Miniaturized bioactivity screening of complex samples

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1. Introduction

1.1. Drugs from nature

The origin of many conventional small-molecule drugs can be traced back to natural products, which have been used for medicinal purposes in the form of roots, vines, bark, and plants since thousands of years [1]. The transition from these “herbal drugs” to modern drugs may be considered to start in 1805, when Sertürner isolated morphine from opium [2]. This is a milestone in modern medicine and drug discovery, as from this moment drugs from natural products could be purified, studied, and administered in a precise dosage [3]. This lead to the isolation of many important drugs such as the antimalarial quinine from *Cinchona* tree bark in 1820 [4]. The next breakthrough took place half a century later with the synthesis and subsequent mass-scale production of salicylic acid by Kolbe and von Heyden (respectively). This decimated the cost of the natural pyretic and ensured a steady supply of a chemically pure product, which before was only available as willow bark extract (*Salix alba*). Modification of this pioneering synthetic natural product led to the synthesis of aspirin in 1897 by Bayer AG, which is generally seen as the starting point of modern drug discovery [5]. Other prominent examples for drugs derived from natural products include the anticonvulsant butylscopolamine [6], the potent anticoagulent hirudin [7], which is a 6.6-kDa peptide isolated from the leech *Hirudo medicinalis*, and paclitaxel [8, 9], an alkaloid isolated from the *Taxus brevifolia*, which is known for its mitosis inhibition and use in cancer therapy. The discovery of penicillin by Fleming led to the isolation and large-scale production of this and subsequently many other antibiotics [10]. As a result, also fungi and other microorganisms were included in natural product screening campaigns.

By 1990, 80% of drugs were either directly generated from natural products or a derivative of a natural product [3]. Since then, the role of natural products in drug discovery declined as pharmaceutical companies largely abandoned their natural-product discovery lines. In part, this had to do with alternative drug discovery strategies such as rational drug design and combinatorial chemistry as well as with advances in X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) [11]. But it had also to do with limitations inherent to natural-product drug discovery; problems related to the complexity of natural extracts, their batch-to-batch variability, and the challenges for large-scale production of the more complex molecules and the fact that natural products often come in small quantities and require labor-intensive purification procedures [12].

However, as the global registration of new drug entities has declined over the last decade, pharmaceutical industries are pursuing new sources for possible drug candidates, which led to a revival of natural-product screening [13, 14].

This revitalized interest is particularly powered by advances in analytical chemistry and biotechnology. The emergence of microfluidics, improved and miniaturized separation technologies, and advanced (hyphenated) detection methodologies such as mass spectrometry (MS) allows for easier characterization of complex natural extracts. Furthermore, developments in the field of NMR, such as increased sensitivity and the possibility of analyzing mixtures, are of great importance to compound elucidation. These advances

shortened the process of dereplication, isolation, and structure elucidation of crude extracts [12].
Another important development in analytical chemistry comprises its integration with automated biochemical assays, which allows natural-extract screening to become a more viable competitor to or more compatible with high-throughput screening. Because of these advances, compounds can be screened from previously unexplored natural products such as venoms from insects, rare snakes or cone snails, which are available in (sub) milligram amounts [15-18]. As an example, cone snail venom, which contains highly specific ion-channel blockers, yielded one of the latest medicines from nature in the form of Ziconotide (Prialt)[19, 20]. Ziconotide is a 25 amino-acid peptide coming from the *Conus magus* snail, which acts as an analgesic by blocking a Voltage-Gated Calcium Channel receptor ($Ca_v2.2$) [21]. As marine organisms, the cone snails actually open a fourth group of sources for natural-product derived drugs, that is next to plants, fungi and venoms.
Peptide-based drugs such as Ziconotide and hirudin differ from traditional (non-protein) natural-product derived drugs. In 2012, there were 67 therapeutic peptides on the market, about 150 in clinical phases, and more than 400 in the pre-clinical development [22]. As such, natural-product derived peptide drugs nicely fit in current developments towards biosimilars and biopharmaceuticals. In principle, next to natural-extract screening, such (potentially) therapeutic peptides and proteins can be discovered via genome mining [23, 24]. Obviously, therapeutic peptides come with many challenges. Current state-of-the-art in peptide chemistry allows the synthesis of peptides up to 80-100 amino acids long [25]. Obviously, for bioactivity, additional issues as appropriate secondary and tertiary structure (folding, disulfide bridges) and possibly post-translational modification are of pivotal importance. In this respect, biosynthesis approaches are of interest including the before mentioned ribosomally synthesized peptides [23], which also enables inclusion of posttranslational modifications which were formally very hard to synthesize [21]. Issues with (oral) bioavailability and stability are currently addressed by chemical modifications (e.g., [26]) and advanced drug delivery strategies [27], including nanoparticles [28].

1.2. Towards this thesis

The research described in this thesis involves further improvements of analytical technologies applicable in the field of natural-extract screening, in order to provide robust, fast and sensitive analytical screening technologies which can better cope with the aforementioned challenges in natural-extract screening. We have undertaken efforts to further miniaturize and integrate analytical technology and bioassays. This includes the development of nano-liquid chromatographic (nano-LC) separation hyphenated to miniaturized screening assays, and the development of miniaturized fractionation approaches for both LC and gas chromatography (GC). The latter is particularly useful when screening targets which demand long incubation times or in cellular assay [29].
Besides for their medicinal use, the characterization of natural compounds may be crucial for unraveling the physiological aspects of the human body. If we take the human central nervous system (CNS) as example, this aspect is illustrated by nerve receptors named after the natural compounds to which they exhibit affinity, such as the opioid receptor, the cannabinoid

receptor, or the nicotinic or muscarinic acetylcholine receptors. Therefore, affinity profiling of compounds derived from plants, fungi, animals and marine organisms may lead to the discovery of either new compounds bioactive towards known CNS receptors or even new CNS receptors and processes. An obvious choice for this type of research would be the analysis of extracts from sources known to contain psychoactive compounds. Recently, several natural compounds, such as trace amines, have also been detected to be endogenously present in the mammal nervous system [30]. These low-molecular-weight trace amines are non-catecholic biogenic amines and are found as trace constituents of various vertebrate and invertebrate tissues. This involves, for example, the serotonin-like tryptamine neurotransmitters *N*,*N*-dimethyltryptamine, which is one of the most powerful hallucinogenic compounds [31-33], and bufotenine [34], found in the skin secretions of the toad *Bufo alvarius*, which exhibits binding to the serotonin receptors. However, their binding to other CNS receptors is largely unexplored.

Not only the low-molecular-weight compounds, but also peptides and proteins may exhibit bioactivity towards CNS receptors. The snake-venom-derived bungarotoxin has proven to be an important tool in the discovery of the α7 nicotinic acetylcholine receptor (nAChR) [35]. Some recent examples for the successful identification of peptidoid natural products include, next to the conopeptide Ziconotide (Prialt) already mentioned, the conopeptide ACV1 (from Metabolic Pharmaceuticals Ltd), which is active against the α9α10-type nAChR [36], and the conopeptide-based drug candidate, xen2174 (from Xenome Ltd), which increases norepinephrine levels in the spinal cord [37]. The therapeutic potential of conopeptides was recently reviewed by Vetter and Lewis [38].

We have developed a robust on-line high-resolution screening (HRS) assay for the α7 nicotinic acetylcholine receptor (nAChR), which is based on a water-soluble analogue acetylcholine binding protein (AChBP) showing enhanced fluorescence with a novel reporter compound DAHBA. We used this HRS platform to screen animal venoms from snakes, predatory snails and toads from which we could identify new natural peptides and alkaloids which have not been previously identified as binders of the neuronal nAChR.

Furthermore, we investigated at-line fractionation technologies both for LC- and GC-based separations of complex mixtures. Hereby, we aimed to apply highly integrated and automated miniaturized analytical techniques with biochemical assays which need (relatively) long incubation times. Additionally, by integrating both LC and GC into these integrated platforms, we hope to make miniaturized at-line screening applicable to both the more polar and (extremely) apolar compounds (which are often investigated in environmental chemistry).

1.3. Screening approaches for natural extracts

Traditionally, natural extracts have been separated using LC and fractionated using large amounts of plant or animal material, up to the kilogram scale. Two famous examples are the isolation of ethinyl estradiol from horse urine and the isolation of insulin from pig pancreas. In the first case, milligrams of ethinyl estradiol were isolated from hundreds of liters of horse urine. For the large-scale production of insulin from pig pancreas, tons of pig pancreas had to be extracted. Clearly, these approaches are not applicable to very scarce plant material and especially not to venoms from rare species and/or small animals, such as cone snails, snakes

or toads. In addition to this, the applied analytical strategy has moved away from the detection and isolation of (any) novel natural compound from a natural extract towards an early identification of bioactive compounds. This approach is known as bioactivity-guided fractionation or effect-directed analysis, depending on the application area [39]. Compounds are separated by HPLC (typically reversed-phase HPLC, RPLC) after which compounds are fractionated based on the chromatogram acquired using ultraviolet or fluorescence detection. These fractions are then subjected to a biochemical assay with the target of interest. Compounds in fractions that show bioactivity, the so-called 'hits', are further structurally characterized using methods such as MS and NMR. In most cases, this screening strategy involves an iterative process, as the (rather large) fractions collected usually contain a (complex) mixture of compounds. Thus, relevant fractions must be further separated and fractionated to pinpoint the actual bioactive compound. Thus, the process is rather time consuming and requires significant amounts of starting material. As an alternative to these fractionation approaches, hyphenated at-line and on-line screening approaches have been developed. Particularly, advances of analytical techniques and their integration into traditional approaches allow the development and application of new concepts for the screening of natural extracts [40]. The main driving force of innovations within this field is the on-line coupling of HPLC and spectroscopic or spectrometric detection techniques like NMR and MS. Advances in the field of separation sciences have enabled the dereplication of active compounds in reasonable amounts of time using minute sample material. In addition, screening approaches are nowadays combined with bioactivity assessment at early stages of the discovery process. This is accomplished by HPLC-based fractionation followed by bioactivity testing of the collected fractions (off-line bioactivity screening). In order to avoid limitations in fractionation speed and fraction size as well as possible sample losses during the fractionation process, more advanced on-line (HRS) and at-line screening techniques have been developed [41]. In these approaches, the bioactivity testing is done in parallel with the structure elucidation process, resulting in a more efficient screening process.

1.3.1. On-line screening approaches

In order to best explain the process of on-line screening, Figure 1 shows a classical setup with a receptor as target protein and fluorescence enhancement as the bioassay principle [40]. A natural extract sample is loaded onto an analytical column at which compounds are separated by RPLC. A two-way split, incorporated into the analytical setup, allows for simultaneous MS and biochemical detection. Typically, ~90% of the eluent is guided towards the MS, while the remaining eluent is subjected to the in-flow homogenous bioassay featuring a (water-soluble) receptor (or enzyme). The receptor protein is infused into the flow path by means of a 'superloop', which basically is a large syringe where the plunger is driven by an LC pump. The separated compounds are allowed to incubate with the receptor in a reaction coil, which consists of a piece of coiled tubing to enhance mixing of separated compounds and the dissolved receptor. The incubation time is determined by the flow rates and the dimensions of the tubing. Next, by another 'superloop', the reporter ligand is infused and mixed with the flow and allowed to interact in another reaction coil. As, in this example, the bioassay is based on fluorescence enhancement, the reporter ligand is a compound of low fluorescence, which upon binding to the target receptor exhibits an enhanced fluorescence signal. However, when

the binding pocket(s) of the target receptor is occupied by a bioactive compound in the LC eluent, fluorescence enhancement does not take place, which manifests itself as a decreased fluorescence signal that is a “negative” peak monitored by the fluorescence detector.

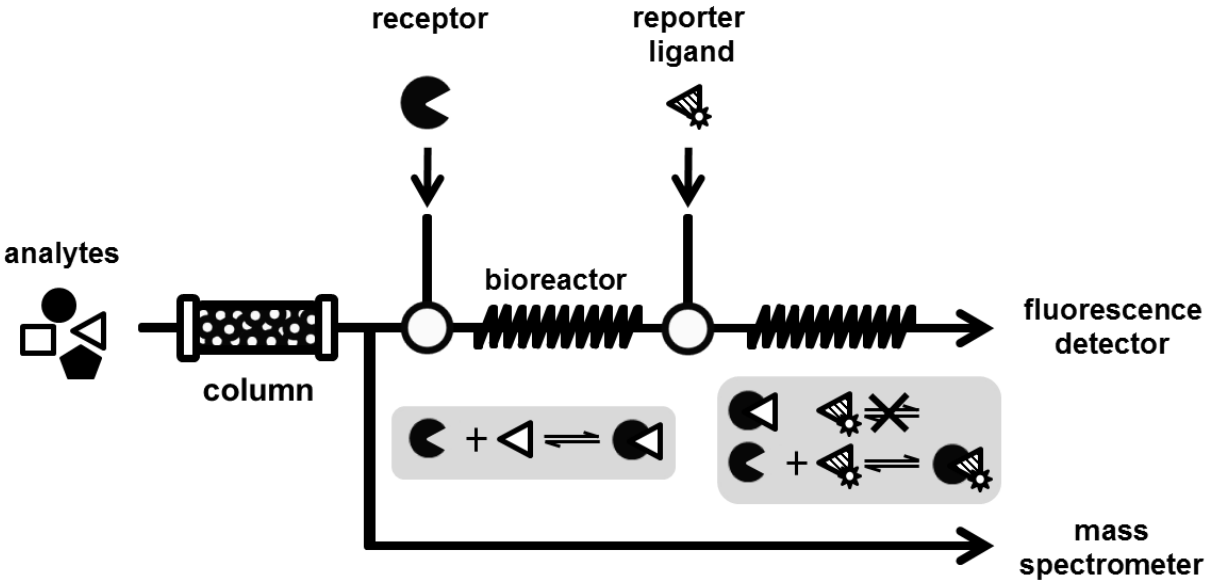

Figure 1. Schematic representation of a typical on-line screening setup for high-resolution screening [40].

The main advantage of this approach is that a single integrated screening platform can be used, which unlike most off-line approaches provides high resolution at the stage of bioactivity assessment. It is therefore that on-line screening has been coined high-resolution screening (HRS). However, there are a number of limitations as well. In order to avoid excessive peak broadening in the post-column reactor, the maximum incubation time is ~3 min per reaction coil. Thus, target selection is restricted to a fast converting enzyme or a receptor with a low ligand dissociation rate (K_{on}). Consequently, cell-based and (gene)reporter-based assays cannot be applied in on-line screening approaches. Furthermore, the receptor is required to be both water-soluble, which rules out membrane-bound target proteins, and stable in solvents with up to 15% of methanol and acetonitrile (solvents which are typically used in mobile phases for HPLC). Another drawback is related to the relatively large consumption of assay constituents and, more importantly, high sample consumption. This is especially relevant when screening samples with limited supplies such as venoms from rare animals or insects. Finally, the log-P range of analytes is restricted to the range of RPLC, as the high organic modifier content of normal-phase and/or hydrophilic interaction chromatography would certainly disturb the bioassay. Even the separation of highly lipophilic compounds by RPLC may require too high concentrations of organic modifier for the bioassay.

Various ways to overcome some of these limitations have been explored in this thesis. These include on-line chip-based bioassays and at-line (micro)fractionation for both HPLC and GC. The developments make the concept of integrated separation, (ligand) identification and bioactivity profiling more applicable to a wider range of analytes and target proteins.

1.3.2. On-line chip-based screening approaches

For some natural extracts, the main limiting factor in on-line screening is the small sample amount available for screening. This especially applies to the screening of venoms from small and/or rare animals. To solve this bottleneck, we scaled down the complete on-line screening platform from the micro- to the nano-scale by the implementation of nano-LC, microfluidics, and sensitive micro-optics [42]. The system was optimized to run at a total flow of 5.4 µL/min. The concept was first demonstrated by nano-LC with UV detection coupled to the microfluidic assay. Later on, a post-column split was implemented to enable on-line MS detection [43]. A schematic diagram of the nano-LC with microfluidic bioassay and parallel MS detection is provided in Figure 2. Sub-µL samples are injected onto a nanobore column operated by a gradient nano-LC system running at 400 nL/min. Like all the integrated screening systems mentioned before, a post-column split divides the effluent between the mass spectrometer and the microfluidic bioassay. In this case, the split ratio was 1:1, that is 200 nL/min of the nano-LC effluent was hyphenated to the mass spectrometer equipped with a nano-electrospray ionization (nano-ESI) source, while the remaining 200 nL/min was introduced to the microfluidic chip containing a 4 µL incubation chamber. There, the column effluent was mixed with a solution containing both the soluble receptor and the fluorescent reporter ligand, infused by a single syringe. The exit of the chip was coupled to an in-house built miniaturized LED-induced fluorescence (LIF) detector [42]. The LIF detector, featuring a bubble cell with a dead volume of only ~250 nL, was able to monitor fluorescent tracer concentrations down to 10 nM with very little band broadening. This setup was applied to screen for compounds bioactive against the nAChR-analogue AChBP in snake, snail and toad venom samples. Typically, <5 µg of sample was injected (see Section 2.1; [44]).

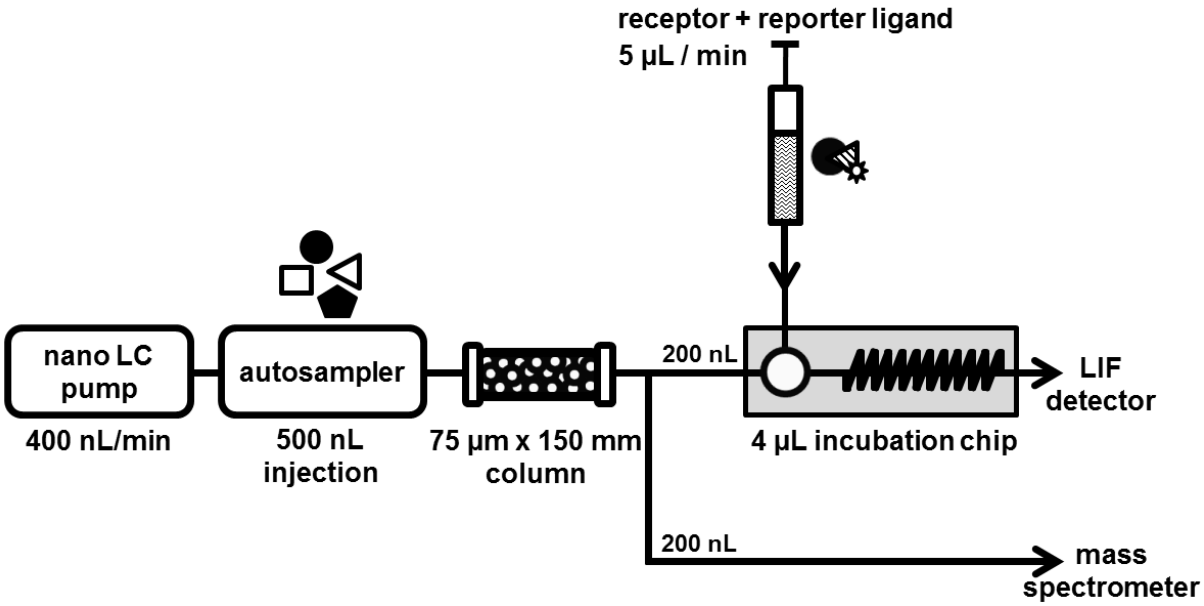

Figure 2. Schematic diagram of the on-line nano-LC microfluidic screening system.

1.3.3. At-line (micro)fractionation and screening approaches

Performing the bioassay in effluent fractions collected after (micro)fractionation addresses a number of the limitations of on-line bioassay screening approaches [29, 45, 46]. By at-line screening after fractionation, it should in principle be possible to apply much longer incubation times, also with non-soluble receptors, e.g., in cell-based and (gene)reporter-based assays. In addition, by the incorporation of a freeze-drying step, the solvent composition during the bioassay can be independent of that used in the HPLC separation. In order not to compromise the high resolution, characteristic to on-line post-column bioassays, much smaller fractions must be collected than generally applied in EDC or similar fractionation strategies. This can be achieved by collecting assay constituents and analytes in a microtiter plate (386 and 1536 wells) and utilizing a microplate reader for fluorescent readout of the bioassay after incubation [46]. Miniaturization of the complete system by using a micro-LC system would allow for smaller sample amounts used. Figure 3 shows a schematic diagram of the microfractionation/at-line bioassay system.

The analytical setup of this system is actually very similar to the on-line system, shown in Figure 1, with the major difference being the integration of a micro-LC system, allowing for a reduction of the injection volume down to 1 µL. In this system [29], 30% of the effluent was guided towards the MS, while the remaining effluent was send to the microfractionation part of the system. To the low-flow effluent, an enzyme or receptor solution and a substrate or reported ligand solution were infused by means of two syringe pumps, with the added benefit of being a cheaper means of assay infusion than the superloops driven by two LC-pumps utilized in on-line system. These three liquid flows were mixed and directly deposited onto a 386 or 1536 microtiter plate by a fraction collector/auto-injector equipped with an x,y,z arm. As this system is able to deposit fractions as small as 2 µL with a resolution of as little as 2 s, the resolution of the analytical separation was hardly compromised. Furthermore, the post-column void volume was minimized by omission of the reaction coils, making the system more robust. As the incubation time is no longer restricted by the dimensions of the reaction coils, a wider range of bioassays can be applied, including slow converting enzymes, receptors with a low K_{on}, heterogeneous assays and even cell-based assays [47, 48]. In addition, if the bioassay reagents are pipetted into the microtiter plate after fractionation rather than prior to fractionation, a solvent evaporation step, e.g., by freeze-drying, may be incorporated for bioassays that are sensitive to the organic solvents used in the HPLC mobile phase [49]. Further miniaturization of the fractionation can be achieved by a technology which spots the nano-fractions inside the microtiter plate, thereby eliminating possible problems with analyte evaporation and relatively high surface tension of nano-droplets on the fractionation outlet line [45].

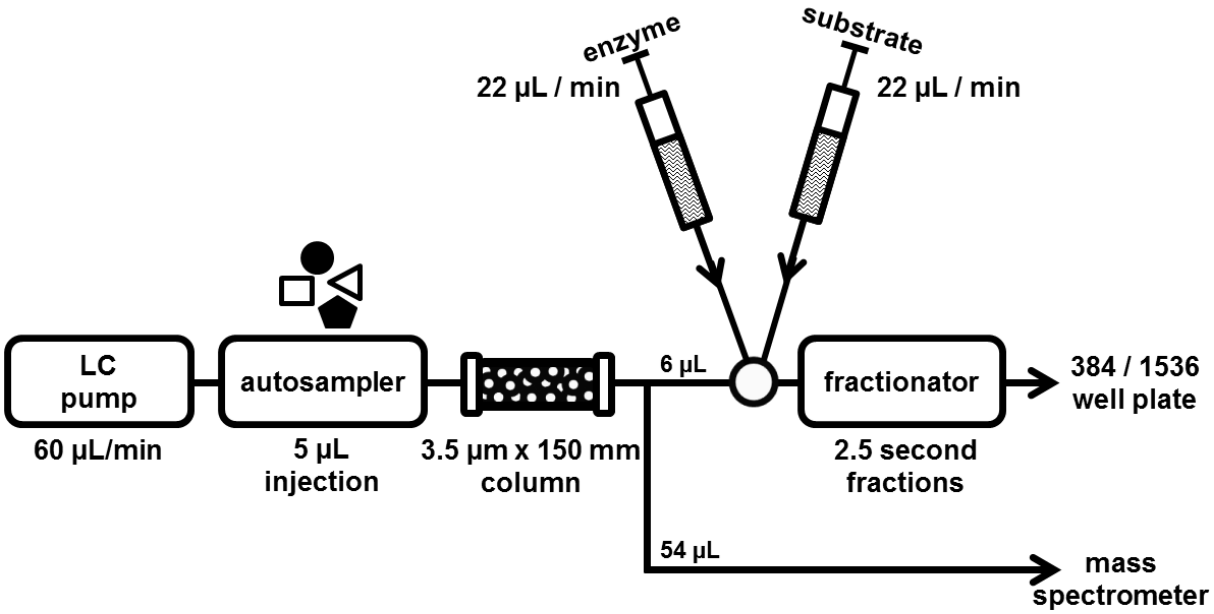

Figure 3. Schematic diagram of a typical setup for post-column fractionation and an at-line bioassay [29].

1.3.4. At-line fractionation after GC separation

For many analytical applications involving the separation of rather polar molecules, HPLC is most widely applied. Hundreds of solid-phase chemistries have been developed over the last decades, which can be applied as stationary phases in HPLC. They provide an analytical solution to a broad range of applications and for many compound classes. However, for some compound classes, HPLC is not ideal, e.g., given their volatility and/or lipophilicity. For many of these compounds, GC is a vastly superior separation technique. In line with the discussion in this chapter, it would be highly interesting to enable post-column bioactivity/bioaffinity screening for analytes and mixtures amenable to GC analysis. Therefore, we developed an off-line GC-based post-column fractionation technique [50]. Figure 4 shows a schematic diagram of the developed setup. Compounds separated by the GC column are post-column mixed in a connector with a solvent (hexane or acetonitrile) which is introduced to the GC oven by means of a syringe pump. The analytes and solvent vapors are then flushed outside of the oven by a transfer line and collected and fractionated in a condensed state into a 96-well plate, using a fractionation system similar to the at-line HPLC-based microfractionation system, shown in Figure 3. Next, the solvent is evaporated and the analyte fractions can be subjected to a bioassay. A proof of concept was demonstrated by profiling mixtures of polycyclic aromatic hydrocarbons, some of which are bioactive towards the dioxin receptor, with a mammalian gene reporter assay which allowed dioxin receptor bioactivity to be measured directly after fractionation [50]. An intrinsic limitation of this approach is the relatively poor sensitivity of the bioassays given the small amount of sample that can typically be injected into a GC system. Obviously, this platform is only applicable to the screening of volatile, thermostable compounds.

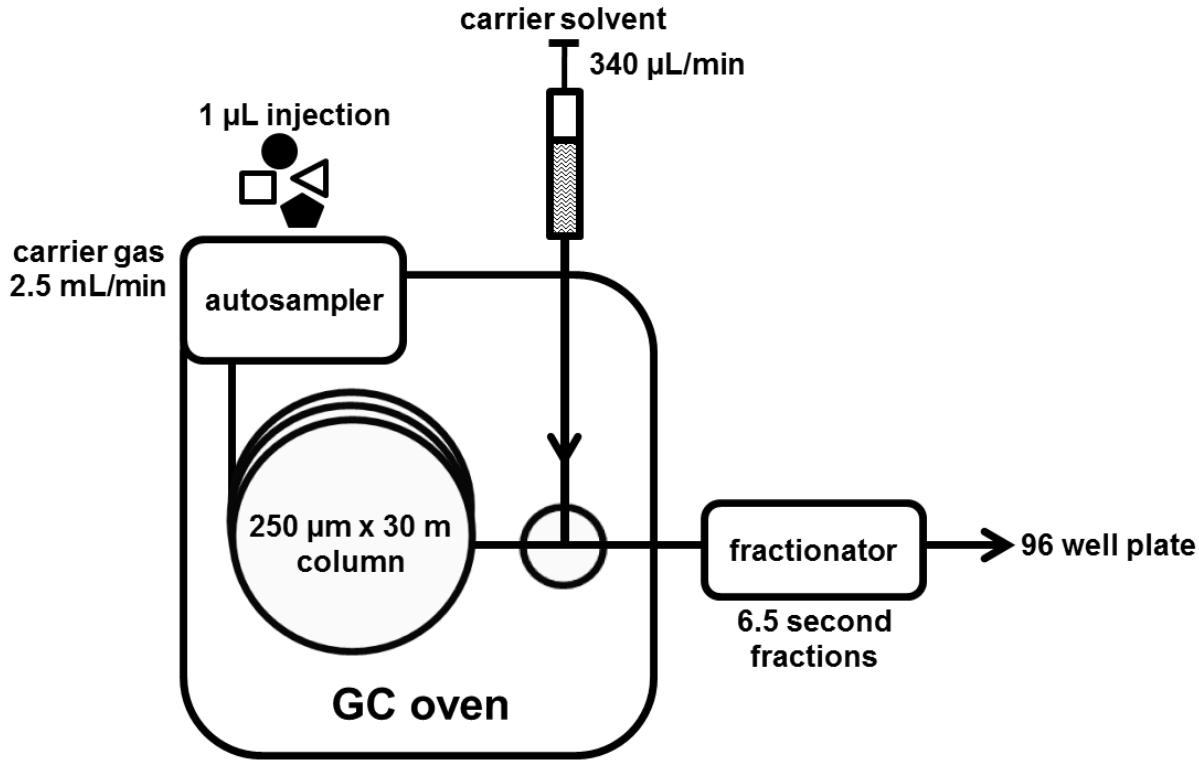

Figure 4. Schematic diagram of the at-line fractionation system enabling a GC-based screening platform [50].

2. On-line (high-resolution) screening approaches for the detection of novel bioactive substances targeting the acetylcholine binding protein (AChBP)

A wide variety of targets have been investigated in the course of development of on-line screening methods [41]. This includes antigen-antibody interactions (biotin-streptavadin, digoxin-antidigoxin), enzyme-inhibition screening [51] (cathepsin B protease, angiotensin-converting enzyme, acetylcholine esterase (AChE) and acetylcholine `binding protein (AChBP), MAP kinase p38α (p38), protein kinase A, soluble epoxide hydrolase), affinity against estrogen receptor (phytoestrogens in natural extracts, endocrine disruptors, bioactive drug metabolites). Initial results with microfractionation and at-line bioassays were achieved in enzyme-inhibition screening involving protein kinase A [29] and AChBP [45]. As a substantial part of the work described in this thesis is directed at AChBP, both in on-line and at-line screening, special attention is paid to this below.

2.1. Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are in the superfamily of cys-loop, ligand-ion gated receptors, which are widely expressed in the nervous system where they participate in a variety of physiological functions, including regulating excitability and neurotransmitter release, as well as neuromuscular contraction [52]. These membrane receptors are triggered by their endogenous agonist acetylcholine (ACh) as well as by nicotine. These receptors are

pentameric assemblies of five subunits, with each subunit arranged around a central pore. The binding of ACh between two subunits of the extracellular ligand-binding domain induces channel opening. The molluscan ACh-binding protein (AChBP) is a soluble protein analogue of the extracellular ligand-binding domain of the nicotinic acetylcholine receptors (α7 nAChRs) [53]. It has become a target to identify novel ligands for nAChRs. The recent 4-Å resolution of the *Torpedo* nAChR, and the elucidation of the crystal structure of the AChBP has provided significant insights in the structure of the ligand-binding domain, the channel pore, and the structural rearrangements upon channel opening [52]. Nicotinic acetylcholine receptors (nAChRs) constitute potential pharmaceutical targets against pain relief, Alzheimer disease, Parkinson disease, epilepsy, anxiety, and several cognitive and attention deficits [54].

2.2. Venom analysis

Venomous reptilian, insect, and snail species evolved their venomous arsenals to immobilize prey by targeting a myriad of neurotoxic and/or hemotoxic receptors and enzymes with derivatives of peptide toxins and enzymes with often high affinities [55]. Some of these proteins have shown potential in cancer treatment [56], and in pain treatment [57]. Therefore, the highly variable toxic peptides in these venoms may be very interesting candidates for drug discovery. A bottleneck for drug discovery from venoms is the need for an efficient and accurate method to screen for relevant bioactive peptides and elucidate the structure/identity of the individual peptides showing bioactivity. As discussed above, bioactivity-guided fractionation approaches are today's standard for drug discovery from natural products [40]. Usually, this process consists of (preparative) LC, fractionation and biochemical assays. Discovering and elucidating ligands via this laborious process from venoms can be quite challenging. One reason for this lies in the high complexity of these venoms, which can comprise up to several thousands of toxic peptides. Moreover, these peptides can partly share a highly similar amino-acid sequence, which challenges the separation procedure. An example of this are the so-called the three-finger toxins (3-FTX) found in snake venoms that often form broad peaks and additionally are highly homologues [58, 59]. An additional challenge is the large sample amounts that are often required for this traditional bioactivity guided fractionation approach. As a typical (crude) venom sample is only several milligrams for most species, this approach can be quite troublesome. This is especially true for venom samples originating from rare species or insects.

Considerable work has been done in the characterization of snake venoms. Whereas initial approaches required very elaborate workflows, the advances in analytical chemistry, and especially in the proteomics workflows involving LC–MS and tanden mass spectrometry (MS–MS), greatly facilitate an efficient identification of venomous peptides and proteins. A good overview of the current conventional MS-based workflow in snake venomics is provided by Calvete et al. [60]. The first step of the protocol involves the fractionation of the crude venom by RPLC. Each fraction is initially characterized by a combination of *N*-terminal sequencing, gel electrophoresis (GE), and determination of molecular masses and the cysteine (SH and S–S) contents by MS. If a protein fraction shows a single band in GE, the molecular mass and *N*-terminal sequence can be assigned to a known protein family by means of BLAST analysis. For heterogeneous protein fractions or proteins with blocked *N*-termini, the

bands of interest are subjected to reduction, alkylation, and in-gel tryptic digestion. The resulting tryptic maps are then analyzed by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and amino-acid sequencing using MS-MS [60]. The advent of high-resolution MS greatly facilitated the MS-related part of the workflow.
However, not only snake venoms may be relevant sources for bioactives for drug discovery. Other species are known to exhibit venoms as well, e.g., cone snails, scorpions, spiders, skin excretions from toads. The complexity of these proteomes in most cases remains to be explored. In the case for cone snails, for instance, it was until recently believed that these venoms contained only ~150 compounds, whereas in 2009 using an optimized LC–MS method more than 1500 peptides were discovered in cone-snail venoms [61]. However, next to detection and chemical characterization of the peptides in such complex mixtures, an extensive biochemical and pharmacological screening of these peptide mixtures is needed in order to assess whether useful bioactive peptides are available for specific drug targets. In addition, these proteins may not only serve as a source of potential medicines, they also provide more insight into our own physiology. A good example are the bungarotoxins, derived from krait venoms, which also initially led to the discovery of the neuronal nAChR [35]. The screening of these venoms poses severe analytical and biochemical challenges, mainly related to the sample complexity, the nature of the peptides and/or proteins they contain, and the generally small amount of venom available. As indicated, the nature of the peptides, often involving rigid serine bonds and several sulfur-sulfur bridges, poses a challenge to their characterization by the general proteomics workflow.
The work described in this thesis greatly facilitates the complete venomics workflow, not only in relation to peptides and proteins present in snake venoms but also in venoms of other species [43, 44]. Our miniaturized HRS systems facilitate finding peptides or proteins in the complex venom mixture with bioactivity against a particular target. The on-line bioassay after RPLC separation allows relevant bioactive peptides to be indicated, whereas the simultaneous MS analysis in parallel provides (provisional) identification of the relevant peptides. In addition, the on-line bioassay enables bioactivity- and MS-guided fractionation for further off-line structural and pharmacological characterization [62].

2.3. Alkaloids from frogs

The skin secretions from toads and frogs are complex mixtures containing large amounts of tryptamines, steroidal compounds and small peptides that might be a natural source for bioactive compounds. The tryptamines are derivatives from serotonin, being the archetype ‘scaffold’ [63]. These compounds, being excreted from glands in the skin of these toads, serve to deter animals, function as anti-predation toxins, and are known to cause convulsion, hallucinogenic effects and (even) death [64]. Toad skin excretions from Cane toad skin (*Bufo marinus*) and the Colorado River toad (*Bufo alvarius*) may also exhibit AChBP affinity. The screening of toad skin excretions indeed revealed a number of bioactives towards AChBP [44].

3. Scope of the Thesis

Over the past years, the high-resolution screening (HRS) approach has been developed in our laboratory [41]. This approach enables the screening for bioactive compounds in complex mixtures. It features LC separation of the complex mixtures followed by parallel continuous-flow bioaffinity or bioactivity detection and (tandem) mass spectrometry based identification. This thesis addresses a number of evident shortcomings and/or limitations of the conventional HRS approach.

Being a continuous-flow biochemical detection strategy, HRS involves the continuous infusion of the receptor protein or enzyme investigated as well as of the receptor ligand or reporting substrate. Relatively large amounts of these often expensive reagents are thus needed. Miniaturization of the post-column continuous-flow bioassay would greatly reduce the costs. Somewhat based on preliminary experiments on a microfluidic enzyme inhibition bioassay [65], a post-column microfluidic confocal fluorescence detection system was developed and coupled to nano-LC (400 nL/min flow). The system was applied in a fluorescent enhancement bioassay with acetylcholine binding protein (AChBP) as receptor and an in-house synthesized fluorescent tracer ligand DAHBA. As proof of principle, the activity of a number of small-molecule ligands was studied (**Chapter 2** [42]).

Whereas the high costs of the biochemical reagents may be one motivation for miniaturization of the bioassay, limitations in the amounts of sample or natural extract available for the bioassay is an obvious other motivation. Using the same microfluidic fluorescence enhancement bioassay, involving AChBP, profiling of neurotoxic snake venoms was performed. Snake venoms are complex mixtures of bioactive peptides, of which only small amounts of sample is available. The post-column parallel AChBP bioassay and mass spectrometric detection enables direct correlation of relevant bioaffinity with accurate mass of the bioactives. This enables provisional identification of the bioactive so-called three-finger peptides (3FTXs, 60–80 amino acids, ~7 kDa). The method was developed and evaluated using α-bungarotoxin, eventually spiked in an extract from *Vipera ammodytes*, and its identification power for bioactives was demonstrated in profiles the venom proteomes of *Dendroaspis jamesoni kaimosae*, *Naja annulifera*, and *Naja nivea* (**Chapter 3** [43]). As the usual follow-up upon detection of bioactive peptides in a natural extract, like venom, would require isolation and purification of the bioactive and subsequent pharmacological and structural characterization, LC–MS guided fractionation was added to the platform. The complete workflow of separation of the venom, correlating bioactivity towards AChBP, MS detection, followed by LC–MS guided purification and amino-acid sequencing of the isolated peptides was demonstrated for venom of *Naja mossambica mossambica* (**Chapter 4** [62]). In a third application paper, a similar workflow was demonstrated for a somewhat smaller bioactive peptide (16 amino acids, ~1.7 kDa) from the cone snail *Conus textile*. In addition, small-molecule ligands towards AChBP from skin excretions of the toads *Bufo marinus* and *Bufo alvarius* were investigated in the same workflow, thus enabling structural characterization using NMR spectroscopy (**Chapter 5** [44]).

The post-column continuous-flow bioassay set-up poses limitations in terms of the maximum incubation time permitted, thus restricting target selection to fast converting enzymes or

receptors with low ligand dissociation rates, and in terms of the homologous character of the assay, thus ruling out call-based assays and the use of membrane-bound target proteins. These limitations can be circumvented by the use of post-column microfractionation and subsequence bioassays in 96, 384 or 1536-well microtiter plates and conventional plate readers. In principle, thus would also allow decoupling the solvent composition of the LC separation and the buffer composition in the bioassay. The potential of an at-line bioassay was initially demonstrated for Protein Kinase A inhibitors spiked to or present in natural extracts, separated by LC and fractionated onto a 1536-well plate (**Chapter 7** [29]). Subsequently, a laboratory-built modified fractionation device (nano-spotter technology) was developed and applied in high-resolution bioactivity profiling of compound mixtures using an at-line AChBP bioassay in 1536-well plates (**Chapter 8** [46]).
So far, on-line and at-line HRS has been only applied in combination with LC separation. Bioactivity screening after GC separation may be important in some application areas, for instance in effect-directed analysis (EDA) of environmental samples containing polycyclic aromatic hydrocarbons (PAHs) and pesticides. Post-column high-resolution fractionation of compounds separated by GC can be achieved immersing the GC outlet into a continuous-flow of carrier solvent, which is subsequently fractionated in a 96-well plate (6 s fractions). Such a system was developed and characterized in detail, prior to its application to the analysis of a PAH-containing sample, featuring GC separation, post-column microfractionation, and a DR-LUC bioassay with parallel GC–MS analysis for identity confirmation of the PAHs (**Chapter 6** [50]).

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