Chapter 6

Summary and Future perspectives
Summary

Compounds exerting biological activity, i.e. the capability to interact with proteins and receptors present in a living organism, are produced naturally (by animals or plants), but also anthropogenically (e.g., originating from human activity). In both cases, bioactive compounds can modulate, ameliorate or damage physiological functions. Human exposure to bioactive and potentially toxic compounds often happens through the surrounding environment (e.g., water, air or soil), food, medicines, plants and (venomous) animals. Samples exerting bioactivity, in particular natural extracts and venoms, can be great sources of pharmacologically active compounds and play an important role in discovering new drugs. Hence, studying the bioactive and/or toxic potential of samples originating from a variety of sources expands our knowledge and thus may help in reducing exposures to hazardous substances or discovering new drug candidates and treatments. Such studies are of importance in the pharmaceutical, food, environmental and clinical field.

As explained in Chapter 1, unravelling samples with a bioactive/toxic potential can be a long and complicated process. Often, the starting point is to determine the kind of bioactivity a sample exerts and its level of toxicity. The initial tests are performed with the entire, often complex sample, containing a great number of constituents which can be toxic, non-toxic and also pharmacologically active. Also taking in consideration the possible “cooperation” of sample constituents – leading to a synergic or antagonistic activity – general assessment often provides limited information. In order to improve the bioactivity/toxicity testing, analytical techniques which involve separation and fractionation are required to allow faster and more selective assessment of bioactive components of a sample.

Additional challenges in bioactivity/toxicity assessment can arise from limited sample volumes. For instance, pharmacologically interesting venoms and other extracts derived from poisonous animals may be difficult to obtain due to the small size of the respective species (e.g., insects) or the remote environment it inhabits (e.g., deep sea, desert or the Arctic). Such samples require analytical tools able to handle minute samples.

This thesis comprises studies on the development of methodologies for the fast screening of bioactive/toxic compounds in complex samples, such as drug metabolic mixtures and snake venoms. The pursued approaches combine chromatographic separation with both high-resolution fraction collection for bioactivity assessment, and mass spectrometry (MS). The aim of this thesis was to further develop and apply this ‘nanofractionation’ concept to screen for potent inhibitors of cytochrome P450 enzymes (which are involved in drug metabolism), and toxic proteolytic enzymes, such as metalloproteinases (SVMPs) and serine proteases (SVSPs) present in snake venoms. Another aim of this thesis was to miniaturize the existing nanofractionation platform and the bioassay format, in order to allow analysis of samples
available only in minute quantities. For that, fractionation of the effluent of nano liquid chromatography (nanoLC) on microscopic glass slides was examined. Considering the need for extended selectivity, the potential of trapped ion mobility spectrometry (TIMS) was investigated as well, including its possibilities for separation of isomeric and isobaric compounds.

Chapter 2 describes the development of a nanofractionation platform that comprises a chromatographic separation, UV detection and parallel fractionation and MS analysis for the detection and identification of potent Cytochrome P4501A2 (CYP1A2) inhibitors present in Phase I metabolic mixtures. CYP1A2 enzyme was selected for this study because it is known to be one of the most important enzymes taking part in drug biotransformation (drug metabolism) in the human body [1]. With the nanofractionation system, an in vitro generated metabolic mixture of ellipticine (a potent CYP1A2 inhibitor) was first separated by liquid chromatography (LC), and next, the column effluent was on-line split into two portions of 90% and 10%. The former part was collected onto a 384-well plate with the use of an in-house made fraction collector. The latter part of the effluent was directed to a mass spectrometer for a chemical analysis. After freeze-drying, the collected fractions were exposed to a bioassay based on the conversion of a fluorogenic substrate to its fluorescent product by CYP1A2. Decrease in the conversion of the substrate was indicative of enzyme inhibition. The bioactivity results were presented in a form of a bioassay chromatogram. Collection of 6-s fractions maintained the resolution achieved during the chromatography, which enabled a straightforward correlation of the bioactivity data with the in parallel obtained MS data. Considering the peak shape and retention time of the peaks observed in the bioactivity chromatogram and MS data, drug metabolites of ellipticine with potent inhibition toward CYP1A2 were detected and identified. Validation of the method was performed with five potent inhibitors of CYP1A2, viz. ellipticine, 9-hydroxyellipticine, alphanaphthoflavone, rutaecarpine and fluvoxamine. For all compounds the inhibitory potential (IC50 values) toward CYP1A2 determined with the new method were in agreement with values obtained using conventional methods and those reported in literature. The strength of the method lies in the ability to perform bioassays that require long incubation times. These are needed, for example, to study drugs and their metabolites exhibiting time dependent inhibition (TDI). In the presented research, the aim was to develop a rapid screening method, and therefore the pre-incubation step, that is necessary to correctly measure TDI, was omitted. In future studies, a pre-incubation step could be included to fully exploit the developed methodology.

Considering the global burden of snakebites and the many issues associated with the lack of effective snakebite treatments [2], profiling and understanding chemical venom composition can aid in the development of new, toxin-specific envenomation treatments. The specific and potent activity of snake venom constituents toward a number of drug targets present in vital organs, also makes venoms a great source of potential templates for lead compounds for drug
development [3]. Chapter 3 aimed at the development of a screening method for the detection and identification of the key proteolytic enzymes responsible for the hemorrhagic effect of snake venom, i.e. SVMPs and SVSPs. Simultaneously, the method was applied for the detection and identification of compounds with potential pharmacological activity: inhibitors of plasmin, a key enzyme in the fibrinolytic process. Crude venoms were first separated with reversed phase (RP) LC, and next using a flow split, a parallel collection of 6-s fractions of the effluent onto a black 384-well plate and MS analysis was performed. The evaporated fractions of the effluent were then exposed to a fluorescence-based bioassay. The bioassay was based on the enzymatic conversion a fluorogenic substrate to its fluorescent product by plasmin. Lack of conversion to the fluorescent products was indicative of plasmin inhibition and the presence of antiplasmin activities. An induced conversion of the substrate was observed when a sample contained proteolytic enzymes, such as SVMPs or SVSPs. The bioassay data were presented as bioactivity chromatograms which were correlated to obtained MS data for chemical identification. By performing two bioassays on a well-plate containing fractions of a single venom sample (one injection), a higher throughput of the method was achieved. In addition, a bioactivity-guided proteomics approach was used to identify bioactive peptides and proteins. The presented method aided in the differentiation between zinc- and calcium-dependent SVMPs and facilitated the identification of bioactive compounds. The usefulness of the methodology was demonstrated by analyzing venoms of the snakes Daboia russellii (Dr) and Crotalus basiliscus (Cb). In Dr venom, plasmin inhibitors in a mass range of the Kunitz-type serine proteases were detected and identified. In Cb venom, enzymes exerting fibrin(ogen)olytic activities similar to plasmin, were found. The overall method was shown to be robust and high-throughput.

Methods to screen for bioactive/toxic compounds present in complex mixtures such as natural extracts or venoms often are limited to samples that are available in relatively large quantities, allowing injection volumes of ~50 μL. The requirement of such large sample volume can disqualify a great number of venoms and other animal extracts to be studied for their bioactivity and toxicity. In order to tackle this problem, a picofractionation platform, which employs a miniaturized version of nanofractionation analytics, was developed (Chapter 4). For the picofractionation system, nanoLC was hyphenated to a high accuracy nano-volume liquid dispenser allowing fractionation of nL/min flow rates on a glass slide with a hydrophobic coating. This system enabled deposition of 10-s fractions of nanoLC effluent in a serpentine fashion generating a microarray of effluent droplets. After drying, the fractions were exposed to a fluorescence-based bioassay that was dispensed in a spot-on-spot fashion using the same high accuracy nano-liquid volume dispenser. A bioassay measuring activity against plasmin that was developed in Chapter 3 was adapted to the microarray format. To prevent bioassay droplet evaporation – which was one of the main difficulties in the miniaturization of the bioassay format – encapsulation with mineral oil was used. Spotted
A microarray bioassay readout was performed kinetically with an in-house modified inverted fluorescence microscope. With the picofractionation platform, the inhibitory potential (IC50) for plasmin’s known inhibitors – aprotinin and leupeptin – were accurately determined. The feasibility of the picofractionation method, that included the separation capabilities, was studied with the analysis of a mixture of leupeptin diastereoisomers, and four snake venom samples. The venoms were screened for the presence of compounds with both anti-fibrinolytic (i.e. inhibition of fibrin formation) and fibrinolytic-like (i.e. venom compounds converting the plasmin substrate) properties. A baseline separation of the two diastereoisomers of leupeptin was obtained allowing both forms to be tested for their bioactivity. In the analyzed snake venoms protease activity was observed, and compounds with both anti-fibrinolytic and fibrinolytic-like properties were detected. In general, the results obtained were in good agreement with available literature.

In Chapter 5, the usefulness of trapped ion mobility spectrometry mass spectrometry (TIMS-MS) for the study of gas-phase ions of fourteen pharmaceutically relevant compounds with a molecular weight ranging from 270 to 645 Da was explored. The group of ions selected for this study differed, for example, in molecular size, shape, and number of rotational bonds. TIMS offers tunable and relatively high resolving power and may separate molecules with very similar collision cross sections (CCSs). Additionally, TIMS analysis is done at low electric field, limiting ion heating and potential fragmentation. In this study TIMS was used to investigate the stated analytes and their adducts with cations (sodium, cesium, silver and lithium) in the gas phase, and find correlations between ion gas-phase behavior and molecular shape/geometry. Such correlations could be useful in compound structural elucidation. Separation of two isomeric compounds, i.e. tetrahydrocannabinol (THC) and cannabidiol (CBD), was also studied.

TIMS analysis showed that the formation of an analyte-metal ion adduct could lead to the appearance of multiple gas-phase ion conformations. It was suggested that this was induced by the presence of several double bonds in the structure of the analytes, as well as the presence of other interaction sites that caused the metal ion to bind at different positions of the molecule. In general, a positive correlation between the CCS of the ion-metal adduct and its size was observed. Flat (planar) and rigid molecules were shown to form dimers more readily than more flexible molecules (containing rotating bonds). This tendency was also observed when studying THC and CBD, where dimer formation occurred mostly for THC, which shows a more rigid structure than CBD. By optimizing the range and speed of the electric field gradient and ramp, respectively, the separation of THC and CBD was achieved by employing metal adduct formation.
Future perspectives

In Chapter 2, a method to screen for potent inhibitors of CYP1A2 was developed. Although the method was shown to successfully assess inhibitory properties of drugs and their metabolites toward CYP1A2, there is opportunity for extension of its applicability. In the metabolic pathway of a drug, often multiple CYP isoforms are involved, requiring multiple bioassays for testing. Considering the high number of compounds to examine – as usually encountered in a drug discovery phase – a “1 sample 1 bioassay” approach will come with a low throughput. Assay multiplexing, as was introduced in Chapter 3, can improve throughput. The picofractionation system (Chapter 4) has the potential to accommodate up to four glass slides (so far, tests were performed with 1 glass slide only). By further decreasing the volume of the collected fractions as well as the volume of the bioassay, a further increase of the throughput can be envisioned, as it will provide the possibility to generate screening panels for a number of targets.

Considering the complexity of the human body, even a biotransformation of single drug can lead to a number of outcomes. As mentioned earlier, it can involve a number of CYP isoforms, but also drug transporters (membrane proteins that are necessary for classes of drugs/xenobiotics to enter and leave a cell [4]). Furthermore, drug metabolism can lead to the generation of metabolites that can possess further inhibitory/inducing properties toward other CYPs/drug transporters, and generation of reactive metabolites that will interact with critical cell receptors and organelles leading to toxicity. Hence, in order to obtain a comprehensive pharmacokinetic, pharmacodynamics and toxicity information on a drug, we foresee the nano- and picofractionation analytics to be potentially used together with more advanced cell bioassays, such as 3D human hepatocyte cultures, advanced liver culture systems (organ-on-a-chip, which include other hepatic cells important for functioning of the liver) and even multi organ (body or human-on-a-chip) systems based on microfluidics technology.

In Chapter 2, one of the difficulties was to assign the hydroxylation site of the in vitro generated hydroxylated metabolites of ellipticine (i.e. structural isomers), as identification based on MS and MS/MS data did not suffice. Considering that the structural isomers differ in their shape and size, future research involving structural assignment of isomeric metabolites should include the use of IMS-MS technology bringing additional selectivity which will aid in their structural elucidation by identifying the exact site of attachment of a functional group (Phase I metabolism) or a conjugate (Phase II metabolism). In addition, an attempt to hyphenate TIMS to the current nanofractionation as well as picofractionation platform should be made. Further confirmation of a metabolite structure should be followed by nuclear magnetic resonance (NMR) method.
In Chapter 5, using IMS-MS technology, small molecular ions in complexes with cations were observed to have multiple gas phase conformations. The ability to detect different gas phase ion conformation will be of great value in the analysis of proteins and peptides (e.g., venoms), where the conformation of a protein is related to its activity. TIMS has already been shown to successfully separate peptides with isobaric post translational modifications (PTMs) [5]. PTM in case of snake venom toxins may influence their biological activity, toxicity and structural stability. Massonnet et al. [6] showed that peptides containing different number of disulfide bridges can be separated and detected with IMS-MS in a fast manner. In addition, they showed that IMS-MS can differentiate peptides containing the same amino acid sequence (isomers), but different cysteine connectivity in their disulfide bridges.

Considering that snakebite envenoming is a tropical neglected disease there is an on-going need to study snake venoms and develop methodologies aiding the development of new treatments for snake bites. Currently, administration of antivenom is the only effective snake bite treatment. However, antivenom production is expensive, and there is a risk for serious side effects (e.g., anaphylactic reactions) and low efficacy (due to the choice of an inappropriate antivenom for treatment). Therefore, other, innovative treatments are needed. The method described in Chapter 3 successfully detected compounds with proteolytic activities, i.e. SVMPs and SVSPs, which are the key toxins responsible for amongst others the devastating hemorrhagic effect of snake envenomation. Knowledge on the bioactivity profile of a venom and detection of the responsible toxins will help in developing effective snake-bite treatments that will be toxin/pathology specific and not venom specific [7]. Further research should therefore aim at expanding the nanofractionation method to search for toxin-specific treatments by exposing already tested venoms to for example compounds with a neutralizing potential against the detected toxins (e.g., small-molecule therapeutics [5]), or to study the effectiveness of existing antivenoms. This could be performed by incubating collected fractions of crude venoms with selected compounds or antivenoms prior to a second bioactivity measurement using the same bioassay. This would give an indication of the neutralizing potential of the agents against the toxins present in the analyzed venom. Of course, the method would be used only as an initial screen providing a fast overview of the effectiveness of new and/or already established snake bite treatments.

The picofractionation platform described in Chapter 4 still requires further development. These include hyphenation to MS, parallel to microarray spotting of the effluent. Following the approach used in the nanofractionation platform, a flow splitter can be implemented, directing a part of the column effluent to the liquid dispenser, and the other part to a mass spectrometer. In an earlier study performed by Heus et al.[8] a split ratio of 50:50 for nanoLC effluent with a flow rate of 400 nL/min was achieved with the use of a low dead volume T-connector. In their study, however, an on-line bioassay was performed. Nevertheless, they proved that such approach is feasible. For further implementation of bioassay multiplexing
performing more than one bioassay on a single sample/glass slide), the addition of in-house developed pressurized containers could be studied. These containers can hold up to 16 vials of different sizes, i.e. 10-mL Greiner tubes and 2-mL and 0.5-mL Eppendorf tubes. These will give the possibility to use several bioassay solutions, cleaning and washing solutions, and other necessary liquids, such as mineral oil, in an automated manner. Additional cooling of the containers would help preventing sample thermodegradation.

As presented in Chapter 4, the picofractionation platform enables deposition of nanoLC effluent on a glass slide with a hydrophobic surface. However, the deposition is not limited to a glass slide. This potentially would allow MALDI-MS analysis on fractions on a MALDI plate. Matrix solution could be applied before or after collection of fractions, enabling co-crystallization. Although the idea of interfacing droplet microfluidics with MALDI-MS is not novel [9], it shows that the picofractionation analytics can be used for a number of applications in the research area of, e.g., drug metabolism, bioactivity screening and proteomics.
References


