into an assay chamber. Pumps and valves are fully integrated on-chip and pneumatically actuated, which allowed delivery of nanolitre volumes of reagents to the cell assay chamber. The system was used and tested for cell viability and calcium flux assays [83].

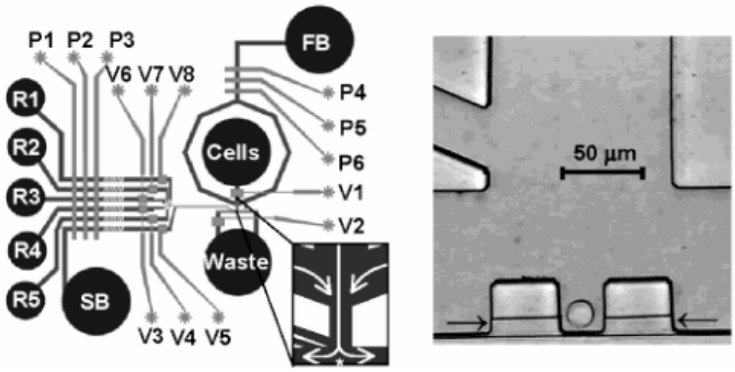


Figure 14. Microchip with integrated micropumps and valves fabricated in poly(dimethylsiloxane). R1 to R5 are reactant inlets, SB and SF are shield and focusing buffer inlets, respectively. Valves are actuated by applying pressure to inlets V1 to V8. Pumps are controlled by actuating P1 to P3 or P4 to P6 in series, which results in a peristaltic-like pumping mode. The cell suspension is housed in the Cell reservoir. The right inset shows a detailed photo of the cell assay chamber having one cell loaded [83].

Most of the reported microfluidic cell assay systems make use of suspended bacterial [94, 186] or mammalian cells [81, 83, 84, 183] and only a few are based on immobilised mammalian [82, 185] and bacterial cells [184]. In contrast, most mammalian cell lines used in molecular biology and in cell based microwell assays are in fact adherent, meaning that they

need to grow on a solid surface in order to feel well and multiply; this is one important aspect addressed in this thesis (paper V). Apart from immobilisation or continuous pumping of suspended cells, systems having an array of microchambers for confinement of cells for continuous assaying bacterial growth have also been reported [187-189].

Except from transport, handling and assaying cells, a quite extensive literature is available on how cells behave (*i.e.*, grow and promote) on chemically [175, 190-193] or physically [194-196] structured surfaces. These investigations cover a range of different perspectives and interests, for example, engineering cellular microenvironments to improve cell-based drug testing [175], site-specific immobilisation of cells for biosensors [197] and fundamental molecular biology of cell attachment and proliferation [190-194], tissue engineering [198, 199], and how cells attach to microstructured implants used in surgery [196].

6. SUMMARY OF PAPERS

In this thesis, flow-through silicon microchips have been used as platforms in the development of microfluidic systems for assaying antibodies (paper I and II), enzymes (paper III) and living cells (paper IV and V). The goal has been to show the feasibility, but also problems, of developing and using bioanalytical microfluidic detection systems.

Biosensing in combination with fluidic sample and reagent handling has several advantages. The fluidic format allows repeated measurements on the same population of immobilised components, which saves expensive reagents and is thus cost effective. It is easier and less expensive to automate and to integrate with preceding sample preparation systems than a corresponding batch assay. More importantly it allows development of detection systems for monitoring dynamic processes, *e.g.*, dynamic changes of a sample composition with time.

The microchip platform used for immobilisation of antibodies and enzymes is seen in Figure 11, and consists of highly porous silicon with parallel U-shaped channels used as the coupling matrix for immobilisation of biomolecules and cells. Another microchip design, shown in Figure 15, with inclining walls and V-shaped parallel channels, was used for the immobilisation of cells. This structure was developed since the U-shaped channels were believed to be unsuitable for cell immobilisation because the vertical walls did not allow any attachment sites (the cells were immobilised by sedimentation from a suspension). The alternative could of course have been a flat surface, but here the V-structured microchips are better since they have a larger surface area for cell attachment, due to their folded geometry.

CL was the chosen detection principle throughout all developed systems. The reason for this was that an external light source is not necessary. Compared to fluorescence detection, Raman and Rayleigh scattering, as well as background noise and stray light from the source, are minimised [135, 136, 200]. CL thus enables the use of higher photomultiplier tube (*i.e.*, dynode) voltages and potentially results in a better signal-to-noise ratio. In practice this means that very low detection limits may be obtained with linear calibration plots (sometimes in the log-log format) that may span several orders of magnitude [135, 136, 200]. The developed enzyme assays and immunoassays (paper I-IV) are based on the well-known luminol-

hydrogen peroxide CL system, using HRP as catalyst and PIP as enhancer [201] (see also Figure 2). In the last paper (paper V), a human cell line modified with a reporter gene that produces intracellular luciferase (LUC) [36] was used. The signal was generated by supplying the cells with luciferin, which was oxidised by luciferase in the presence of oxygen and adenosine 5'-triphosphate (ATP) while emitting light.

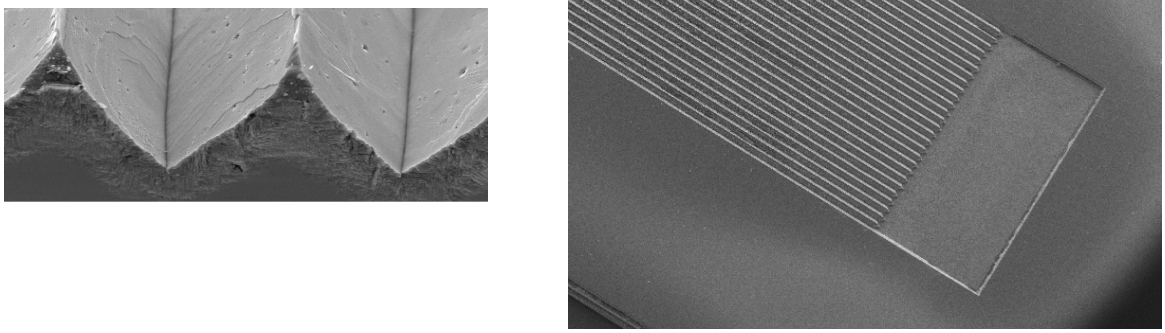


Figure 15. Microchip construction used as support for immobilising living cells (paper IV and V), consisting of 28 parallel V-grooves, 10 mm long, 100 µm wide at the top and 71 µm deep, with each end falling into inlet and outlet basins. The total volume was approximately 1.9 µl.

Paper I and II – Microfluidic immunoassays

Immunoassays are mainly found in clinical analysis [28] and to a lesser extent in environmental monitoring, although, the interest in the latter field is increasing where its potential is seen primarily as a screening tool due to the need of very little sample clean-up [202]. In the developed competitive microfluidic immunoassays, the aim was to investigate different assay formats, immobilisation chemistries and how these affected the short- and long-term assay stability and detection limits. Atrazine was used as a model analyte (antigen) that was competing with an HRP-labelled antigen (tracer) for the antibody binding sites. Two different immunoassay formats were used, either immobilised anti-atrazine antibodies (paper I, see also Figure 3 in Chapter 2), or immobilised protein A or G (paper II). In the former, the analyte and tracer were pre-mixed and then injected into the microfluidic system, competing

for on-chip antibody binding sites. In the latter, protein A or G with the inherent ability to bind the Fc region of IgG antibodies without affecting the analyte binding sites [20] was immobilised. Antibodies, analyte and tracer were pre-mixed, and in some tests also pre-incubated, before injection into the microfluidic system, where the antibody complex was captured by protein A or G. The main advantage of using immobilised protein G or A compared to antibodies is that the system has a generic feature, since antibody assays for different analytes can be developed without changing the immobilised layer on the microchip.

Two different approaches for tethering the proteins to the microchips were employed, either direct attachment using silanisation followed by glutaraldehyde (GA) activation, or indirect by first coating the microchips surface with a polymer matrix and then attach the proteins. The tested polymers were polyethylenimine (PEI), dextran (DEX), polyvinyl alcohol (PVA) and aminodextran (AMD). In the direct attachment 3-aminopropyltriethoxysilane (APTS) was used as silanisation agent and was followed by GA treatment, which is a very well known procedure and has been performed during many years [123]. This results in a rather short spacer between the proteins and the microchip surface. Silica is known to interact strongly with proteins [203, 204]. It has also been suggested that short spacers, such as APTS, are not sufficient to keep the large antibody molecule away from the surface and overcome steric hindrance from the vicinity of the support [205]. In paper I and II, the APTS-GA chemistry was used as a reference method due to its frequent applications. In comparison, hydrophilic polymers (PEI, DEX, PVA and AMD) were applied as immobilisation matrix. In several reports, these and other polymers have shown to enhance activity, binding capacity and stability of the sensing layer [205-210]. In paper I, two different forms of PEI were used in various ways. However, a mistake was recently discovered in that both polymers, denoted as linear PEI (LPEI) and branched PEI (BPEI) in paper I, both are branched and only differ in molecular weight; LPEI 750 kDa and BPEI 25 kDa. This misjudgement of structure was due to conclusions when the authors studied in some earlier publications about PEI, where the structure was drawn as non-branched, and second, at that time the chemical vendor did not supply structure information in the catalogue. The notations used in paper I are however, kept in order to easier follow the discussion. This mistake does not, however, affect the quality of the data, but it does affect the speculations of the difference in performance between LPEI and BPEI. Actually, now the difference in analytical performance may be easier to interpret as both polymers are branched and only differ in molecular weight. In general PEI polymers are known to adsorb very well on anionic surfaces [211] and was here accomplished via the

negative charge of hydrolysed silanol groups. The LPEI is 30 times larger than BPEI, which is probably the reason why the former has a better performance than the latter when comparing LPEI-GA and BPEI-GA chemistries. Due to its larger size, LPEI may simply both provide a better shielding of proteins to interact with the microchip surface, and a stabilising microenvironment. Additional polymers were tested in paper II, PEI (750 kDa), DEX, PVA and AMD, for immobilising both protein A and G. In both papers, it was found that the polymeric supports were superior in stability and assay characteristics compared to the APTS-GA chemistry. The limit of detection (LOD, defined here as 90% tracer binding [212]) for antibody-based microchips in paper I were 1.63 ng/l (LPEI-GA) and 0.83 ng/l (GOPS-BPEI-GA) for PEI-based chemistries and 24 ng/l for direct attachment via APTS-GA. In paper II, protein G-based microchips showed better performance than protein A based, likely due to difference in affinity towards the sheep anti-atrazine antibodies [39] and thereby the main focus was put on protein G microchips. The LODs obtained for polymer-immobilised protein G (paper II) was 6 ng/l (PEI), 5 ng/l (DEX) and 65 ng/l (PVA). For comparison, the European Union (EU) requirements for drinking water the threshold concentration is 100 ng/l, which is above all obtained LODs in both paper I, and II.

One important issue addressed in these papers is the regeneration of the biosensing layer without destroying its function. In general, this is done at quite harsh conditions and may cause denaturation of the immobilised proteins after multiple regenerations. In general, protein A and G are known to withstand the harsh conditions during regeneration better than antibodies. This can be exemplified by comparing the calibration characteristics of antibody chips (Figure 8 in paper I) with protein G chips (Figure 7 in paper II). The latter show calibration curves and IC_{50} values that coincide fairly well over a period of more than 200 days, while the antibody microchips show similar behaviour only during a period of 11 to 14 days. In either case, the stability is quite remarkable in spite of the treatment.

The DEX-based protein G chips in paper II were also tested for applications to natural surface water and orange juice samples. All samples were spiked with different concentrations of atrazine. To overcome the matrix effects the surface water was diluted 1:4 and orange juice 1:400. The dilution of the samples in this way is usually a problem due to lack in assay sensitivity, however, in this case the assay is so sensitive that the dilution should not be a major problem. The mean recoveries were in the range 87-102% for surface water (1:4 dilution) and 88-124% for orange juice samples (1:400 dilution). According to the US EPA

recommendations for environmental samples the recovery should be in between 70-120% [213].

Paper III – Optimisation of enzyme based μ-biosensors in a micro sequential injection analysis (μSIA) system

Paper III and IV are written as a series (part 1 and 2). In part 1 (paper III) enzyme μ-biosensor chips are optimised and characterised for monitoring glucose and ethanol in a μSIA system and in part 2 (paper IV) these μ-biosensors are applied for monitoring glucose and ethanol released from μ-chip immobilised yeast chips (see next section).

SIA was used in this paper for sample and reagent handling. All components, including syringe pump, multiposition valve (MPV) and photomultiplier detector, were computer controlled resulting in a highly automated μSIA. However, there was slight conflict between the available equipment; the MPV had a bore diameter of 0.25 mm and was thus not compatible with decreasing the connecting tubing inner diameter from 0.25 to 0.13 mm, which would have been feasible for decreasing the flow rate and thus also increase the CL signal from the enzyme microchips. This is an example of how the commercial available equipment sets a limit to further miniaturisation.

The aim of part 1 (paper III) was to investigate the performance of two co-immobilised enzyme microchips, making use of the CL reaction of luminol and hydrogen peroxide, catalysed by HRP and enhanced by PIP (see Figure 2). Two enzyme systems were used and evaluated: first, glucose oxidase (GOX) co-immobilised with HRP, and second, alcohol oxidase (AOX) and HRP. Detection of the two analytes (glucose or ethanol) took place in a two-step reaction: first hydrogen peroxide was generated from the analyte via catalysis by GOX or AOX, second, the formed hydrogen peroxide participated in the CL oxidation of luminol catalysed by immobilised HRP (see Figure 2). The latter is a very complex reaction system between HRP, luminol, hydrogen peroxide and PIP [201] where PIP is added to enhance the light emission [27]. The CL oxidation of luminol takes place in a series of equilibrium reactions, where there also are competing non-light generating reactions [201, 214]. Since hydrogen peroxide was generated by the immobilised oxidases, and HRP was co-

immobilised on the same chip, the optimum conditions for the CL reaction needed to be investigated.

A 2^3-factor experiment was the starting point for investigating the conditions for the CL reaction. The three investigated factors were luminol and PIP concentrations and flow rate. These factors were evaluated for GOX-HRP microchips using 100 µM glucose and signal peak area as the response. All three factors and several interactions were found to be significant (Student's *t*-test with 95% confidence limit, see Table 1 for brief presentation and Table 1 and 2 in paper III for details). Given the directions from the factor experiment a number of combinations of luminol and PIP concentrations were investigated (see Table 1). The flow rate (the third factor) was found significant in the factor experiment, indicating that a lower rate governed the CL reaction, probably due to more complete substrate conversion of the coupled enzyme reactions, and longer presence of excited reaction products. However, since the analysis time is highly dependent upon the flow rate a compromise had to be made and the flow rate was fixed to 18 µl/min. The maximum signal in the luminol and PIP concentration combination experiment was found for 250 µM luminol and 150 µM PIP, which coincided well with the factor experiment (see Table 1 in paper III).

Table 1. Brief summary of the results obtained from the factor experiment and the combination experiment for luminol and PIP concentrations.

Parameter	**Investigated ranges**		**Optimal values**
	Factor exp.	Combination exp.	
Luminol (µM)	50-200	25-400	250
PIP (µM)	150-350	0-350	150
Flow rate (µl/min)	5-60	Fixed flow rate to 18 µl/min	

The calibration and stability characteristics of GOX-HRP and AOX-HRP microchips were investigated using the optimised conditions. Linear log-log plots were obtained for both PEI- and APTS-based GOX-HRP microchips in the range 10 to 1000 µM glucose. These could be used during at least eight days without any noticeable decrease in activity. Similarly, the PEI- and APTS-AOX-HRP sensors were calibrated for 1 to 10 mM ethanol, and resulted in linear log-log calibration plots. AOX is well known to exhibit poor stability [215-217], and did so in

this system as well. The half-life of AOX-HRP sensor chips of both immobilisation chemistries was more or less the same, 2-3 days.

Both GOX and AOX microchips prepared from the PEI immobilisation route in general responded with higher signal intensity than corresponding sensors prepared by the APTS method. However, as the response changes with concentration and for comparing the sensor sensitivity, the ratio of PEI and APTS sensor signals was calculated and plotted. This revealed that the PEI-based GOX-HRP microchip sensor sensitivity increased with concentration relative to the APTS-GOX-HRP sensor (see Figure 4b in paper III). The opposite was found for the AOX-HRP sensor, were the sensitivity decreases with increasing ethanol concentration (see inset in Figure 5 in paper III). However, in both cases (GOX and AOX), the PEI sensors were found more sensitive than the APTS counterparts. These findings are not easy to explain but probably stem from the complex nature of the luminol reaction [201, 214]. Due to the better performance of PEI-based enzyme microchips these kinds of sensors were applied throughout the work in paper IV for monitoring the release of glucose and ethanol from microchip-immobilised yeast.

Paper IV and V – Microfluidic systems for cell monitoring

The two last papers show the use of microfluidic systems for assaying the activity of living cells. As already mentioned in the two first chapters in this thesis, the cell-based approach is gaining more and more attention, which is primarily fuelled by developments in the drug discovery [10-14]. Cell assays are one important strategy in the challenge to identify the sequences in the human genome that are likely to be therapeutic targets [10, 218]. Once the potential targets have been found they are evaluated towards libraries of compound derivatives, where the cell assays will aid the identification of the most therapeutically relevant targets [10, 218]. Here the cell-based approach has the advantage to distinguish between agonistic and antagonistic binding events, compared to solution-based assays [11].

The first cell-based system (paper IV) is using yeast and the second (paper V), a reporter cell line (HFF11) based on HeLa cells (human cancer cells). Both cell types were immobilised on V-type microchips (Figure 15), but since yeast is adapted to live in suspension it had to be adsorbed on the chips after treatment with PEI. In contrast, the HFF11 cell line is adherent

and needs to grow on a surface in order to promote well, which is the case for most mammalian cell lines. From a fluid technical point of view, to adsorb yeast is a drawback since cells are potentially lost if the flow rate is too high. The HFF11 cells are more firmly attached since they grow on the surface, are larger and thus have a larger surface contact area (see SEM images of adsorbed yeast cells in paper IV and HFF11 cells in paper V). To make the microchips biocompatible with the HFF11 cells the microchips were modified with a layer of covalently attached polylysine.

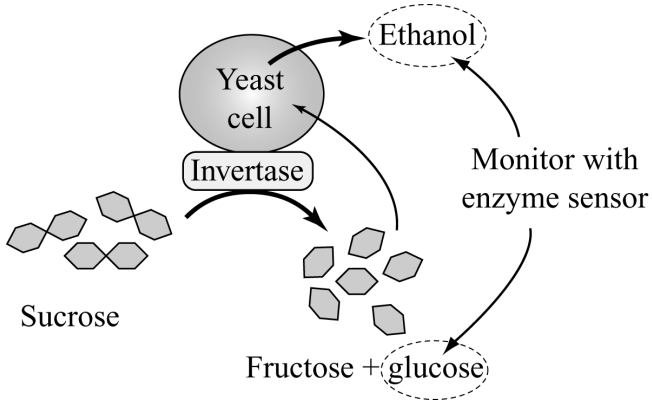


Figure 16. Yeast cell model used in paper IV. The cells are stimulated with sucrose, which is broken down by the periplasmic enzyme invertase into glucose and fructose. The two latter sugars are transported into the cell and consumed, resulting in formation of ethanol. Both invertase activity (via glucose) and ethanol production is monitored continuously using silicon microchip enzyme sensors.

The very first question we asked ourselves was if the silicon microchips could be used for housing and assaying cells in a continuous flowing environment. Yeast was used in our first attempts, since it is a robust organism, well characterised and easy to cultivate without extensive clean-room facilities. The cell model is schematically drawn in Figure 16, showing that when yeast is stimulated with sucrose, the periplasmic enzyme invertase catalyses the hydrolysis of sucrose to fructose and glucose. The two latter sugars are then transported into the cell in which they are consumed during fermentation under the formation of ethanol, which once formed, will diffuse through the cell membrane and out in solution. Both the formation of glucose and ethanol are monitored in the microfluidic system, using GOX-HRP and AOX-HRP enzyme μ-biosensor chips (developed in paper III). Initially the plan was to control the system with the μSIA liquid handling, as developed in paper III, but it was soon

changed to a μFIA configuration because of too slow computer software for syringe pump and MPV control, which was unable to process two tasks simultaneously. Further, the μSIA system sometimes introduced air in the system, which could not be removed without disturbing the immobilised cells. Since the AOX-HRP chip lost activity with time, it was continuously calibrated with an ethanol standard to compensate for this during monitoring of ethanol release from the yeast μ-chip. The results showed that both invertase activity (via glucose) and ethanol production could be followed in real-time using the enzyme sensors in the microfluidic system. In SEM pictures it was shown that virtually no yeast cells were lost after a full assay cycle of approximately 35 min.

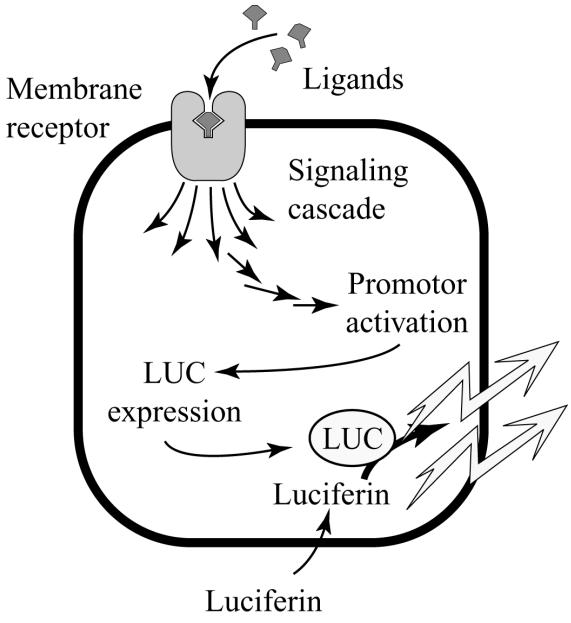


Figure 17. A simplified picture of the HFF11 cell line and its reporter gene system. When a ligand binds and activates the membrane receptor a cascade of intracellular reactions start, which finally will lead to promotor activation, transcription and expression of the reporter gene protein luciferase (LUC). The reporter gene activity is assayed by supplying the cells with luciferin in its protonated (i.e. uncharged) form at pH 6.0, which there pass the cell membrane and is oxidised in the CL reaction catalysed by LUC.

In the next step we proceeded to the more complex HFF11 cell line system (paper V), which was constructed for screening of ligand to 7TM receptors [35, 36], which are potential drug targets [37]. A simplified picture of the HFF11 cell model is shown in Figure 17. When a

ligand binds and activates the membrane receptor a G-protein-coupled cascade of intracellular reactions will occur, which ends with the expression of the two reporter gene proteins LUC and enhanced green fluorescent protein. The latter is only used for clone selection and LUC for the receptor-ligand assay due to better signal-to-noise ratio [36]. In the presence of ATP and oxygen, LUC catalyses the CL oxidation of luciferin. In the microfluidic system LUC expression was monitored in two ways, either invasively by lysing the cells through injecting luciferin mixed with a detergent, or non-invasively by injecting solely luciferin in pH 6.0 buffer. The invasive format is analogously to that when the cell assay is performed in microtiter plate assays [35, 36], and since the cells were lysed, one microchip resulted in one data point. In contrast, the non-invasive format left the cells intact on the microchip. In the slightly acidic solution (pH 6.0), luciferin was uncharged and could thereby pass the cell membrane and be oxidised in the CL reaction inside the cell. The main focus was put on monitoring of intact cells.

The goal in paper V was not to develop a system for ligand screening but rather to investigate and develop a microfluidic system for continuous long-term monitoring of microchip-immobilised cells and to determine which factors were important to make this possible, often unprejudiced in the trial-and-error format. Compared to yeast, the HFF11 cell line places much higher demands on both the physical and chemical environment, where small perturbations may end up with unspecific expression of the reporter gene. The assay time is much longer for the HFF11 system than in the previous described yeast assay, because it involves both signal transduction and expression of LUC. Cultivation of human cells needs extensive sterile facilities, and this and the following immobilisation step were performed in sterile clean-rooms. However, as soon as the immobilised microchips were ready it was preferred to run the assay in a normal analytical laboratory, which requested that the system provided a barrier towards the outer world for intruding organisms and contaminants. Furthermore, the cell medium must contain all vital components that the cells need for living. The yeast (paper IV) was assayed in a water solution of 0.9% sodium chloride at room temperature, while the HFF11 cells needed a complex medium and maintenance at 37°C. The medium itself is a very good ground for microbial growth, especially at this temperature. Supplementing the medium with an antibiotic, bacterial growth can in principle be circumvented, however with the drawback that contamination during the cell preparation may be hidden. The need for sterile environment and handling techniques are usually not met in ordinary analytical laboratory praxis, which during the first long-term measurements (18 h)

resulted in invasion of bacteria on the microchip. Thereby, special considerations had to be taken to sterilise the whole microfluidic system before insertion of a cell microchip, the injection port was equipped with a 0.22 µm filter that trapped all bacterial contaminants in the injected sample.

Several steps were found to cause an elevated expression of LUC. For instance, the immobilisation step resulted in a very high background expression of LUC, which was due to removal of the cells from stock cultivation flask into a suspension. Since the HFF11 cell line is adherent the de-attachment and suspension-based environment during transfer from cultivation flask to the microchips is a highly stressing event. Thereby, the immobilisation was allowed to proceed for three days during which the cells returned to normal steady-state conditions. Moreover, insertion of a freshly prepared cell microchip into the microfluidic system caused an increase of LUC activity. This effect probably stems from the change of stagnant environment in the petri dish, where the cells were immobilised for three days, to the microfluidic format in which fresh medium, delivered by the carrier flow, continuously surrounds the cell. Under the circumstances, this event could not be overcome but was simply allowed to pass for 18 h.

After all these considerations were made, the microfluidic system could be used for keeping and assaying a cell microchip up to 30 h without any contamination problems. Another important issue was cell loss due to the continuous flowing environment and was evaluated by SEM images but showed not to be any problem during the tested period. The final protocol for the continuous long-term measurements of the LUC activity was initiated by insertion of a microchip with immobilised cells in the microfluidic system, followed by approximately 18 h adaptation of the cells to the new environment. The expression activity of LUC was then monitored for a few hours to check that the activity was not increasing. Finally, the cells were stimulated with ATP to trigger the LUC expression via the cells endogenously expressed ATP receptors. The ligand was injected in a stop-flow mode as the ligand reached the cell chip (3 times with 2 min stop-flow). In the case when only the background expression was monitored, the ligand was excluded from the stimulating solution and injected in stop-flow mode (no effect was found). In both cases (specific expression and background monitoring) the LUC activity was monitored for the following 10 to 12 h, which means that in the end of an assay the cell chip had spent almost 30 h in the microfluidic system.

In conclusion, both paper IV and V show how microfluidic systems can be used for monitoring real-time dynamic changes of cellular events of the same cell population over a prolonged time-period. In this way, reagents can be added in discrete portions whereas fresh cell medium can be added on continuous basis to the cells, while any waste products produced from cell metabolism are removed by the flow, thus keeping the cells in an attractive and relatively constant environment. In a microtiter plate counterpart, most commonly used in cell analysis, an added reagent can only be removed by emptying the whole content of the well. In addition, the cells' environment is constantly changing due to consumption of cell media, metabolism, and accumulation of waste products, which in the end leads to cellular stress. Moreover, a feature (not very much explored in the developed system), is the possibility to not only control the chemical environment though microfluidic handling but also the possibilities to tailor the cell's physical microenvironment by micromachining and chemical patterning, which may create an *in vivo*-like milieu and considerable improve cell-based testing [175].

7. CONCLUSIONS

Analytical chemistry is getting smaller. From what has been mentioned so far, microchip technology is appearing everywhere in analytical chemistry (and other chemistry disciplines as well). We have probably only seen the very beginning of a new generation of analytical systems miniaturised onto microchips. The main focus of this thesis has been to develop and apply analytical techniques and methods on microchips in a flow-format. During the period when this research has been performed many interesting and elegant microfluidic analytical systems have been reported in the literature, however, the attention is often drawn mainly to the hardware set-up and construction. The systems developed and shown in paper I to V are technically simple but show the reality of applying and handling biological elements, such as living cells, enzymes and antibodies on silicon microchips. Regarding enzyme assays and immunoassays (paper I to IV) the immobilisation methods proved to be of outmost importance, affecting the stability of the biosensing components. As a consequence of miniaturisation, the available surface for immobilisation decreases and thus special attention must be paid to keep the bioelements in good shape through engineering their microenvironment via the immobilisation chemistry. In the cell monitoring systems for yeast and HeLa cells (paper IV and V), these display some of the advantages of using microfluidic system, *e.g.* continuous monitoring in real time and fluid handling format, which stands in great contrast to the very common microtiter plate assays. However, the demands on yeast cells compared to the HFF11 cell line stand in bright contrast, where the latter has highly specialised requirements on the physical and chemical environment. The aspects highlighted in paper V is very rarely encountered in analytical chemistry and to people in the molecular biology field these are too obvious even to be discussed. However, to meet the increasing demands on more sophisticated biological analysis, analytical chemistry has to develop and consider these aspects as well.

In general, the greatest contest for the microchip-based analytical systems is their commercialisation – if this is successful then they may conquer large and new areas in the point-of-care analysers, remote sensors, and in-situ/in-field monitoring. But to realise this, the basic system hardware such as pumps and valves have to be more developed. Electrokinetic fluidic control, which is widely used today, might not be convenient due to need for high

voltages and other limitations. Since the microfluidic regime does not behave as its "macroscopic" counterpart non-conventional solutions to problems might be needed, and as proven many times in history before, non-conventional matters have a problem with getting accepted and gain attention. Furthermore, most micromachining knowledge is still possessed by engineers to whom for example a valve is not the same as a valve for an analytical chemist, which means that they have to interact more to gain better and new systems and techniques. Moreover, these two subject fields must interact with the areas of biochemistry, biotechnology, molecular biology and medicine where the potential developments for more advanced systems for chemical and biological analysis are located. However, it will never be successful if the biochemists, biologists, physicians etc. do not dare to step away from their conventional procedures (microtiter plates, petri dishes etc.) and try new concepts. Right now, a lot of attention in the research community is paid to nanotechnology, which is engineering at the molecular level. Even though the field of microchip analytical systems is not mature yet, the combination of this with nanotechnology might reach fascinating potentials. One example is combination of microfluidic cell handling systems and nano-engineered tools for probing cellular activity on the molecular level [219].

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