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Electrospray Ionization Mass Spectrometry for Determination of Noncovalent Interactions in Drug Discovery

Kurt Benkestock

Doctoral Thesis

School of Chemical Science and Engineering

Department of Chemistry

Division of Analytical Chemistry

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To my children; Antonia, Karl and Vilma. I hope that this work will inspire you in the future.

Sammanfattning

Mass spectrometri (MS) är ett kraftfullt analytiskt vertyg som används i stor omfattning inom området för utveckling av läkemedel. Sedan i början av 1990-talet har elektrospray jonisering (ESI) varit en teknik av central betydelse för karakterisering av substanser med både låg och hög molekylvikt.

Syftet med arbetet beskrivet i denna avhandling har varit att undersöka möjligheterna att analysera icke kovalenta komplex inom läkemedelsutveckling mha ESI-MS. Bildning av icke kovalenta komplex har en avgörande betydelse i många biologiska system där biomolekyler interagerar reversibelt med en partnermolykel . Proteiner (receptorer) som är involverade i biologiska processer har ofta ingen biologisk aktivitet utan en partner, en ligand. Biologiska signaler uppstår när proteiner binder till andra proteiner, peptider, oligonukleotider, metalljoner, polysackarider, fettsyror eller andra små organiska molekyler.
Några av nyckelfrågeställningarna inom läkemedelsforskningen handlar om att förstå hur dessa icke kovalent bundna komplex hänger ihop. Vi har fokuserat vår forskning på följande processer: ligandscreening, kompetitiv inbindning och ”off-target” inbindning.

Den första uppsatsen handlar om den komplicerade joniseringsprocessen i elektrospray när icke kovalenta komplex analyseras. Vi har föreslagit en teoretisk modell som kan förklara hur jämvikten mellan proteiner och proteinkomplex förändras under joniseringsprocessen. Detta kan ha avgörande betydelse för tolkningen av experimentella data.

I det andra arbetet har ett automatiskt mikrochipbaserat nano-ESI system utvärderats som ett vertyg att studera vilka ligander som binder in till olika receptorer. Data framtaget med MS har jämförts med data erhållna med kärnmagnetisk resonans (NMR). NMR har sedan länge varit en etablerad metod för dessa analyser. Korrelationen mellan data från MS och NMR var utmärkt. Slutsatsen är att nanoESI/MS har stor potential som komplement vid HTS (high throughput screening) av ligander. Alternativt kan metodiken användas i en tidig fas av läkemedelsutvecklingen när endast en liten mängd av en framrenat receptor finns tillgänglig, framförallt pga av den generellt höga känsligheten och selektiviteten hos masspektrometern.

I det tredje arbetet har mikrodialys använts som ett verktyg för att erhålla högre känslighet och upplösning vid analys av icke kovalenta komplex. En befintlig mikrodialysenhet modifierades samt ett nytt sätt att studera kompetitiv inbindning demonstrerades.

Vissa läkemedel har en högre grad av inbindning till andra proteiner än till det tänkta målproteinet (receptorn). I det sista arbetet har en metodik tagits fram för bestämma var inbindning sker på humant serum albumin (HSA). Metodiken har baserats på kompetitiv inbindning.

***Sökord**: Masspektrometri, elektrospray jonisering, läkemedelsutveckling, icke kovalenta interaktioner, komplex, humant serum albumin (HSA), fettsyrabindande protein (FABP), ribonukleas, ligand screening, kompetitiv inbindning, mikrodialysis, aktivt bindningställe, mikrochip.*

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Abstract

Mass spectrometry in general and electrospray ionization mass spectrometry (ESI-MS) in particular is an invaluable analytical tool that is used in major areas of the intricate drug discovery process. The objective of the work has been to investigate some of the possibilities offered by electrospray mass spectrometry, to study noncovalent interactions.

Noncovalent interactions are involved in many biological processes in which biomolecules bind specifically and reversibly to a partner. Often, proteins do not have a biological activity without the presence of a partner, a ligand. Biological signals are produced when proteins interact with other proteins, peptides, oligonucleotides, nucleic acids, lipids, metal ions, polysaccharides or small organic molecules. Some key steps in the drug discovery process are based on noncovalent interactions. We have focused our research on the steps involving ligand screening, competitive binding and 'off-target' binding. The first paper in this thesis investigated the complicated electrospray ionization process with regards to noncovalent complexes. We have proposed a model that may explain how the equilibrium between a protein and ligand changes during the droplet evaporation/ionization process.

The second paper describes an evaluation of an automated chip-based nano-ESI platform for ligand screening. The technique was compared with a previously reported method based on nuclear magnetic resonance (NMR), and excellent correlation was obtained between the results obtained with the two methods. As a general conclusion we believe that the automated nano-ESI/MS should have a great potential to serve as a complementary screening method to conventional HTS. Alternatively, it could be used as a first screening method in an early phase of drug development programs when only small amounts of purified targets are available.

In the third article, the advantage of using on-line microdialysis as a tool for enhanced resolution and sensitivity during detection of noncovalent interactions and competitive binding studies by ESI-MS was demonstrated. The microdialysis device was improved and a new approach for competitive binding studies was developed.

The last article in the thesis reports studies of noncovalent interactions by means of nanoelectrospray ionization mass spectrometry (nanoESI-MS) for determination of the specific binding of selected drug candidates to HSA. Two drug candidates and two known binders to HSA were analyzed using a competitive approach. The drugs were incubated with the target protein followed by addition of site-specific probes, one at a time. The drug candidates showed predominant affinity to site I (warfarin site). Naproxen and glyburide showed affinity to both sites I and II.

***Key words**: Mass spectrometry, electrospray ionization, drug discovery, noncovalent interaction, complexes, human serum albumin (HSA), fatty acid binding protein (FABP), ribonuclease, ligand screening, competitive binding, microdialysis, binding site, microchip.*

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Table of contents

1. List of original papers

The thesis is based on the following papers, which are referred to in the text according to the following numbers:

I. Influence of Droplet Size, Capillary-Cone Distance and Selected Instrumental Parameters for the Analysis of Noncovalent Protein-Ligand Complexes by Nano-ESI Mass Spectrometry. Benkestock, K., Sundqvist, G., Edlund P.O. and Roeraade, J. *Journal of Mass Spectrometry.* 2004; **39**: 1059-1067

II. Automated Nano-Electrospray Mass Spectrometry for Protein-Ligand Screening by Noncovalent Interaction Applied to Human H-FABP and A-FABP. Benkestock, K., Van Pelt, C.K., Åkerud, T., Sterling, A., Edlund, P.O. and Roeraade**,** J. *Journal of Biomolecular Screening*. 2003; **8**: 247-256.

III. On-Line Microdialysis for Enhanced Resolution and Sensitivity During Electrospray Mass Spectrometry of Non-Covalent Complexes and Competitive Binding Studies. Benkestock, K., Edlund, P.O. and Roeraade, J. *Rapid Communications in Mass Spectrometry*. 2002; **16**: 2054-2059.

IV. Electrospray Ionization Mass Spectrometry as a Tool for Determination of Drug-Binding Sites to Human Serum Albumin by Noncovalent Interaction. Benkestock, K., Edlund, P.O. and Roeraade, J. *Rapid Communications in Mass Spectrometry*. 2005; **19**: 1637-1643.

The contributions of the author of this thesis to these papers are:

I Part (½) of the experiments and part (½) of the writing
II The major part of the MS experiments and the major part of the writing
III All the experiments and major part of the writing
IV All the experiments and major part of the writing

2. OTHER PUBLICATIONS AND PRESENTATIONS RELATED TO THE WORK, MENTIONED IN THIS THESIS

Papers

Determination of Dissociation Constants for Protein-Ligand Complexes by Electrospray Ionization Mass Spectrometry. Tjernberg, A., Carnö, S., Oliv, F., Benkestock, K., Edlund, P.O., Griffiths, W.J. and Hallén, H. *Analytical Chemistry*. 2004; **76**: 4325-4331.

Investigation of Multiple Binding Sites on Ribonuclease A, Using Nano-Electrospray Ionization Mass Spectrometry. Sundqvist, G., Benkestock, K. and Roeraade, J. *Rapid Communications in Mass Spectrometry*. 2005; **19**: 1011-1016.

Lectures

Noncovalent Interactions Determined by Electrospray Ionization Mass Spectrometry: a Powerful Tool in Drug Discovery. Benkestock, K, Edlund, P.O. and Roeraade, J. Presented at the 3rd Workshop of Nanochemistry, 2002, Stockholm, Sweden.

Utilizing the Full Potential of Mass Spectrometry – in the Area of Native Protein Mass Spectrometry. Benkestock, K., Edlund, P.O. and Roeraade, J. Plenary lecture presented at the Swedish Mass Spectrometry Society in Feb., 2005, Stockholm, Sweden.

Posters

Micro-Dialysis for Enhanced Sensitivity Competitive Binding assay in Drug Screening Analysis Using nano-ESI-MS. Benkestock, K. and Roeraade, J. Presented at the 25th ISCC, 2002, Riva del Garda, Italy.

Noncovalent Interaction Determined by Electrospray Ionization Mass Spectrometry: a Powerful Tool in Drug Discovery. Benkestock, K., Edlund, P.O. and, Roeraade, J. Presented at the 16th IMSC, 2003, Edinburgh, UK.

3. ABBREVIATIONS

ADME	Absorption, distribution, metabolism and excretion
amu	Atomic mass unit
CADD	Computer aided drug design
CD	Candidate drug
CID	Collision induced dissociation
C2MP	Cytidine-2'-monophosphate
CTP	Cytidine triphosphate
Da	Dalton
DNA	Deoxyribo nucleic acid
DMPK	Drug metabolism and pharmacokinetics
ESI	Electrospray ionization
ELISA	Enzyme linked immunosorbent assay
H-FABP	Heart fatty acid binding protein
A-FABP	Adipose fatty acid binding protein
FT-ICR	Fourier transform ion cyclotron resonance
FWHM	Full width height measurement
H/D	Hydrogen deuterium exchange
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HTC	High throughput chemistry
HTS	High throughput screening
i.d.	Inner diameter
IMS	Ion mobility mass spectrometry
K_D	Dissociation constant
m/z	Mass-to-charge ratio (Thomson)
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCN	Sodium cyanide
NH_4HCO_3	Ammonium hydrogen carbonate
NH_4OAc	Ammonium actetae
nanoESI	nano-electrospray ionization
NMR	Nuclear magnetic resonance
OD	Optical density
RNA	Ribo-nucleic acid
RNAse	Ribonuclease
SBDD	Structure based drug design
SAR	Structure activity relationship
S/N	Signal-to-noise
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOF	Time-of-flight
Q-TOF	Quadrupole time-of-flight

4. PREFACE

The development of a new therapeutic drug requires immense efforts. The typical lead time for the development of a new drug is 10 -12 years, with associated costs, amounting to 1000 million $ or more. Intensive efforts are ongoing to reduce the lead time, and for this purpose, many new methods and technologies are being developed. The objective of the work, described in this thesis has been to investigate some of the possibilities offered by electrospray mass spectrometry, to study noncovalent interactions. Noncovalent interactions are involved in many biological processes in which large molecules bind specifically and reversible to a partner. The major part of the work has focused on improved technologies. The study of noncovalent interactions by mass spectrometry is a challenging and rapidly growing field, which should have a great potential in the process of drug discovery. The thesis starts with an introduction of some of the principles of drug discovery (chapters 5.1 - 5.8), followed by a brief description of some general principles of mass spectrometry (chapter 6.1). Electrospray ionization, which is a central issue in the thesis, is discussed in more detail in chapter 6.2, followed by an overview and discussion of electrospray ionization mass spectrometry for the determination of noncovalent complexes (chapter 6.3). Chapter 7 summarizes the work, described in the ***papers I to IV***. Finally, some future outlooks of the field are discussed in chapter 8.

5. THE DRUG DISCOVERY PROCESS

5.1. Noncovalent interactions in vivo

One of the fundamental processes in living cells is based on a continuous formation and dissociation of noncovalent complexes. The specificity of noncovalent interactions of biomolecules is a basic principle for molecular recognition in nature and one of the most fundamental regulatory mechanisms in the cell.[1] Often, proteins do not have a biological activity without the presence of a partner, a ligand. Biological signals are produced when proteins interact with other proteins, peptides, oligonucleotides, nucleic acids, lipids, metal ions, polysaccharides or small organic molecules. Examples of events, triggered by noncovalent interactions are signal transduction, the cell cycle, protein trafficking, targeted proteolysis, cytoskeletal organization and gene expression.[2, 3] It has been proposed that all proteins in a given cell are interconnected in a huge network. [4-7] Figure 1 shows the complicated network found in a relatively simple cell, the yeast cell. Another example of protein interaction is protein oligomerization, which is believed to cause an improved stability against proteolysis and thermal degradation.[8] Interactions between species not involving proteins are also very common, such as between DNA-DNA, DNA-RNA, DNA-peptide(s), DNA-drugs etc.[9] Important functions of such interactions are transcription and replication.[10, 11]

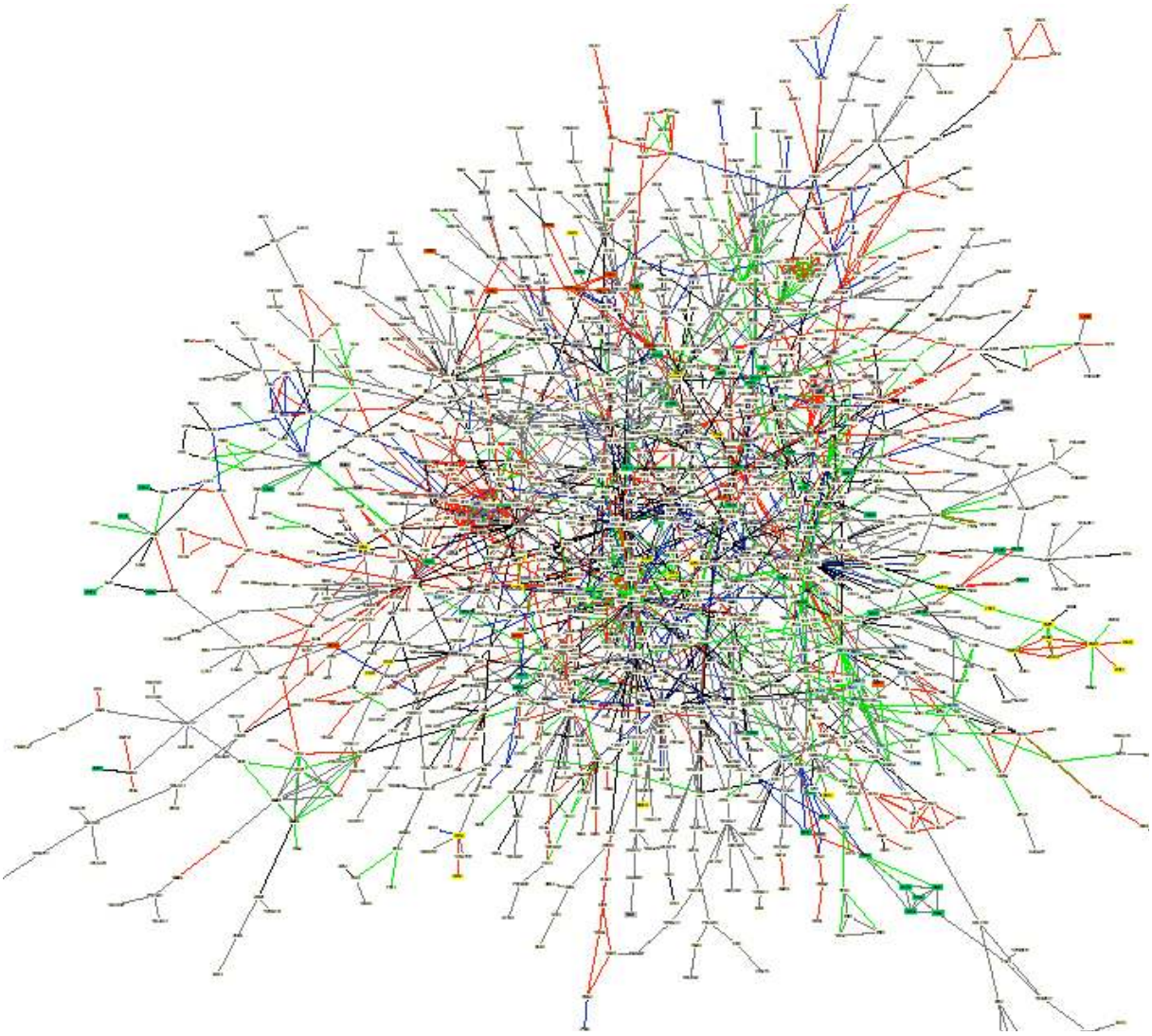

Figure 1. The protein-protein interaction network in yeast. A summary of the network of the yeast proteome assembled from published interactions. The map contains 1,548 proteins (boxes) and 2,358 interactions (connecting lines). Proteins are colored according to their functional role: proteins involved in membrane fusion (blue), chromatin structure (gray), cell structure (green), lipid metabolism (yellow), and cytokinesis (red). Reprinted with permission from Schwikowski *et al.*[4]

5.2. Drug discovery

Drug discovery refers to the research process, with the goal to identify molecules with desired biological effects and minimal side effects, for use as new therapeutic drugs in humans.[12-14] Drugs either act by stimulating, or by blocking the activity of their targets.[15, 16] There are two main approaches in drug discovery. The traditional approach is implemented by pharmaceutical companies that have been engaged in screening for a long time. The basic concept is simple; each company has its own in-house compound library, often consisting of up to millions of entries, known for their therapeutic advantages, which is employed for screening against different target diseases. The other approach which is more systematic, can be referred to as "the bottom-up"

approach, and uses structural and molecular biology to obtain a better understanding of the structures, functions and interactions of genes, proteins and cells that are involved in a specific disease.[16] The early steps of the "bottom-up" drug discovery process involve target evaluation, hit identification, lead identification and lead optimization, followed by pre-clinical and clinical trials (Fig 2). The total drug development time including registration and launch for a typical drug is approximately 8-12 years and the associated cost amounts to approximately $ 80-100 million per year.[17]

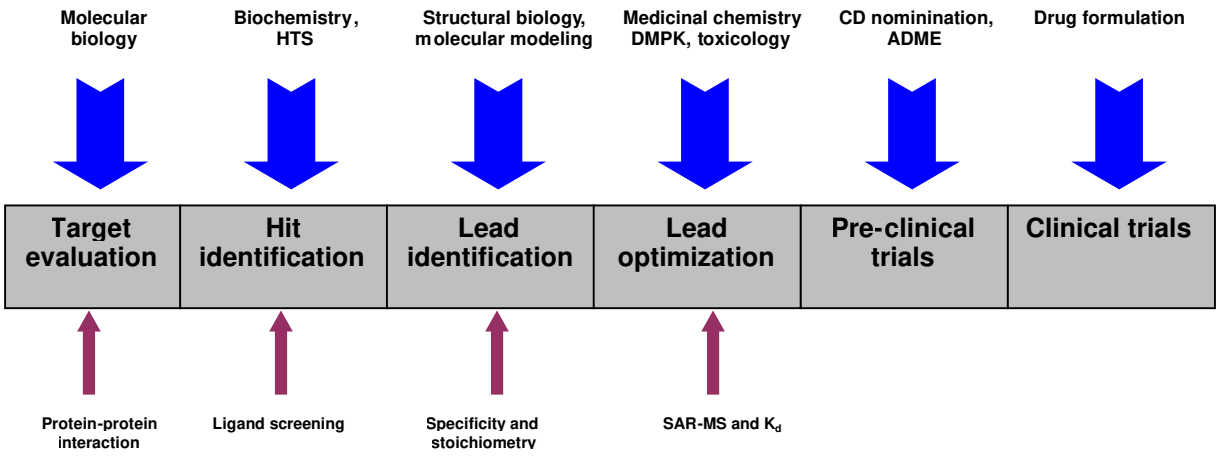

Figure 2. Basic overview of the drug discovery process.

5.3. Target evaluation

Target evaluation includes target identification, target characterization and target validation.[18, 19] The aim of target identification is to find a biological target that is connected to a specific disease. The identification of therapeutic targets requires knowledge of the cause of the disease and the biological systems associated with it. Molecular biology has a large impact in this stage of the drug discovery process.[20, 21] The combined contribution of genomics, proteomics and bioinformatics allows for a rapid and precise discovery of the genes and/or proteins involved in the mechanism of certain diseases.[22] The most important information is to understand the course of action by which the active site of a receptor selectively binds a ligand. In order for a drug compound (ligand) to bind specifically to the target receptor, it must contain a specific combination of atoms that presents the correct size, shape, and charge composition. The ligand activates or deactivates the receptor either by inducing a conformational change of the target or by a competitive binding against the natural ligand (biological) in order to enhance or interrupt key pathways associated with the disease, thereby resulting in a therapeutic effect. Protein-protein interactions are often revealed by the use of the yeast two-hybrid system, which is a molecular genetic tool for investigating intercellular and intracellular signaling pathways.[23, 24] The understanding of these

processes, so called functional proteomics, is very important for establishing which proteins are involved in a disease.[25, 26] In this context, mass spectrometry has a lot to offer.[27] The technique can provide detailed information of the stoichiometry and topological arrangement of subunits in protein complexes and also on interactions and dynamics with other proteins and protein complexes.[8, 28, 29] Equipped with this information, researchers can be more selective in their screening strategy, or even design synthetic compounds for a specific target. Suitable targets for therapeutic development are proteins (enzymes, receptors), DNA or RNA. Currently, there are around 400-500 known drug targets that are used in drug therapy.[30, 31] Examples of drug targets (and related diseases) are G-protein coupled receptors (autoimmune/inflammatory disorders),[32] ligand-gated ion channels (neurological, immunological and cardiovascular diseases),[33] tyrosine kinase receptors (rheumatoid arthritis, Alzheimer, diabetes, cancer),[34] nuclear hormone receptors (diabetes, heart diseases, cancer, asthma and arthritis),[35] proteases and polymerases (infectious diseases, inflammation, cancer).[36] However, there are at least 10 times as many potential targets predicted from the human genome, that can be exploited for future drug therapy.[31] Functional genomics[37] studies of the drug targets gene function such as the analysis of regulatory networks, biochemical pathways, protein-protein interactions, the effects of gene knockouts or gene up/down regulation is referred to as target characterization. The final step of target evaluation is target validation, where it is determined whether modulation of a target is feasible and likely to lead to modification of a disease progression. Validation involves studies, comprising intact animals or disease-related cell-based models that can provide information about the response of an organism to a pharmacological intervention and thereby help to predict the profile of new drugs in patients.

5.4. Hit identification (hit to lead identification)

After evaluation of the relevant therapeutic target, scientists must find compounds that interact with the therapeutic target to induce the desired therapeutic effect, i.e. a drug that inhibits the effect causing the disease. In order to find these compounds, high throughput screening (HTS) can be employed to select compounds from an in-house library.[38, 39] This process is highly automated, using sophisticated methods and laboratory equipment such as bioassays, robotics and data handling. Before HTS can take place, biochemical efforts have to ensure that there is a sufficient amount of purified target to perform the analyses. These activities include cloning, expressing, purifying of the target and finally assay development.[40, 41] After successful development of an assay, screening of compound libraries can be performed. Primary screens will identify so-called hits. Screening up to several hundred thousand compounds is common (for

large pharmaceutical companies) and such a campaign often yields 100-300 hits from which leads eventually are generated. The contemporary ligand screening process is a very streamlined procedure.[42] However, there are some bottlenecks that may be an issue. First, the method development time is often relatively long; a time period of several months is not unusual. Second, the protein consumption during method development and final screening is frequently on the order of multi-milligram quantities. Third, labeled compounds are often used which may alter the binding properties of the interacting species. In this context, mass spectrometry is a very interesting technology for ligand screening due to high sensitivity and speed of analysis.[43] The aim of primary screenings is to find compounds, which serve as a good starting point for development of the optimal ligand. Many screening methods exist, but few methods have the broad dynamic affinity range, starting at millimolar level up to nanomolar level, as mass spectrometry.[43, 44] This feature is very important, in order to keep the discrimination of ligands at a minimum, at this early stage. In ***paper II***, an approach is presented to start the screening process early in the drug development program, for cases where only a small amount of the purified target is available. A prerequisite of such an approach is to have highly sensitive analytical systems and simple method development strategies. These features are combined in the presented method which is based on noncovalent interaction detected by automated chip-based nano-electrospray ionization mass spectrometry.

5.4.1. High throughput screening

The vast number of new drug candidates produced by high throughput chemistry (HTC) requires fast and efficient methods both for characterization of the individual compounds and their affinity to target molecules.[45, 46] The aim of high throughput screening (HTS) is to test large compound collections for discovering potentially active compounds (hits). Often HTS methods [47, 48] based on biological assays with fluorescence detection, radioactive dyes, enzyme-linked immunosorbent assay (ELISA), receptor/binding and enzyme-substrate assays, luminescence etc., are used to investigate the binding properties of a large number of library compounds against a target molecule such as a protein, an enzyme, DNA etc. However, other technologies, using scintillation proximity assays, time resolved fluorescence, NMR, and mass spectrometry are becoming more and more popular as tools for ligand screening.[49, 50] The development of a high throughput screening setup is often a very time consuming (weeks to months) process. On the other hand, a large number of analyses can be performed in parallel, resulting in a high final throughput with low protein consumption per analysis. One way to accelerate drug discovery and thus, to decrease the overall time frame of the drug development process, is to perform as much

screening as possible at an early stage of the process. A prerequisite for such an approach is the availability of sensitive analytical methods.

Biochemical assays used in HTS to identify lead compounds in drug discovery have been formatted using a wide variety of detection techniques. These include radiometric, colorimetric, fluorescent, and ELISA-based methods.[51] Each technique has its strengths and weaknesses. Radiometric assays can be made on a homogeneous basis, with no separation steps involved, requiring washing or filtration. Such techniques include the scintillation proximity assay (SPA), where the scintillant is present in a bead, or ScintiPlates or FlashPlates, where the scintillant is present in the microtiter plate itself. These radiometric techniques are well established, with a wide variety of assays available from many different vendors.[52] The drawbacks of radiometric assays are that the signal-to-background ratio of these assays is typically less than other techniques and that the use of radioactive materials is hazardous to the scientist and the environment. When feasible, colorimetric assays are generally easy to format and to run, though often, a very broad dynamic range is not obtained, since absorbance can typically only be accurately measured in a narrow range (0.1-1.5 OD). Fluorescent techniques offer a wider dynamic range, and are often very sensitive, making it possible to measure low-analyte concentrations. Even so, some compound libraries contain fluorescent compounds, which can interfere with the fluorescence of the label, resulting in false-negatives or -positives depending on the assay format. This problem can partially be avoided by using time-gated detection, since in many (but not all) cases, the compound fluorescence is short-lived compared with that of some labels. Techniques such as ELISA and the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) are also very well-established and have a wide dynamic range.[53] However, these methods can be very time-consuming, because many washing steps must be included in the assays. With the ever-increasing compound library sizes used in drug discovery, time-consuming assays are undesirable.

In-vitro assays can be divided into two groups. Cell free assays measure the biological activity of the candidate drug on a relatively pure drug target, whereas cell-based assays assess the activity of the candidate drug by monitoring a biological response of a wild type or genetically modified cell in which the target resides.[54]

5.5. Lead identification

The hits from the primary screen (leads) are used in a secondary screen, where the target is investigated in more detail. For example, dose-response curves are obtained or time-based measurements are performed. The dose-response is expressed as an IC_{50} in enzyme-, protein-, antibody-, or cell based assays, or as an EC_{50} in in-vivo experiments.[55, 56] For competitive binding assays and functional antagonist/inhibitor assays, the most common summary measure of the dose-response curve is the IC_{50}, i.e. to find the concentration of substance which provides 50% inhibition of the target. For agonist/stimulator assays, the most common summary measure is the EC_{50}, i.e. the concentration which gives 50% of the maximal response of the compound. It is of central importance to perform competitive binding studies where it is verified, that the observed noncovalent binding between the receptor and the ligand is specific, i.e. that the two ligands (drug and biological ligand) bind at the same site. Such data can be obtained by means of mass spectrometry, as is described in ***paper III***, where a novel set-up involving a short dialysis fiber was presented. The advantage of using this approach is enhanced sensitivity. Proteins are often dissolved in non-volatile buffers such as PBS, urea or phosphate buffers, and a desalting step has to be performed prior to the MS analysis. This is not necessarily when on-line microdialysis is applied. The process that identifies leads from the pool of hits is referred to as "hits-to-leads".[56] In this stage, the number of hit compounds has been reduced to approximately 5%. The overall goal for this phase is to obtain proof for mechanism of action (MOA).[57]

5.6. Lead optimization

Hits from the screening procedures are further investigated by an iterative scheme which has been termed "lead optimization". Lead optimization is a process used to increase the understanding of the structure-activity relationship and to facilitate the incorporation of all desirable properties required to make an efficient drug. One of the goals is to identify compounds with a high binding affinity to the target molecule, which would enable the use of low dosages of the drug, thus minimizing potential side effects. At this stage, some basic indicators of metabolism, permeation, drug interactions, pharmacokinetics, bioavailability and toxicity may be considered in an attempt to eliminate potential failures as early in the drug development process as possible. Also at this stage of drug discovery, native protein MS offers valuable applications. Indirect toxicity of compounds can be evaluated using the DOLCE-MS (detection of oligonucleotides-ligand complexes) approach.[58] This high throughput screening method identifies compounds that bind to DNA and thereby exhibit a high toxicity potential. Furthermore, as demonstrated in ***paper IV***, determination of ligand binding to off-target

proteins like human serum albumin (HSA) is important as well. By using site-specific binders (warfarin, digitoxin and iopanoic acid) in separate competition experiments, the binding site at HSA for new drugs could be determined. The lead optimization phase requires heavy support from medicinal chemistry. Promising leads are subjected to iterative cycles of design and assessment, with the goal of developing one or more candidates for pre-clinical evaluation (Fig. 3). These compounds should have optimal drug-like properties which is a best compromise between activity, selectivity, bioavailability and safety. To facilitate the lead optimization process, approaches such as computer aided drug design (CADD)[59] may be very helpful. The lead optimization process is also often supplemented by more intuitive approaches like structure-activity relationship (SAR)[60] studies, trying to maintain activity while improving specificity, physiochemical and ADME properties, or toxicity profiles. Recently noncovalent interactions, determined by MS have been successfully used for SAR to find strong binding motifs by the discovery of individual building blocks that can be linked together to form ligands with higher affinity.[61, 62] Further synthesis may then be required to provide a variety of compounds, which are structurally related to the lead. These sub-libraries must then be screened against targets in order to select optimal structures. Also, relative or absolute dissociation constants can be determined by noncovalent interaction, detected by ESI-MS, to rank the order of ligands. However, the gas-phase data of ESI-MS do not always correlate well with solution-phase binding energies. On the other hand, new reports are continuously published on how to acquire, evaluate and calculate data, based on gas-phase experiments.[63-66] Finally, the formulation and delivery of drugs is an important part of the drug discovery and development process.[67] Formulation issues influence the design of the lead molecules and information is fed back into the iterative lead optimization cycle. This phase is concluded by selecting a promising compound - a candidate drug (CD). All interesting drugs and/or compound scaffolds must be covered by an intellectual property (IP) application to ensure worldwide proprietary ownership.

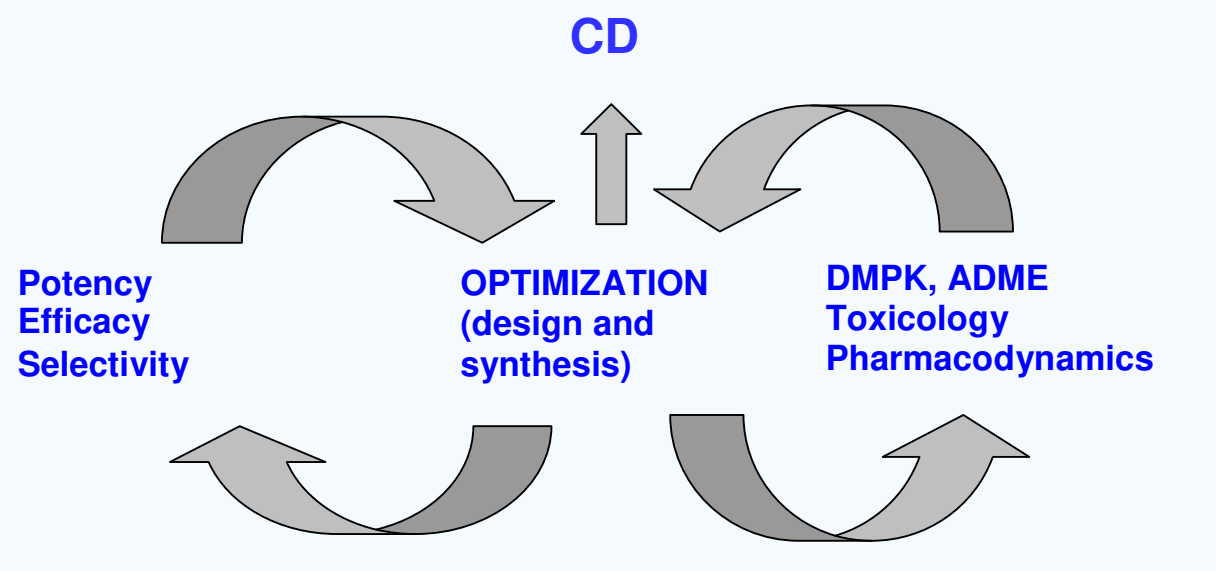

Figure 3. Overview of the iterative lead optimization process.

5.7. Pre-clinical trials

After a CD has been selected, the process continues with the authority-regulated pre-clinical phase (in-vitro and in-vivo laboratory animal testing) through studies intended to assess the pharmacokinetic properties and pharmacodynamics of the substance.[68] The pharmacokinetic evaluation examines the drug absorption and metabolism, the toxicity of the metabolites of the CD, and the speed by which the drug and its metabolites are excreted from the body. The pharmacodynamic studies evaluate the impact of the substance on the body, whether the desired effect is achieved and whether undesirable side effects arise.[69]

5.8. Clinical trials

A clinical trial is a research investigation in which human volunteers and/or patients are tested with new health treatments.[70] Clinical trials are divided into four phases:[71]

- Phase I trials are the first stage of testing with human subjects and involve a small group of healthy volunteers. They are designed to evaluate the pharmacokinetic and pharmacodynamic properties as well as safety profile of the new drug treatment. In the case of new drugs, a safe dose range is established and any side effects are identified. The study usually lasts a few months.

- Phase II trials involve a larger group of participants (20-300) and are designed to test the effectiveness of the new treatment. This is the first study in which affected volunteers (rather than healthy volunteers) are tested. Phase II trials may take up to two years. These

trials are usually randomized, (double-blind studies) to ensure objectivity. Phase II studies are sometimes divided into Phase IIA and IIB. Phase IIA is specifically designed to assess dosing requirements, and Phase IIB is specifically designed to study efficacy.[72]

- Phase III trials are similar to phase II trials but involve hundreds or even thousands of participants. This allows much more information to be collected concerning the safety and efficacy of the treatment. Biostatistics is an important tool used to evaluate the large databases obtained from the studies.[73, 74] Phase III trials may take several years. Once the trials are completed, the treatment can be approved for general therapeutic use.

- Phase IV trials are also known as post marketing surveillance trials and are designed to detect any rare or long-term adverse effects over a much larger patient population and longer time period than was possible during the Phase I-III clinical trials.

At the end of the clinical development phase, most of the investigational new drug candidates will have been eliminated on safety or efficacy grounds, and only very few compounds will be submitted to the regulatory authorities as a new drug application (NDA) issue, which includes permission to market.

6. MASS SPECTROMETRY

6.1. General

Mass spectrometry is one of the most powerful analytical tools. It has revolutionized many areas due to the combination of very high sensitivity and the ability to identify and/or obtain structural information of unknown components. Examples, where mass spectrometry is used extensively are:

- Biochemistry: analysis of proteins, peptides, oligonucleotides, polysaccharides, lipids, virus particles[75-79]

- Pharmaceutical: drug discovery, drug metabolism, combinatorial chemistry, pharmacokinetics[80-84]

- Clinical: neonatal screening, diagnosis of diseases, hemoglobin analysis, drug testing[85-87]

- Environmental: PAHs, PCBs, water and air quality, food contamination, heavy metals (as well as organic chemistry in general)[88, 89]

- Geological: isotopic composition, oil composition[90]

- Forensic: identification of unknown samples[91]
- Industry: monitoring of process streams[92, 93]

A mass spectrometer generates a characteristic qualitative and quantitative pattern of ions derived from a chemical compound, often including an ion due to the molecular mass of the compound. The process includes ionization, ion separation, and recording of the ions according to their mass-to-charge ratio (m/z) and abundance. Thus, mass spectrometers can be divided into three fundamental parts: the ionization source, the mass analyzer, and the detector. These parts also include lenses for focusing and acceleration stages for transportation of the ions. Part of the system (analyzer and detector) is working under high vacuum and the whole system is controlled by an advanced data system (Fig. 4).[94-96]

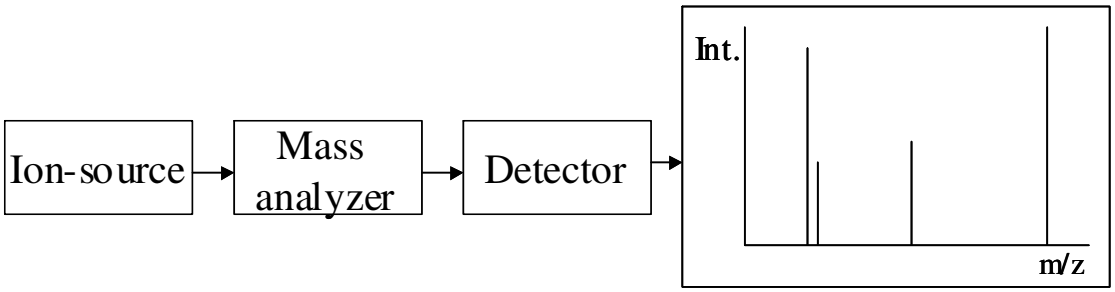

Figure 4. Basic elements of a mass spectrometer

Molecules that are introduced into the ion source are converted to ions either by gain or loss of an electron or by protonation or deprotonation. There are many types of ion sources available and the choice is made, based on the sample nature and/or what kind of MS data is required. Samples can be introduced as a solid, liquid or gas. The generated gas-phase ions are electrostatically extracted into a mass analyzer and separated according to their mass-to-charge ratio and finally detected.[97-100] The result of this process yields a mass spectrum that provides molecular mass or even structural information.[101-104] The fundamental platform for mass spectrometry was developed by J.J. Thomson during his studies of electrostatic and magnetic deflection of cathode rays in 1897.[105] Major contributions to the history of mass spectrometry have been made by e.g.: Aston,[106] Herzog,[107] Stephen,[108] Paul,[109] Dole,[110] McLafferty,[111] Hillenkamp,[112] Tanaka[113] and Fenn.[114]

6.2. The electrospray ionization process

Electrospray ionization (ESI) is the most commonly used ionization method when dealing with liquid samples. The technique is suitable for the analyses of both low mass molecules and biomolecules. John B. Fenn, the inventor of electrospray, was awarded the Nobel Prize in chemistry 2002 for "The development of methods for identification and structure analyses of biological macromolecules".[114-116]

The unique features of electrospray ionization are: 1) the ability to produce multiply charged ions; 2) it is a soft ionization technique; 3) the method is very versatile; 4) it is a solution based technique. Electrospray ionization involves a complex series of events, all occurring during a fraction of a second including electrolysis at the spray tip, the establishment of charge gradients, a charge-based separation, solvent evaporation, acid-base reactions and droplet fission.[117] Furthermore, the chemical/physical properties (droplet size, charge, surface activity and ion-pairing) of the analytes, buffer and solvents are also important for the electrospray process.[118] Basically, the electrospray ionization mechanism can be divided into the following steps: [118] a) a production of charged droplets at the electrospray capillary tip; b) an evolution of the charged droplets by repeated droplet disintegration and formation of very small highly charged droplets capable of producing gas-phase ions; c) the actual mechanism by which the final ions are produced.

6.2.1. Generation of charged droplets

When a high voltage, typically 3-4 kV, is applied onto a flowing conductive liquid in a metal capillary, positioned in front of the mass spectrometer inlet (the counter electrode), a very high electric field strength is created between the end of the capillary tip and the inlet. This electric field leads to a partial separation of positive and negative ions of the electrolyte. In positive ion mode, positive ions are enriched at the capillary tip (the meniscus), whereas negative ions are driven towards the inside of the capillary. The mutual repulsion causes the positive ions and liquid to move downfield. This expansion creates a Taylor cone[119] at the capillary tip. The least stable point at the Taylor cone extends into a liquid jet (filament) that breaks up into individual highly positively charged droplets. The onset potential of the electrospray that leads to destabilization and disruption of the meniscus is defined by:[120]

(Eq. 1) $$V_{on} \sim (r_c \gamma \cos 49° / 2\varepsilon_0)^{1/2} \ln(4d/r_c)$$

where γ is liquid surface tension, cos 49° is the Taylor cone half-angle, ε_0 is the permittivity of vacuum, r_c is the o.d. of the capillary, and d is the distance to the counter electrode. It can be noted as an example, that the onset potential for a liquid with a high surface tension like water is approximately twice as high as for methanol.

6.2.2. Charged droplet evolution

Solvent evaporation of the charged droplets produced by the electrospray process will decrease the radius of the droplets, while their charge remains more or less constant. As a result, the electrostatic (Coulombic) repulsion of the charges at the droplet surface will increase until the droplets reach the Rayleigh stability limit:[121]

(Eq. 2) $$q_{Ry}=8\pi(\varepsilon_0\gamma R^3)^{1/2}$$

where q is the charge, ε_0 is the permittivity of vacuum, γ is the surface tension and R is the droplet radius. When the Coulombic repulsion between the surface charges overcomes the surface tension, droplet jet fissions occurs. The generated offspring droplets have a radius that is approximately 10% of the parent droplet (~ 1μm). Furthermore, the offspring droplets carry 2% of the mass and 15% of the charge compared to the parent droplet.[122] The offspring droplets themselves undergo further fissions and this process is repeated until very small droplets (< 10 nm) remain. The process is schematically shown in Figure 5.

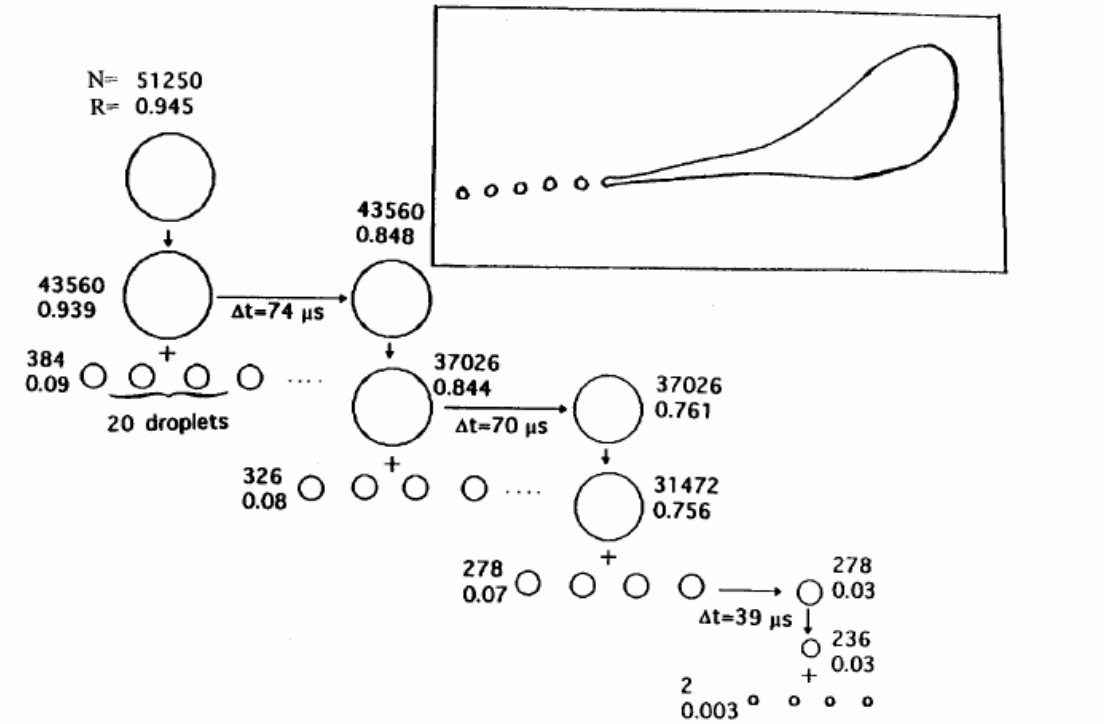

Figure 5. Droplet evolution scheme showing the time history of parent and offspring droplets for the three first successive fissions. N=number of elemental charges, R= droplet radius in μm and Δt=time required to reach the next fission. The top right insert represents the shape and starting point for fissions from an initial droplet. Reprinted with permission from P.Kebarle and L. Tang.[123]

6.2.3. Formation of gas-phase ions from small highly charged droplets

There are two basic mechanisms proposed for the formation of desolved gas-phase ions from highly charged droplets, the charged residue model (CRM) and the ion evaporation model (IEM). According to the first mechanism, suggested by Dole and coworkers,[110, 124] droplet fission continues until the droplet reaches such a small size, that fission is not possible anymore. In an ideal world, this would mean one analyte ion per droplet (charged residue). A final solvent evaporation from such species will lead to a desolved gas-phase ion. The CRM has been suggested as the preferred mechanism for macro-ions such as proteins.[118, 125] The IEM model, reported by Iribane and Thomson[126, 127] eight years after the proposal of the CRM, is based on a different scenario, which involves a continuous ion evaporation process. When the droplet has reached a radius in the range of 10 nm after solvent evaporation and jet fission, direct "emission" of analyte ions into the gas-phase is initiated. The suggested explanation is that the charge of the droplet required for ion evaporation is lower than the charge required for the droplet jet fission process. As a result, the ion evaporation process replaces the fission process. It is widely known that the IEM is suggested to be the dominant mechanism for ionization of small molecules. Fernandez de la Mora[128] came to the conclusion that the charged residue scenario was

predominant for molecules having masses above at least 3300 Da. The mechanism debate is still ongoing and has been alive for over a decade. However, some reports suggest that both mechanisms are involved to some extent during the electrospray ionization process, depending on the size of the species.[129, 130]

6.3. ESI-MS for determination of noncovalent interaction

Many reports have demonstrated that dissolved macromolecules, subjected to ESI have the capability to retain a memory of their conformation in solution, despite the 'dynamic' process of ESI.[131-134] There is obviously a clear distinction between folded and unfolded protein structures determined by ESI, but to which extent can unfolded proteins preserve their viability to interact with a partner during ESI? Does the ESI-MS response of a complex reflect the behavior of the complex situation in solution? In the excellent review by Breuker[135] the structural information from MS gas phase experiments is discussed in depth. The gentle desolvation and ion transfer of ESI that minimizes energetic activation cannot prevent changes in the stability of higher-order interactions, as a result of solvent removal. Upon dehydration, the competition of water molecules for hydrogen bonds is eliminated, and very strong bonds can be formed with a thermal strength higher than for covalent bonds. Robinson *et al.* reported that removal of water drastically weakens hydrophobic bonds which leads to an increased strength of electrostatic forces within the protein structure.[136] It has been shown that stability of higher order interactions is altered upon the transfer of the molecules into the gas phase and it is expected that some bonds are broken, while new ones may be formed.[137, 138] Therefore, it is unlikely that the solution structure is preserved in the gas phase, and obviously this may as well affect the binding interface of a protein-ligand system. But minor structural rearrangements may not necessarily result in a complete dissociation of the complex.

In 1991, Ganem, Li and Henion showed the first evidence that ESI-MS had the potential to become useful as a tool for detecting noncovalent interactions. In their article "Detection of noncovalent receptor-ligand complexes by mass spectrometry"[139] complexes between FK binding protein (FKBP) and the ligands FK506 and rapamycin were shown. They came to the conclusion that detection of noncovalent complexes is highly dependent on many parameters, including pH, buffer additives, cone potentials, temperatures and flow rates. Katta and Chait reported observations of the heme-globin complex in native myoglobin by ESI-MS[140] They conclude in their article that the observed data suggest that noncovalent associations of proteins and cofactors in solutions can be preserved in the gas phase and observed by mass spectrometry

but do not imply that the exact solution conformation of myoglobin is preserved in the gas phase environment. Despite of the seventeen years that have gone by since these articles were written, the same questions and issues are still hot discussion topics. Some of these issues will be discussed later in chapter 7 (***paper I***). How can specific noncovalent interactions be distinguished from non-specific interactions? Observation of peaks in a mass spectrum which indicate the possible existence of a complex does not imply that there actually is a specific complex. Aggregation in a solution or in the gas-phase may provide non-specific interactions. Smith and Light-Wahl proposed criteria for evaluating whether complexes observed in the gas phase do mirror specific interactions in solution.[141]

The first issue is the *stoichiometry of the complex*. Specific complexes (M+L) formed in solution should be observed without comparable contribution from random associations like: M+M, L+L, M+L_2, M_2+L etc. However, it should be noted that random association could be promoted in solution as well when dealing with high analyte concentrations. Therefore, it is recommended to use as low concentrations as possible.

Gas phase lability is the second parameter that can be used to differentiate the binding type. This can be achieved by increasing the energy in the ion-source or by means of tandem MS experiments. Normally, non-specific interaction complexes dissociate more easily than specific ones. However, nucleic acids (DNA, RNA) that form multiple noncovalent bonds with a partner may result in very strong complexes that survive collision energy levels which break covalent bonds.[142]

Dissociation due to modification of solution conditions such as change of pH, temperature, adding of organic solvents or salts etc may weaken or disrupt specific complexes in solution and a corresponding change in the mass spectra should be obtained. Helicobacter pylori urease converts urea to ammonia in H. pylori. Pinkse *et al.* showed how the α and β urease subunits form a macromolecular complex consisting of $\alpha_{12}\beta_{12}$ with a molecular weight over 1 MDa. This huge complex was shown to dissociate into $\alpha_3\beta_3$ subunits upon slight heating *in vitro* which was consistent with X-ray crystallography data.[143]

Sensitivity to structural modifications is probably the most unambiguous demonstration of the existence of a specific noncovalent complex. Modification of one of the complex compounds present will produce a significant change in the relative intensity of the complex. An elegant

example was given by Gao *et al*. They studied the relative gas phase stabilities of noncovalent complexes of carbonic anhydrase II (CAII) and benzenesulfonamide inhibitors by ESI FT-ICR MS.[144] In the binding pocket of CAII there is a Zn^{2+} ion chelated by three histidine residues in a tetrahedral configuration. A polar interaction between Zn^{2+} and the negatively charged ligand is crucial for the complex association. They compared the dissociation energetics of the complex, using CAII and apoCAII, where the Zn^{2+} ion is removed from the active site, and obtained same results as in solution, i.e. no ligand binding was observed without the Zn^{2+} ion.[145]

Furthermore, a binding study of isomeric inhibitors to CAII was performed, in view of the fact that the binding pocket of CAII is very sensitive to the position of substitution on the benzenesulfonamides. Para- and ortho-NO_2-benzenesulfonamide was incubated with CAII whereby the E_{50}-value was determined by SORI-irradiation (CID). The E_{50}-value is defined as the amount of energy (amplitude of irradiation) required to dissociate 50% of the complex. The relative stability of CAII with para-substituted (63.7 V) ligand was significantly higher than with the more bulky ortho-subtituted (62.9 V) counterpart. These gas phase data correlate very well with solution data. Hanch[146] showed that a bulky group such as $–NO_2$ or –COOH in the ortho position of benzenesulfonamide has unfavorable steric interactions with the binding pocket on CAII and thus reduces the binding affinity relative to the para-substituted inhibitor. These data demonstrate clearly that steric interaction observed in solution was maintained in the gas phase and that the active site of CAII retains at least some similarity to solution conditions.

Solution phase hydrogen/deuterium (H/D) exchange is a powerful method to elucidate if the association to a complex is formed in solution or in gas phase. By comparison of the average number of H/D exchanges for the individual compounds in the complex with the number of H/D exchanges in the complex itself, provides information about the complex origin[147] (i.e. information whether the complex was formed in the solution or if the association was an effect of non-specific binding during the ionization process).

Comparison with solution-based methods (CD, NMR, calorimetry etc). There are a number of articles, where a high correlation has been demonstrated between complexes, measured with solution-based methods and ESI-MS.[64, 148] However, Robinson *et al.* published an article in 1996 where the relationship between solution and gas phase data could not be established.[136] In studies of complex formation, involving acyl CoA binding protein (ACBP) and acyl CoA derivates with variation in the hydrophobic acyl chain (C_8, C_{12} and C_{16}) it was shown that the derivate with the longest chain was less stabile in the gas phase. There is a large difference in dissociation

constants, measured in solution, $K_d=10^{-6}$, 10^{-8}, and 10^{-12} M for the C_8, C_{12} and C_{16} in complex with ACBP, which is related to the increased hydrophobic effect of the hydrocarbon chain. As pointed out above, hydrophobic interaction is weakened in the gas phase when the water is removed. This suggests that the nature of a bond, stabilizing a complex, can differ significantly between solution and gas phase conditions, and that interpretation of noncovalent complexes, observed by ESI-MS always should be approached with caution.

At present time there are more than 300 articles published, which concern the area of noncovalent complexes and ESI, and the area is still under intensive development. The following reasons explain some of the great interest in the topic. Biophysical techniques including ultracentrifugation, SPR, calorimetry, fluorescence, circular dichroism (CD), raman spectroscopy, electron spin spectroscopy, chromatography, NMR, X-ray crystallography, IR, UV, SDS-PAGE etc are the conventional and mostly used methods to study noncovalent interactions.[149] However, the general drawbacks of these methods are long analyses time and the need for relatively large amounts of protein, while targets or ligands often need to be modified by labeling or need to be bound to a surface. Method development is usually very tedious.

ESI-MS based methods may have the following features; direct measurement of the observed complex signal, the mass of the complex is unique, a high mass measurement accuracy, no modification of the ligands is needed (fluorescence labeling or covalent binding to a surface), high sensitivity, a high speed of analysis and, the ability to obtain stoichiometric information. The gas phase behavior of noncovalent complexes has been discussed by Smith *et al.* and others[135, 141, 150] and it has been shown that the technique has the potential to determine dissociation constants (K_D) of complexes. However, there are several pitfalls and limitations to be aware of. Firstly, (apart from the ones discussed above), problems with non-specific clustering or oligomerization (i.e. formation of dimers and trimers etc.) This may be a problem, especially if high concentrations of ligands containing functional groups that show a tendency to oligomerize are used. Secondly, careful optimization of the MS parameters is important to reduce nonspecific binding and to minimize adduct ion formation. Also, the macromolecule must be soluble in a volatile buffer like NH_4OAc or NH_4HCO_3, which may be a problem for hydrophobic proteins. Furthermore, the purity of the protein must be high enough to avoid spectra interpretation difficulties. Finally, the limitation regarding the molecular mass of the target protein and ligand depends on the mass resolution and mass range of the MS instrumentation.

In the papers (I- IV) of this thesis, some of the issues, mentioned above have been studied in further detail, and some examples to determine noncovalent interactions by ESI-MS are shown. The work is summarized in the next chapter.

7. SUMMARY OF PAPERS I - IV

7.1. Paper I: Influence of Droplet Size, Capillary-Cone Distance and Selected Instrumental Parameters for the Analysis of Noncovalent Protein-Ligand Complexes by Nano-ESI Mass Spectrometry

As pointed in chapter 6.2, ESI is a very complex process consisting of a series of events depending on many parameters. The influence of the electrospray process on noncovalent complexes is even more complicated and so far not fully understood. For the analysis of noncovalent complexes with ESI-MS, a variety of different instrumental and sample-derived parameters have shown to affect the relative intensities of the complex ions, derived from the species involved.[151-153] Of particular interest is the droplet size and location of the species in the electrosprayed droplets.[153] In noncovalent systems, the equilibrium between protein, ligand and complex is strongly dependent on the dynamic conditions (concentration changes and droplet radius decrease) of the droplets during the evaporation/fission into the gas phase.

7.1.1. General instrumental parameters

The effects of the following instrumental parameters were investigated: ion-source temperature, drying gas flow rate, spray capillary voltage, cone voltage, collision gas pressure, and the gas pressure between the cone and the extraction lens. In general, increased values for parameters such as drying gas flow rate, ion-source temperature, and capillary tip voltage resulted in a dissociation of all complexes studied. It was therefore concluded that the values of these parameters should be as low as possible to obtain a high response of the complexes. Desolvation parameters such as the cone voltage, and the pressure between the cone and the extraction lens and the collision gas pressure showed to have a variable influence on optima for different complexes. It has been shown earlier by others, that these parameters are very important for sensitivity, resolution and m/z discrimination.[154] Increased pressure in the different regions (ion-source and collision cell), helps to enhance desolvation and decreases the kinetic energy of the analyte ions (also referred to as a collisional cooling effect). It is fairly straightforward for the MS operator to control the discussed parameters compared to the issues regarding the actual evaporation/fission process. However, it should be noted that the optimum values are instrument dependent due to differences in the design of the ion-source, gas flow path, collision cell, position of the spray capillary, vacuum system configuration etc.

7.1.2. Capillary-cone axis distance

The distance between the nano-ESI capillary needle and the cone was shown to be an important parameter for the absolute response of noncovalent complexes. Two model systems were used: a) RNAse-CMP (polar ligand) and b) H-FABP-oleic acid (hydrophobic ligand). The details about the target proteins are found in ***paper I*** (chapter 12). Our study showed that an increase in capillary to cone axis distance yielded an increased ion intensity of the complex relative to the free protein, when the complex was made up with a relatively polar ligand (see Fig. 6A). For a hydrophobic ligand, the reversed effect was observed, i.e. the ion intensity of the complex decreased with increased distance as shown in Fig. 6B. The S/N responses for the complexes and free protein are plotted in Figure 6C and 6D as a function of the capillary cone distance. These graphs (lower curves labeled with triangles) clearly show that the response of the free protein peak is decreased with the longer capillary cone distance while the capillary voltage was held constant. This indicates that the free proteins are located on the droplet surface and are depleted through the offspring droplets as expected. This effect is more pronounced with increased capillary to cone distance due to the higher number of droplet fissions. When observing the S/N for the individual complexes, an increase in response was obtained for the complex including a polar ligand (6C). The complex containing the more hydrophobic ligand (6D), oleic acid, showed a decreased signal with higher capillary to cone distance.

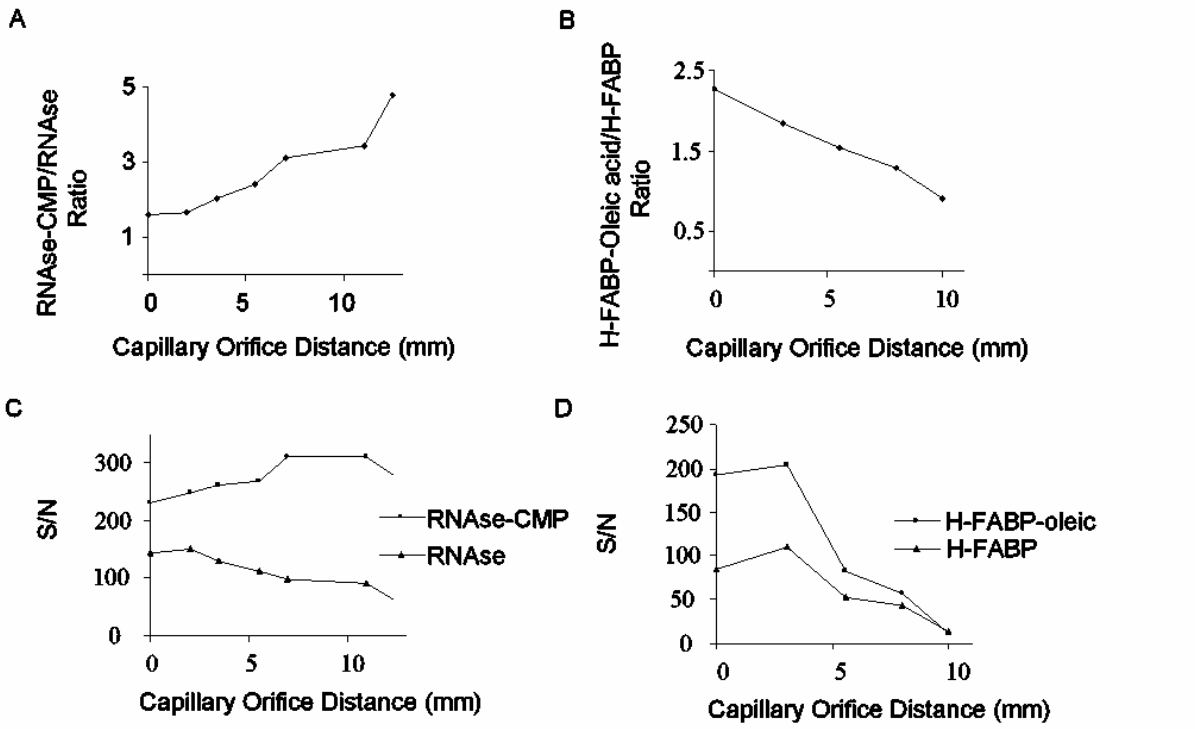

Figure 6. Relative abundance as a function of the distance between the cone (orifice) and the nano-ESI needle for (A) RNase-CMP (22:44 μM) and (B) H-FABP-oleic acid (22:114 μM). Signal-to-noise ratio for protein and complex of (C) RNase-CMP and (D) H-FABP-oleic acid systems.

7.1.3. A proposed model

Based on these results, we suggest a model to describe the overall observed effects on the relative intensity of a noncovalent complex for different positions of the electrospray needle.

Figure 7 shows a schematic diagram that suggests the change in concentration of a protein in equilibrium with a hydrophilic ligand in residual droplets as well as offspring droplets, generated during the evaporation process. To simplify the discussion, an equal molar concentration of the species in the initial droplet is assumed. *Parent droplet* - The horizontal route shows an enrichment of the surface-active compound (P=protein) during the offspring droplet formation. As a result, the mass balance between the complex and free protein is shifted towards the free protein state. *Residue droplet* – The vertical route (longer capillary-cone distance) shows an enrichment of non-surface-active (L) species in the droplet bulk and the concentration ratio of protein to ligand is decreased in the corresponding offspring droplets. Thus the equilibrium is shifted in favor for the complex and as a result this leads to a more pronounced ion intensity of the complex.

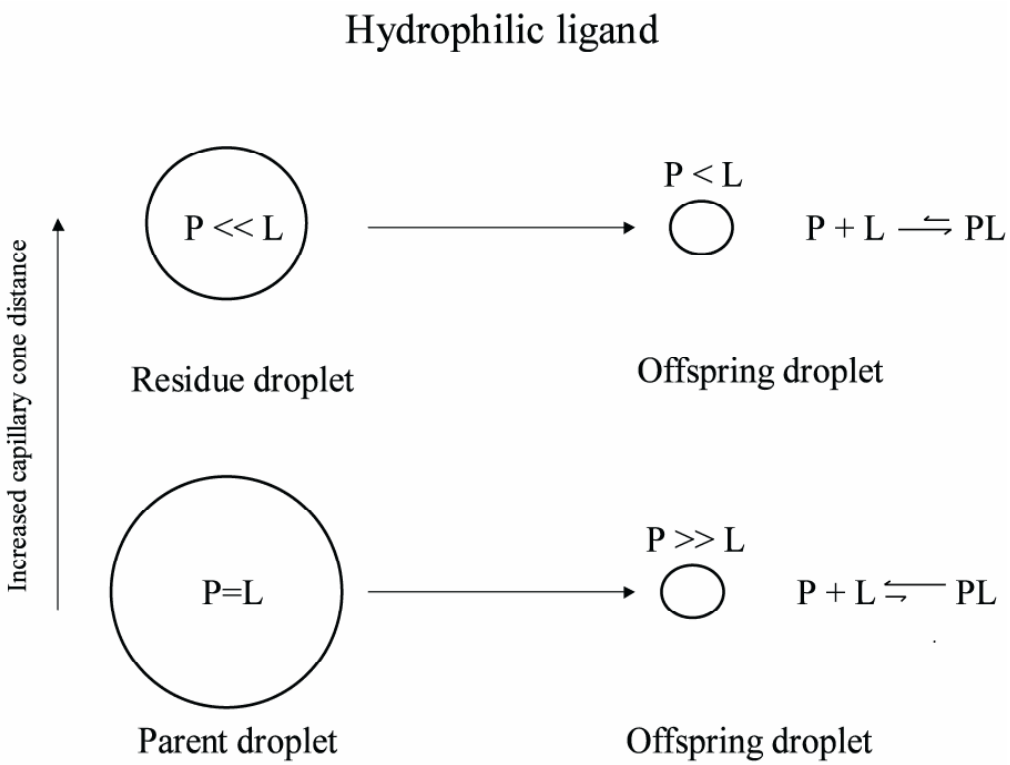

Figure 7. Schematic diagram showing the different ionization routes for parent and residue droplets (longer capillary-cone distance) for a hydrophilic ligand located in the droplet bulk and the protein present on the droplet surface. P = protein; L = ligand; PL = complex.

Our model is only valid for droplet sizes above approximately 10 nm when the droplet fission is the major route for reduction of the droplets radius. Below this radius different ionization mechanisms (as discussed in chapter 6.2.3) start to act in order to fully desolvate the ions. When noncovalent complexes are analyzed it is reasonable to believe that both ionization mechanisms are in play. CRM is the preferred mechanism for the protein and complex species and IEM for the ligand. However, since the time scale is very short to fully desolvate ions (a few μs) it is likely that the depletion of ligand (according to IEM) is not significant, meaning that the equilibrium is fairly constant during the final desolvation process.

7.1.4. Sampling of the electrospray plume

Even though the ionization efficiency is high in electrospray,[155] the overall ion transmission into the analyzer is low, in the range of 0.1-1%. Most of the ions are lost between the ESI source and the first sampling orifice.[156, 157] The obvious question is therefore: is the composition of the sampled ions representative for the composition of the ions in the sample solution and how is the equilibrium of the species (protein and ligand) affected by the phase transition? As pointed out above, the equilibrium between free protein and complex, leading to detectable desolvated

ions, can be very dynamic during the evaporation/fission process. This suggests that it would be advantageous to sample a larger portion of the electrospray plume, both small and larger droplets, in order to obtain an average picture of the equilibrium for the free protein and the complex. Another possibility is to freeze the equilibrium as soon as the droplets have been formed (charged). If the equilibrium has already been changed prior the ionization, it could be due to pH-changes at the tip of the electrospray needle (electrochemical cell) or electrostatic (repulsion, attraction) effects during the initial ionization. Regardless of the fundamental questions about the complex ionization mechanism, many reports have been published, as also discussed in chapter 6.3, where excellent correlation has been achieved between K_D in solution and in the gas-phase. In principle, it would be interesting to transfer the whole electrospray plume into the first sampling orifice. Several attempts have been made to increase the sampling efficiency. One approach is to increase the diameter of the sampling orifice. Other methods include ion lenses between the sprayer and sampling orifice[158] or an ion funnel between the sampling orifice and the nozzle of the mass spectrometer.[159] However, none of these developments showed major improvement in ion transmission. With the use of an ion funnel, the ion transmission efficiency has been increased by over one order of magnitude but this device was only functional under conditions of reduced pressure. This shows that it is very complicated to increase the transmission and it is difficult to know, how these improvements would affect complexes. The other property of an ideal electrospray source would be if droplets with an extremely small, uniform diameter could be generated immediately from the tip of the spray needle (monodisperse "attospray"). This would mean that a minimum number of repeated fissions would take place prior to a complete desolvation of the complex, possibly resulting in a better reproducibility and predictability of the composition of the gas phase ions.

7.1.5. Conclusions

Important issues to consider for determination of noncovalent complexes are the parent droplet size, the chemical/physical properties of the protein and ligand species involved and the capillary-cone distance. Our findings show that the residue droplets play an important role in the ionization during detection of noncovalent complexes. Sampling of late-generation residue droplets benefits from longer capillary-cone distance (i.e. longer flight/evaporation time). The most important issue seems to be the surface-active properties of the species, especially of the ligand.

7.2. Paper II: Automated nano-Electrospray Mass Spectrometry for Protein-Ligand Screening by Noncovalent Interaction Applied to Human H-FABP and A-FABP.

As pointed out in the introduction, ligand screening is a very streamlined procedure but there is a need for improvements. This is especially true when screening is performed in drug development projects at an early stage, as soon as a small amount of purified target is available. This article describes an evaluation of an automated chip-based nano-ESI platform for ligand screening.

7.2.1. Chip-based nano-ESI platform

Nano-ESI offers higher sensitivity and lower sample consumption than pneumatically assisted ESI. The main drawback of nano-ESI is the manual and tedious work to first load the sample into the nanospray needle and then to fit the needle in an optimal position in front of the ion-source, before the actual analysis can be performed. As discussed in ***paper I*** a fixed needle position is of utmost importance for the outcome of the results. Furthermore, there are often problems to onset the spray especially when samples with high water content are analyzed. Moreover, needle clogging can be a problem. Recently, a fully automated chip-based nano-ESI system was introduced by Henion and his group.[160] The core of the system consists of a disposable chip with a 10 x 10 array of nozzles with tip i.d. of 10 µm (Fig. 8).

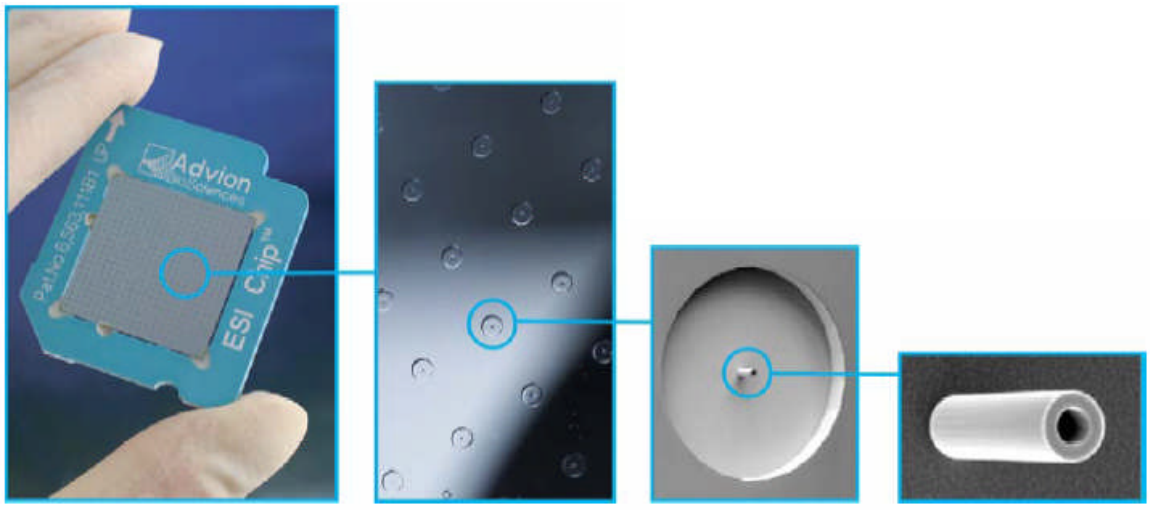

Figure 8. Enlarged views of the ESI chip. Reproduced with permission from Advion Bioscience, Inc.

The instrument first aspirates an aliquot of sample from a conventional 96-well microplate using a disposable, conductive pipette tip and then delivers the sample to the inlet side of the ESI chip. The pipette tip forms a pressure seal around a wafer-based channel on the back of the chip. A gas pressure and a voltage are applied, which initiates the electrospray. A separate nozzle and pipette tip is utilized for each sample, which eliminates cross-contamination between samples.

The system has been shown to work very well for different application such as proteomics,[161] metabolite identification,[162] small molecule quantification,[163] biomarker discovery[164] etc. However, applications in the area of noncovalent interaction had not been reported. The water-based buffers of such samples, as well as the relatively high viscosity and high surface tension make the onset of the spray rather difficult (see chapter 6.2.1). However, due to the relatively large i.d. (10 μm) of the chip-based nozzles (compared to classical nanospray needles: 1-5 μm) the onset of the spray was instantaneous, with high spray stability and reproducibility between nozzles (data not shown). Furthermore, the large i.d of the chip-nozzles reduces the risk of clogging. The spray stability could probably be increased even further if another spray tip geometry is considered. It has been shown by others that a conical tip design performs better than a "flat" tip in terms of spray stability.[165] This is due to the formation of a more reproducible Taylor cone since the width of the Taylor cone is defined by the outer diameter of the tip. In the conical design, an uncontrolled wetting of the tip shaft is avoided.

7.2.2. Speed of analysis

Since MS is a sequential technique the cycle time per sample is an important issue when performing HT protein-ligand screening. Using one of the microdialysis systems with pneumatically assisted ESI, as employed in the work shown in ***paper III*** (Fig. 12), an analysis time of approximately 6 min was achieved. The minimum cycle time obtained with the automated chip nano-ESI system was 1.1 minutes (0.8 min robot time + 0.3 min data acquisition). With the present software, it should be possible to further reduce the cycle time to 0.8 min (0.5 min robot time + 0.3 min acquisition). This means that approximately 600 samples can be analyzed per 8 hours working day, which is adequate for early screening work.

7.2.3. H-FABP ligand screening

FABP's is a group of cytoplasmic (M_w=14-16 kDa) proteins that have a widespread distribution in tissue. The primary function of FABP's is intracellular fatty acid transport and storage.[166] Fatty acids bind to FABP's in both a hydrophobic and electrostatic manner. Two of these proteins were used in this study, A-FABP (adipose) and H-FABP (heart).

The interaction between A-FABP and a number of potential ligands could not be detected when pulled glass capillaries were utilized, but the complexes could easily be evaluated when using the automated nano-ESI chip based system (part of the data is shown in ***paper II***). The observed lower sensitivity when using the pulled glass needles was probably due to a formation of undesired metal adduct ions.

Figure 9 shows the results of noncovalent interaction between H-FABP and potential ligands. The mass spectrum of the solution of the protein alone (P) is shown in Fig. 9A. Not only is the protein peak appearing, but there is also a response from the protein in association with a natural ligand as well as with acetic acid. Acetic acid is normally not a strong binder but due to the high concentration of ammonium acetate (10 mM) in the sample a strong signal is obtained. The natural ligand to H-FABP is not known to our knowledge, but a peak with an increment between the complex and free protein of 256 Da can be noted. Because fatty acids are known ligands to FABP's, palmitic acid, having a molecular mass of 256 Da is suggested as a possible candidate. This compound has a relatively high binding affinity to FABP's.

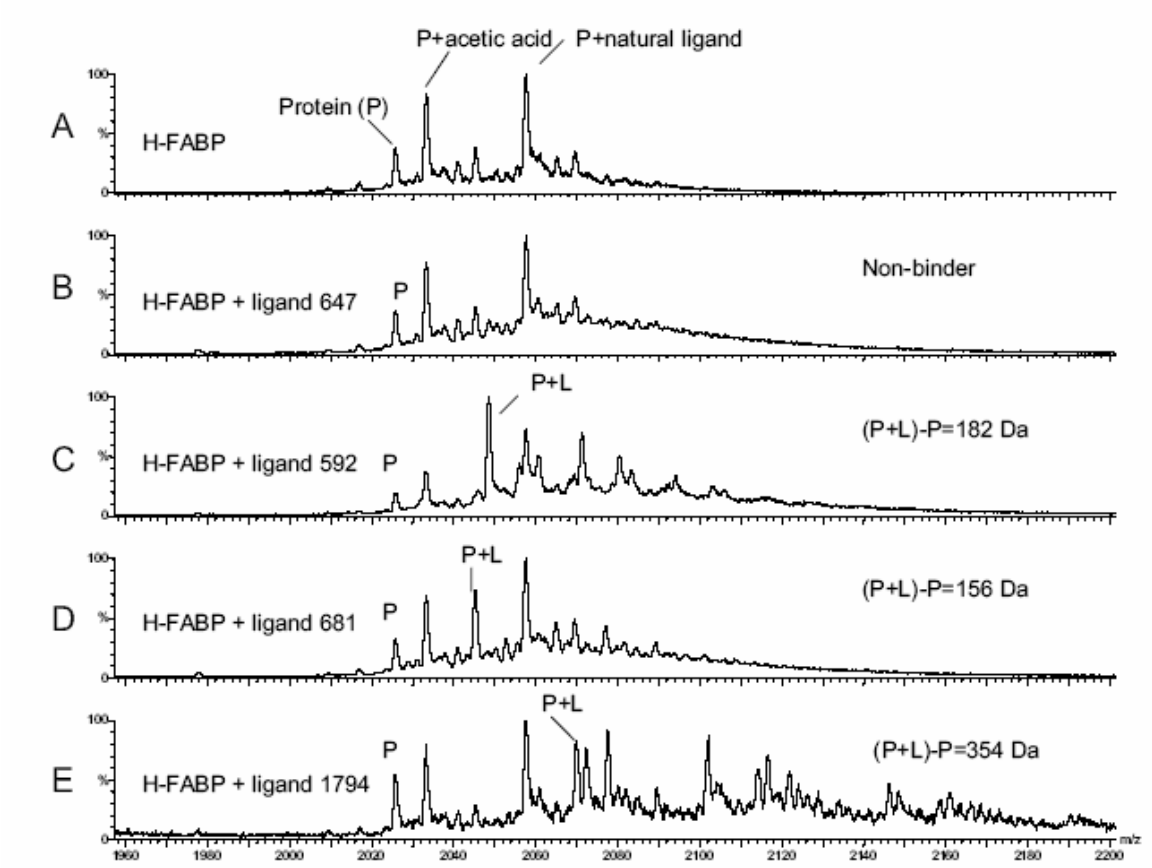

Figure 9. Spectrum A shows H-FABP(23 µM) without ligand. Spectra B-E (blowup of charge state +7) shows potential ligands to H-FABP. P = m/z of H-FABP and P + L = m/z of complex. (P + L) – P = molecular mass of the ligand. H-FABP, fatty acid binding protein (heart).

Figure 9B, shows the same pattern as 9A, indicating a non-binder whereas 9C-E clearly shows association of the added ligands with the target, which provides a new peak labeled P+L. The increments between the free protein peak and those of the complexes correspond very well with the theoretical molecular masses of the respectively ligands (M_w=182, 155 and 354 Da). In competitive binding studies, it is an important issue to verify that the new ligand binds at the same site as the displaced ligand. As mentioned above, a natural ligand is bound to the H-FABP target, which could be good indicator that the added ligands bind to the same active site. All the binders in Fig. 9C-D displaced the natural ligand partially, especially the ligand, shown in Fig. 9C. The new complex peak is the most abundant peak in the spectrum, while the response of the natural ligand complex has decreased. This is an indication of a strong competitive displacement. In these experiments the ligands were added in amounts, 3-4 times in excess compared with the target. In a retro perspective view, the concentration of ligand could have been higher to obtain a clearer recognition of the displacement effect, but the reason for the chosen concentration was to avoid non-specific binding.

7.2.4. MS versus NMR screening

To verify the utility of the nanoESI screening approach, a comparison was made with a recent published work using NMR where the same set of ligands had been employed. Van Dongen *et al.*[167] introduced a concept called structure based screening consisting of the following three steps: 1. A generic binding assay, 2. An affininity ranking by SAR, 3. A chemical optimization of ligands with respect to affinity and selectivity. This approach correlated very well with data obtained by fluorescence displacement determinations in order to establish the IC_{50} values of the ligands. As shown in Table 1, the MS and NMR methods yield similar results except for the ligand 2500, which was identified as a hit in NMR but not in MS. It could be a false positive hit in NMR because it is not an acid, or it could be a nonspecific binder. Alternatively, this may reflect a different affinity cutoff between the two methods.

		H-FABP			A-FABP		
Ligand	$M_{w\ (theor.)}$ (Da)	MS hit	ΔM (Da)	NMR hit	MS Hit	ΔM (Da)	NMR hit
293	200	No		No	No		No
592	182	Yes[a]	0	Yes	Yes[a]	0	Yes
596	153	No		No	No		No
601	174	No		No	No		No
647	123	No		No	No		No
663	147	No		No	No		No
681	155	Yes	+1	Yes	Yes	0	Yes
1379	154	No		No	No		No
1794	354	Yes	0	Yes	Yes	0	Yes
1892	138	Yes	0	Yes	Yes	-2	Yes
2281	163	No		No	No		No
2396	151	Yes[b]		Yes	Yes[b]		Yes
2497	172	Yes	0	Yes	Yes	0	Yes
2500	142	No		Yes	No		Yes
2506	172	Yes	-1	Yes	Yes	-3	Yes
3081	182	Yes	0	Yes	Yes[a]	-1	Yes
3084	202	Yes[a]	-2	Yes	Yes	-1	Yes
3559	197	No		No	No		No
4033	183	Yes[a]	-1	Yes	Yes[a]	-2	Yes
4438	189	Yes[a]	0	Yes	Yes[a]	-2	Yes
4465	225	No		No	No		No
BVT.1960	166	Yes	0	Yes	Yes	0	Yes
BVT.1961	208	Yes[a]	0	Yes	Yes[a]	0	Yes
Compound mixture	168	Yes[a]	+1	Yes	Yes[a]	-3	Yes

Table 1. Comparison of ligand binding between NMR and MS Screening. a. Potentially strong binder. b. Very weak binder.

The above NMR screening method is relative slow compared to the MS screening approach. The generic binding assay step takes approximately 60 min which is 50 times slower than MS for a single compound. Although compound mixtures have successfully been analyzed both in NMR and with our MS approach, the MS provided still 5 times faster analysis.

7.2.5. Summary of the results

- A fully automated nanoESI-MS method was developed for the screening of intact gas-phase noncovalent complexes of A-FABP and H-FABP with potential drug candidates.
- A-FABP complexes that were not detected by conventional pulled glass capillaries were detected using the NanoMate 100 robot and an ESI Chip.
- Results from this work correlated well with NMR screens of the same ligand library.
- The method developed has the capacity to screen 2200 samples/week (1.1 min/sample), in single sample mode, combined with low protein consumption.

7.2.6. Conclusions

The automated nano-ESI/MS method offers a new platform for compound library screening with improved sensitivity. Automated nano-ESI/MS should have a great potential to serve as a complementary screening method to conventional HTS. Alternatively, it could be used as a first screening method in an early phase of drug development programs where only small amounts of the purified target are available. The results regarding the similarity of the NMR and MS screening data are very encouraging but it remains to be seen if to what extent the MS method is generic.

7.3. Paper III: On-line Microdialysis for Enhanced Resolution and Sensitivity During Electrospray Mass Spectrometry of Non-covalent Complexes and Competitive Binding Studies.

The aim of this study was to demonstrate the advantage of using on-line microdialysis as a tool for enhanced resolution and sensitivity during detection of noncovalent interactions and competitive binding studies by ESI-MS.

7.3.1. Adduct ion formation

Many proteins and other biomolecules easily form metal adduct ions which impairs their analysis by MS. The most common adducts obtained with mass spectrometric analyses during non-covalent studies (at physiological pH) in addition to proton adducts are due to sodium ions, which are present in many buffers employed. Furthermore, glass laboratory utensils are another source for contamination of proteins with sodium ions. The formation of sodium ion adducts makes the mass spectrometric analyses more difficult due to decreased sensitivity, difficulties to recognize the molecular mass and irreproducible results. The formation of undesirable adduct ions during the ionization process can be reduced by optimization of ion-source parameters such as the ion-source temperature and the drying gas temperature. Moreover, the kinetic energy of the ions in the ion-source enhances declustering and charge stripping by collisions with gas molecules. However, optimization of these parameters has to be compromised between sensitivity and the amount of adduct ions. Unfortunately, these actions are not always effective to remove trace levels of adduct ions, as observed in ESI-MS.

7.3.2. Sample clean-up by microdialysis

Liu *et al* constructed a microdialysis device that involved two gluing steps to fix a semipermeable fiber with epoxy into T-unions.[168] It has been shown by others that epoxy when in contact with a liquid flow may give raise to slow bleeding of contaminates that can interfere with the analyte.[169] To avoid this as well as the demanding gluing steps, a simple procedure, using standard peek sleeves and two stainless steel T-unions was developed. The setup is shown in Figure 10. The microdialysis fiber (A) was inserted into a PEEK sleeve (D), which was inserted into a short PEEK tubing (C) and tightened by a ferrule and a nut into the Swagelok union (B).

Between the T-unions a 12.5 cm PEEK tubing with an inner diameter of 0.04" was mounted. The space between the fiber and this PEEK tubing served as a channel for the dialysis buffer.

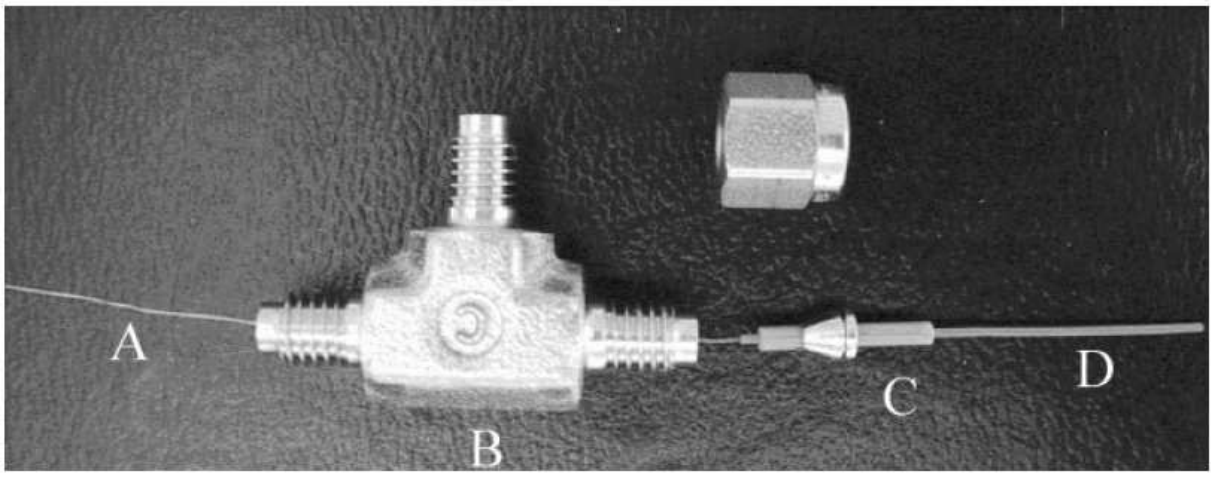

Figure 10. Photograph showing the mounting arrangement of the modified microdialysis device. (A) Dialysis fiber; (B) stainless steel T-union; (C) PEEK sleeve; and (D) PEEK tubing.

7.3.3. Device characteristics

Two devices with dialysis fibers were employed in this study, the commercially available CMA/20 and the modified device. The properties of these devices are presented in Table 2. The commercially available fiber CMA/20 is normally used for in-vivo determination of peptides and other endogenous compounds in living animals. The device is used to sample the analytes inside a tissue and to remove salts, which enhances the sensitivity of the MS analyses.[170] The fiber volume of the commercial device is much less compared to the modified device, 0.8 μL (10 mm in length) compared to 5 μL (160 mm), but the total volume is approximately the same (5-6 μL). Both devices were used with a mobile phase flow rate at 5 μL/min. There are many parameters that affect the dialysis process but the most important issues are the buffer concentration and the linear velocity through the fiber. It was possible to obtain an almost equal desalting effect with the short fiber compared with the effect obtained with the long fiber but the concentration of the NH_4OAc buffer had to be increased from 10 mM to 100-200 mM. Furthermore, the linear velocity in the fibers is different. A low velocity is advantageous for the desalting effect. On the other hand, a low velocity will lead to an increased loss of compounds with a molecular mass lower than the cut off for the membrane.

Device	Fiber Length (mm)	Fiber volume (μL)	Linear velocity in fiber (mm/s)	Dead volume (μL)	Fiber material	M_w cut off (Da)
Modified	160	5.0	2.7	< 1	Regen. cellulose	13 000
CMA/20	10	0.8	1.1	4.4	Polycarbonate	20 000

Table 2. Properties of the two microdialysis devices.

7.3.4. Demonstration of the desalting power

The interaction between Myoglobin (Myo) and cyanide (CN) were used as a model system to demonstrate the desalting efficiency for a complex with low molecular mass ligand. The modified device was used with a dialysis buffer flow (0.3 mL/min) opposite in direction to that of the mobile phase flow. As shown in Figure 11, the effectiveness of the sample clean-up is evident. In the spectrum from the non-dialyzed sample, the signal from the myoglobin-CN adduct was completely unresolved from the signal of the sodium adduct ions, whereas the myoglobin-CN adduct peak was easily detected in the dialyzed sample. Not only sodium ions are removed, but also the concentration of other salts, small molecules and unknown impurities are minimized as well, leading to a spectrum dominated by proton adducts which results in a considerable increase in signal to noise ratio.

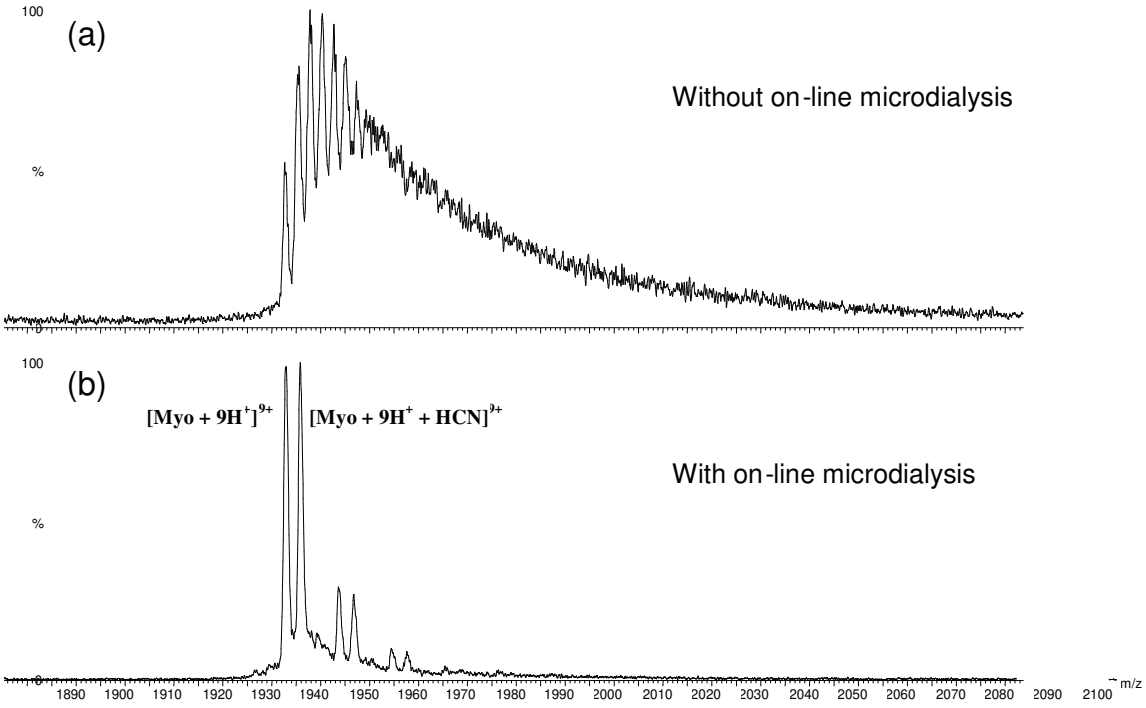

Figure 11. ESI mass spectra of horse heart myoglobin (27 μM) with NaCN solution added to give a final concentration of 0.05 μM. (a) Without on-line microdialysis, and (b) with on-line microdialysis.

7.3.5. Competitive binding studies

We also utilized the microdialysis approach for competitive binding studies, using ribonuclease. The advantages are: (a) reduced problems with metal adduct ions as well as a reduced interference of salts and other unknown low mass molecules in the sample, (b) reduced problems arising from interactions between ligands, such as complex formation or precipitation. The long fiber that was used in the previous experiments (Fig 11) was replaced by a concentric model to offer a more convenient way to administer the competitive ligand through the dialysis buffer (Figure 12). Administering of a competitive ligand via the dialysis buffer pump using the modified device was tested, but the MS response decreased dramatically compared with the results obtained with the short fiber device. It is likely that the MS signal was quenched by the excess of the ligand in the sample.

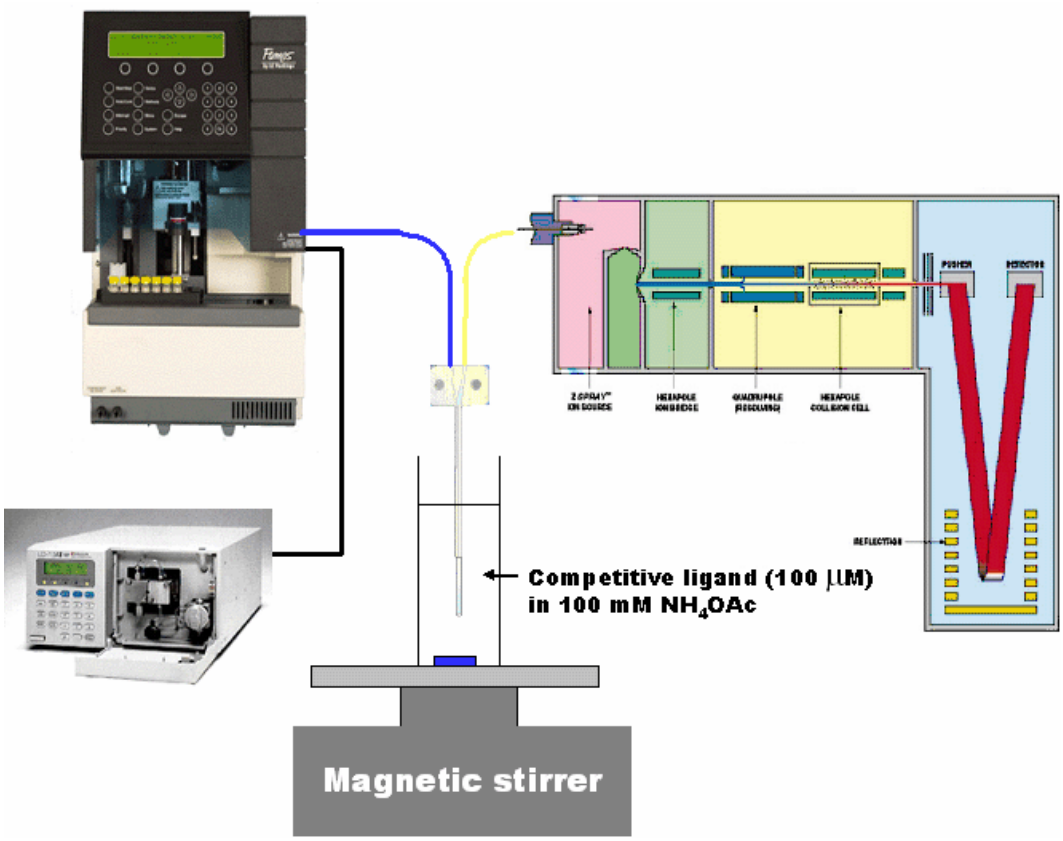

Figure 12. Schematic setup showing the analytical system for determination of noncovalent complexes using microdialysis combined with ESI-MS. The core of the system consists of the CMA/20 probe immersed in desalting buffer and ligand.

Ribonuclease (RNase) is a globular protein that catalyzes the hydrolysis of single stranded acids (RNA).[171] Known ligands are phosphorylated nucleotides like cytidine-2-monophosphate (C2MP) and cytidine triphosphate (CTP).[172] A solution of RNase was analyzed without a ligand added (only NH_4OAc in the dialysis buffer), see the result in Figure 13a. Next, the noncovalent complex between RNAse and CTP was formed in the sample vial prior to injection into the dialysis fiber and analyzed using pure dialysis buffer. The result is shown in Fig. 13b. Finally, the complex was injected into the microdialysis system, where a competitive ligand (C2MP) was administrated in the dialysis buffer. The original ligand was now partly displaced by the competitive ligand via transport through the wall of the dialysis fiber. The intact complexes could be detected by ESI-MS, as shown in Fig. 13c. The molecular cut off of the dialysis probe was 20 000 Da which means that some of the protein (M_W~17 600 Da) and ligand 1 were lost through the fiber. But since the ligand always was present in excess, which means that there was always a sufficient amount of ligand present inside the short fiber to result in complex formation.

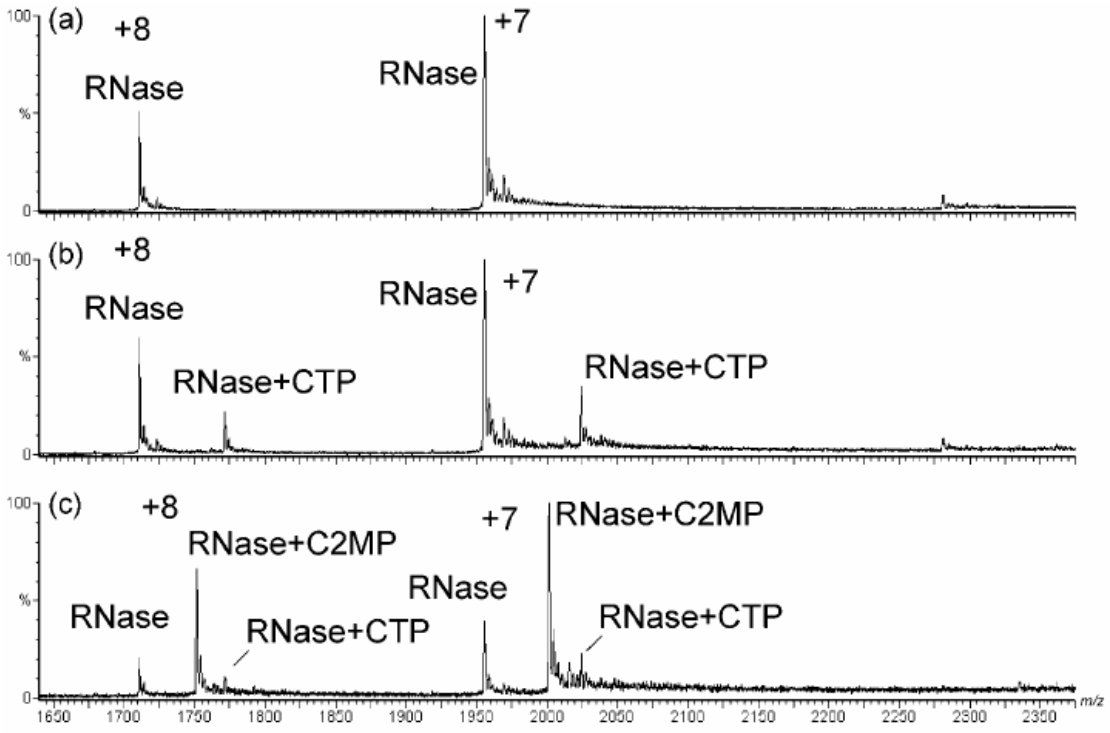

Figure 13. ESI mass spectra of RNase using on-line microdialysis. (a) RNase alone (32 µM); (b) RNase (32 µM) and CTP (50 µM), mixed prior to injection in a sample vial; and (c) same as (b) but with the displacement ligand C2MP (100 µM) administered in the dialysis buffer.

7.3.6. Conclusions

The on-line microdialysis devices clearly demonstrated, that enhanced sensitivity (approximately 5 times) and resolution can be obtained. The microdialysis concept opens many possibilities to study the dynamics of a macromolecule or of a noncovalent complex. The conditions outside the fiber can be changed continuously whereby corresponding changes will occur inside the fiber. Titration curves could be obtained if the concentration of a competitive ligand is changed during the experiment. Such data might be useful to determine dissociation constants of ligands. However, it can be expected that this is not straightforward, due to the different equilibriums of protein and ligands present, both inside and outside the membrane. Not only a competitive ligand can be administrated through the fiber, but also other parameters could be changed, for example: pH, temperature, buffer concentration, buffer salts, content of organic solvent, addition of D_2O for H/D studies etc.

7.4. Paper IV: Electrospray Ionization Mass Spectrometry as a Tool for Determination of Drug Binding Sites to Human Serum Albumin by Noncovalent Interaction.

In ***paper II,*** we performed ligand screening and in ***paper III,*** we utilized competitive binding experiments to ensure that a ligand was bound to the active site of a target protein. In addition to these applications, MS can be used to determine if and where drugs bind noncovalently to off-target proteins,[58] such as HSA. This paper describes an evaluation of a novel approach to determine the 'off-target' binding site on HSA using noncovalent studies detected by nanoESI-MS.

7.4.1. Human serum albumin (HSA)

Human serum albumin (M_w ~66.5 kDa) is the most abundant blood plasma protein and plays an important rule in the transport of both endogenous and exogenous compounds.[173] Reversible noncovalent binding to HSA controls the active concentration of a drug and provides a reservoir for a long-term action, which may affect the pharmacokinetic, pharmacodynamic and toxicological properties of a drug. The HSA molecule consists of three tandemly-fused domains, where each domain consists of a ~200 amino acid folded polypeptide.[174] There are six main binding sites located in the HSA structure that account for most of the ligand binding.[175] Site I binds compounds like warfarin and azapropazone, site II has a high affinity for indole and benzodiazepine-like compounds, site III binds digitoxin, biliary acids and aspirin, site IV binds billirubin, site V binds fatty acids and finally site VI binds metal ions, see Table 3.[176]

Binding site	Amino acid involved	Known ligands
I	Trp 214	Azapropazone and warfarin
II	Arg 145, His 146, Lys 195 region 220-320	Indol and benzodiazepines
III	Lys 199	Digitoxin, aspirin, biliary acids
IV	Lys 240	Bilirubin
V	Residues 422 and 347-351	Fatty acids
VI	His 3	Metallic ions

Table 3. A summary describing the respective key amino acids and known ligands for the various binding sites in intact HSA,compiled from Hervé *et al.*[176]

The target of our study was HSA domain II, since the most important binding sites for low molecular weight drugs are located within this domain.[176] The recombinant HSA domain II (HSA-II) used in this study consisted of amino acids 189-385 (M_w~22 470 Da) from intact HSA. The ligands warfarin, iopanoic acid and digitoxin were chosen as probes for binding sites I-III.

7.4.2. Drug protein binding

Many drugs are strongly bound to plasma proteins. For such drugs, only the unbound fraction of the total amount in plasma is available for diffusion out of the vascular system towards sites where a pharmacological activity can be exerted. Thus, a much higher dose of a drug that is strongly protein-bound has to be administered compared to a drug that is less bound, to reach an equivalent concentration of the unbound fraction in plasma. Many drugs have a very high protein binding level (> 95-99%) leading to a reduced pharmacological action, as well as slow clearance and elimination. During the drug development process it is of great importance to fully characterize the binding properties of new drugs to look for low plasma protein binding properties. In this context, it is also important to consider possible drug-drug competition, since few high affinity sites (in our case of HSA) may cause competitive binding leading to unexpected concentrations effects for a particular drug.

7.4.3. Competitive binding

Our approach for determination of binding sites is based on competitive binding. This procedure has been utilized in a number of reports, using different techniques mostly based on fluorescent probes.[177] A prerequisite is that one of the ligands, either the competitive ligand or the displaced ligand, has fluorescent properties. In some cases the ligands display a native fluorescence. If not, modification of the ligands has to be performed. However, the modification of the ligands may alter the binding properties between the ligand and the protein. Therefore, the use of ESI-MS may offer an advantage.

7.4.4. Results

Figure 14 shows the series of experiments needed to determine the binding site to HSA for a ligand (compound A). At first, compound A with an unknown binding site was incubated with the target protein (Fig. 14A) followed by addition of the site-specific probes, warfarin, iopanoic acid and digitoxin, one at a time (Fig. 14B-D). If the ligand (drug) is bound to one of the three main binding sites it will be partially displaced, as depicted in Figure 14B. The level of drug displacement was determined by comparison of the first protein-drug complex peak ($P+L_1$) and the free protein peak (P) ratio before and after addition of the site-specific probes. The addition of the first site-specific probe, warfarin, to the protein-drug complex resulted in a displacement of approximately 50% of the HSA-II/compound A - peak (at m/z 2544). Simultaneously, a peak corresponding to the HSA-II/warfarin complex appeared at m/z 2537 ($P+L_2$). Addition of the site II or III probes to the complex showed a slight decrease in response for the HSA-II/compound A - peak, but this decrease was significantly less (11 and 13% for site II and III, respectively) compared to the case of the warfarin addition. Compound B showed similar binding properties as compound A. Warfarin acted as a competitive ligand but not the other two probes, see Figure 15(A-D). The data for naproxen and glyburide show binding to both sites I and II, (see Figures 16 and 17). Data presented by other groups confirm the same findings regarding the behavior of naproxen.[178] The overall results for the four drugs are summarized in Table 4.

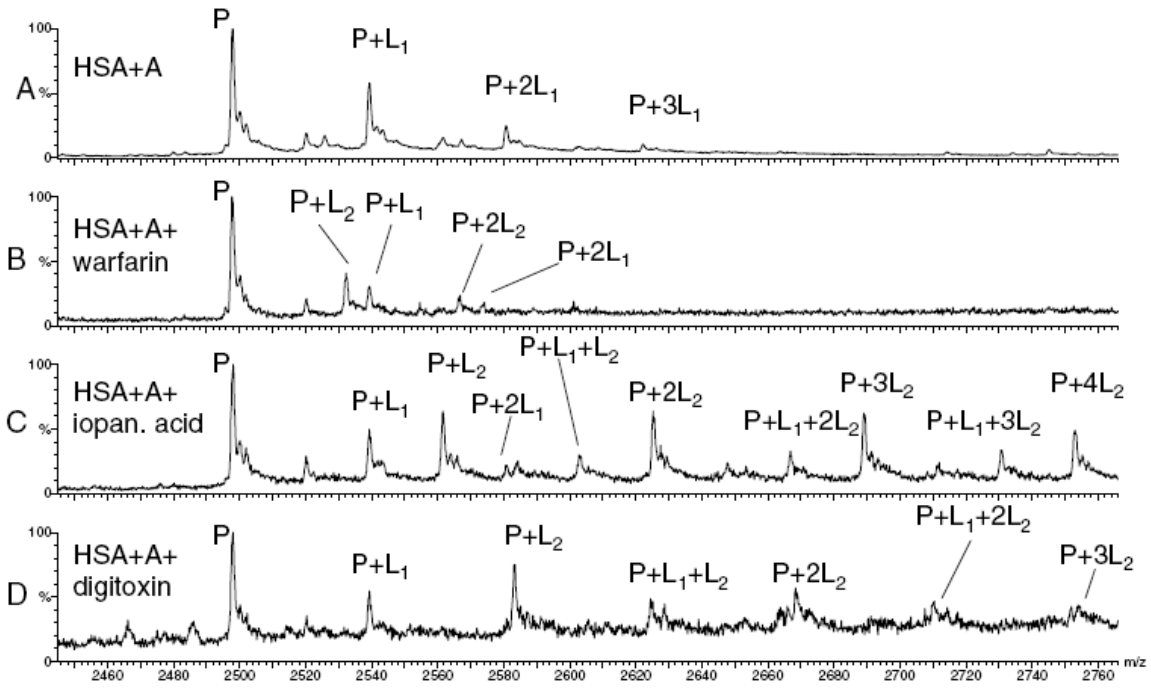

Figure 14. (A) ESI mass spectrum (charge state +9 only) obtained when compound A was incubated with HSA-II (29:247 μM). (B–D) Spectra showing the results of addition of the site-specific probes for binding sites I–III to the sample used to obtain spectrum (A). The concentrations of the species (HSA-II/compound A/site-specific ligand) in (B), (C) and (D) were (23:198:80 μM), (19:165:134 μM), and (23:198:106 μM), respectively.

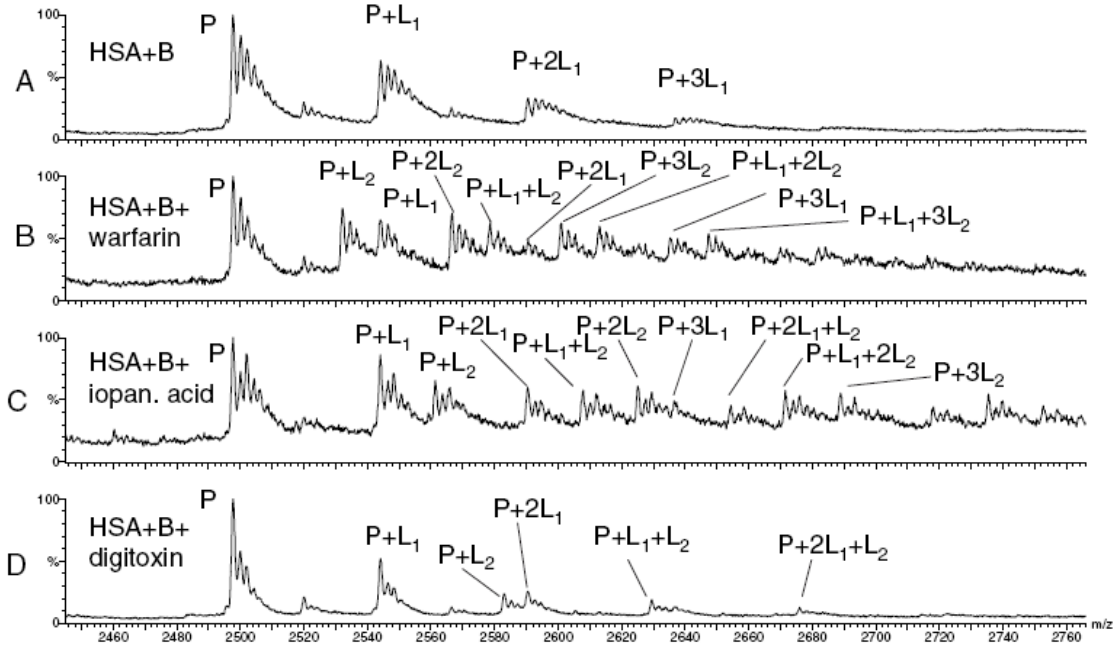

Figure 15. Spectrum 2(A) shows the spectrum obtained when compound B was incubated with HSA-II (28:120 μM). Spectra 2(B-D) show the resultant spectra after addition of the site-specific probes for binding sites I-III to the sample depicted in spectrum 2(A). The concentration of the species (HSA-II:compound B:site-specific ligand) in (B), (C) and (D) were: (28:120:50 μM), (28:120:98 μM), (28:120:65 μM), respectively.

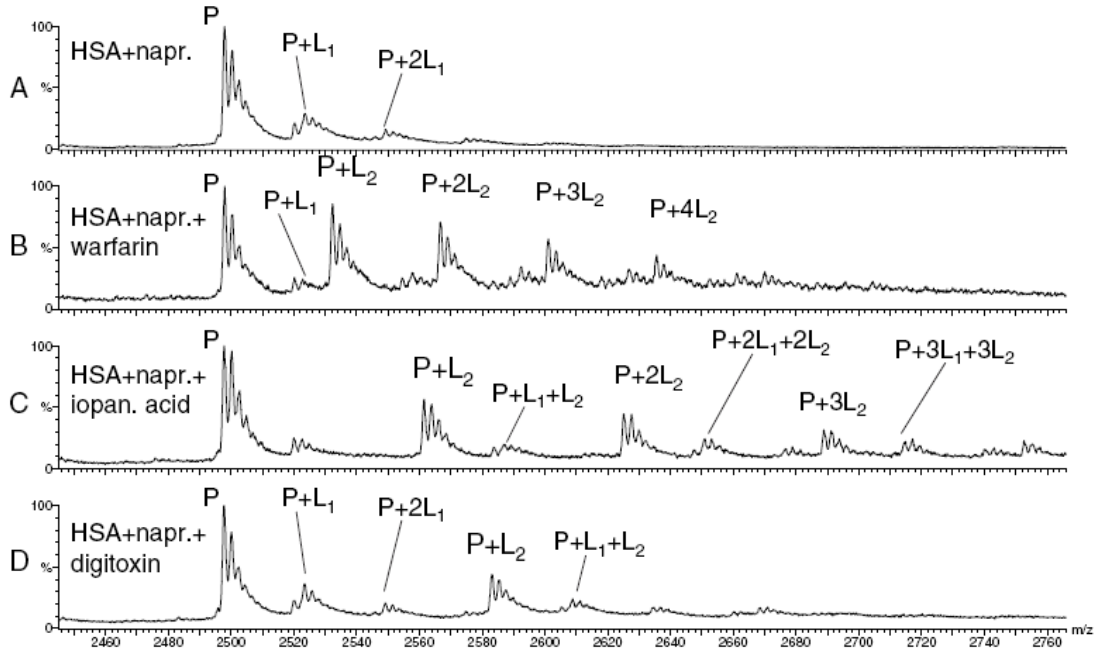

Figure 16. Spectrum 3(A) shows the spectrum obtained when naproxen was incubated with HSA-II (43:274 μM). Spectra 3(B-D) show the resultant spectra after addition of the site-specific probes for binding sites I-III to the sample showed in spectrum 3(A). The concentrations of the species (HSA-II:naproxen:site-specific ligand) in (B), (C) and (D) were: (28:183:72 μM), (28:183:150 μM), (28:183:100 μM), respectively.

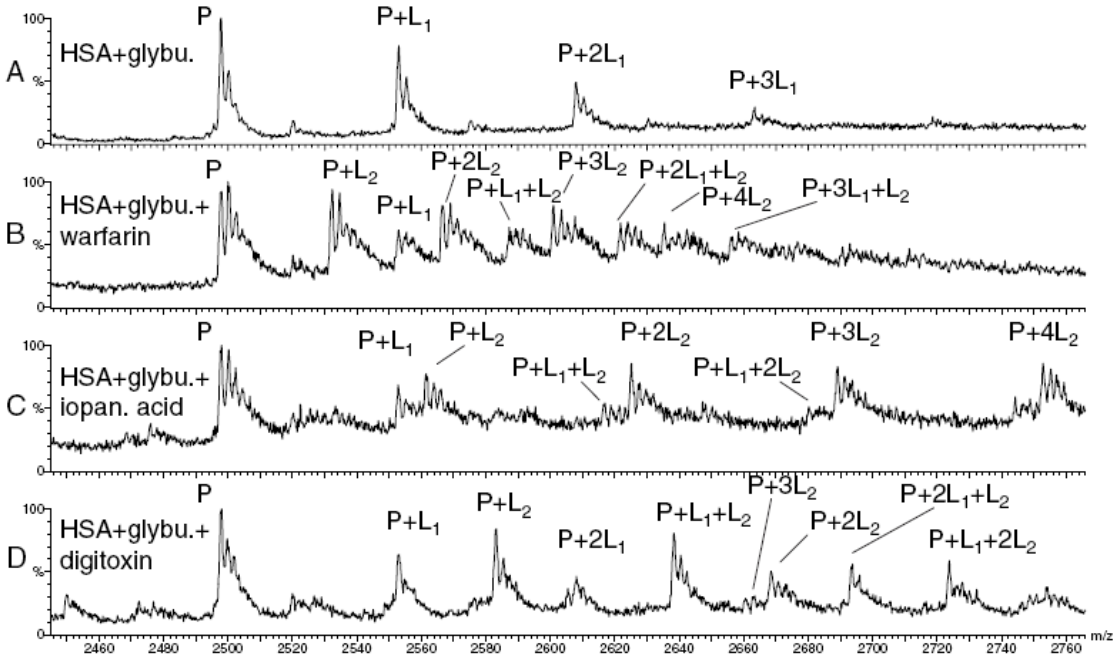

Figure 17. Spectrum 4(A) shows the spectrum obtained when glyburide was incubated with HSA-II (46:200 μM). Spectra 4(B-D) show the resultant spectra after addition of the site-specific probes for binding sites I-III to the sample depicted in spectrum 4(A). The concentration of the species (HSA-II:glyburide:site-specific ligand) in (B), (C) and (D) were: (23:100:42 μM), (23:100:80 μM), (23:100:54 μM), respectively.

Drug	Site I (warfarin)	Site II (iopanoic acid)	Site III (digitoxin)
Comp. A	0.48	0.89	0.87
Comp. B	0.68	1.15	0.82
Naproxen	0.35	0.51	1.00
Glyburide	0.38	0.53	0.79

Table 4. The potential binding location of drugs determined by competitive binding with site-specific probes. The numbers show the ratio of the protein-drug complex peak ($P+L_1$)/free protein peak after addition of the site-specific probes (L_2) compared with the ($P+ L_1$)/free protein peak ratio with only the drug present.

As shown in Table 4, it is clear that warfarin partially displaced all ligands from binding site I. Site I has a preference for compounds consisting of dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge located in the middle of the molecule.[179] Furthermore, the binding pocket seems to be large and flexible and binds compounds with very different chemical structures.[177] This may explain why all the drugs were bound to the site. Naproxen and glyburide were displaced by iopanoic acid from site II. It has been shown that many compounds, including naproxen, have affinity to multiple binding sites.[178, 180] Some of the values listed in Table 4 are in the 'gray zone' between displacement or not, e.g., the displacement of compound B and glyburide from site III. As shown in Table 3, the amino acids involved in the binding sites I, II and III are quite close to each other. The reason for vague displacement could be that the binding sites on HSA-II domain are not completely separated in space and may influence each other. Transformation of the present binding data to the native HSA molecule should be possible if the protein folding and other properties do not differ too much. One other factor that may contribute to the uncertainty of some the displacement data is that there are different levels of sodium adduct formation in the different experiments, see spectra in Fig. 15A and 15D.

7.4.5. Conclusions

We believe that the described approach has the potential to provide a first indication of specific binding for drug candidates. This information together with absolute protein binding data is valuable in an early drug discovery process, which can be followed up by more elaborated studies to fully explore the binding properties, e.g. by using X-Ray and/or NMR.

8. FUTURE OUTLOOKS

The work in this thesis has been focused on some applications for detection of noncovalent complexes detected by ESI-MS. The technique is very promising and new applications are published continuously. There are numerous review articles covering the area, which show the scope of use, especially in the drug discovery area [8, 135, 181-197] However, a better understanding of the factors that potentially lead to differences in the complex composition and/or folding properties between solution- and gas phase conditions has to be investigated in depth. As discussed earlier in ***paper I***, one of the main issues is how to maintain the equilibrium of the species composition constant during the desolvation (fission evaporation) of the electrospray droplets. The goal is to 'freeze' the ionization equilibrium and obtain instantaneous formation of gas phase ions. That could be accomplished if it would be possible to use extremely fast lyophilization of very small droplets. Another approach to eliminate the problem with different droplet sizes could be to make the spray through an ultra fine grid inside a capillary which would be an advantage compared to a needle with only one tip where clogging is a common problem. A variant of this theme would be if the ligand binding could be performed in the gas phase (preferably in an ion-trap) as an alternative in solution. In this case all the issues with the equilibrium dynamics during ion evaporation would be minimized. However, association in gas phase will be different compared to solution conditions, depending on which bonding mechanisms are involved, but it could be interesting to try.

It is interesting to note that quite many of the published articles consist of work on determination of dissociation constants of complexes.[63, 198-200] This means that native protein MS has been established as an alternative method for this purpose and that it has been accepted by the research community that complexes more or less are maintained in the gas phase and thereby reflects the solution equilibrium. The folding in the gas phase may not be exactly the same as in solution phase but high enough similarity exists to maintain the basic properties like ligand binding and overall topology (three dimensional- and quaternary structures).

The use of noncovalent interactions detected by ESI-MS in an early stage of the drug discovery process is a very useful approach, as discussed in ***paper II***. The ultimate goal is to have as much information of noncovalent interactions as possible, already in the target evaluation step. In that respect one very appealing application would be if native protein mass spectrometry (FT-MS

maybe combined with SEC) could be used as a tool for investigating the entire noncovalent network of a whole cell content. This would revolutionize the area of functional proteomics.
The combination of MS and two dimensional (2D) gel electrophoresis is a powerful tool in proteomics to identify proteins.[201] An even more mind thrilling project would be if determination of noncovalent interaction could be performed directly with proteins separated on a 2-D-gel using 'attospray' or 'zeptospray' ESI. Most of today's 2D-gels are run in a denaturated environment which means that the proteins are completely unfolded and are therefore not in a biologically active state. However, there are native 2D-gels available.[202, 203] Alternatively, 2D-gel electrophoresis could be exchanged against multidimensional chromatography[201] and combined with Advions Triversa[204] system operating in fraction collection mode followed by nanospray analysis.

Off-target binding (***paper IV)*** of ligands detected by ESI-MS will probably increase in popularity in the drug discovery process. One approach could be if the desired target is mixed with an 'off-target' (DNA, RNA etc) already in the screening process. This would mean that ligands, which bind to the drug target and to the off-targets can be eliminated early in the process (in view of the risk for high toxicity).

It is my belief that ESI analysis of noncovalent complexes will probably be implemented more routinely in the drug discovery process as a support to and/or in exchange for other more established techniques within the next 3-5 years. The reasons are the inherent properties of mass spectrometry; sensitivity, selectivity and speed. However, more elaborated studies concerning the mechanism involved during formation of solvent free gas phase ions for noncovalent complexes have to be performed to make the technique fully implementable.

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11. ERRATA LIST FOR PAPERS I-III

Paper I, page 1062, Figure 2. Should read: “charge state 8” instead of “charge state 8-”

Paper I, page 1063, Figure 3. Should read: “charge state 7” instead of “charge state 7+”

Paper I, page 1063, Figure 4. Should read: “charge state 7” instead of “charge state 7+”

Paper II, page 250, column 2, line 8 from the bottom. Should read: “not occurred” instead of “occurred”

Paper III, page 2057, column 1, line 4 from bottom. Should read: “1 cm” instead of “2 cm”

12. PAPERS I-IV