This thesis describes the development of new analytical screening platforms, which can be used as tools in the field of discovery of biologically active compounds from natural extracts. Nature provides a rich source of active compounds that are produced by microbes, plants, mushrooms and animals, and these active compounds are known to interact with many different types biological targets, such as receptors, ion channels and enzymes. Therefore, bioactive compounds derived from natural extracts can be valuable drug leads in drug discovery processes. The first phase in active compound discovery from natural extracts is traditionally performed by bioassay-guided fractionation (BGF). This methodology has been applied successfully for the discovery of many important drugs and other active compounds in the past. However, BGF has limitations, which amongst other reasons has caused a decrease in natural-extract based drug discovery programs over the last 20-30 years. BGF requires large quantities of initial sample, is labor intensive and time consuming, and is susceptible to loss of bioactives because of the repeated bioassay steps needed. New analytical methods, which are less labor intensive, require less rounds of bioassaying, and which only need low amounts of sample for analysis, are in demand. This thesis deals with the development and application of on-line high-resolution screening (HRS) analytics for natural extracts, with a focus on venom. HRS setups commonly combine liquid chromatography (LC) with both mass spectrometry (MS) and a post-column continuous flow bioassay. This hyphenated technology provides separation of components of complex mixtures followed by parallel detection of their specific bioactivity and chemical identity. An important advantage of HRS compared to BGF is that from a single HRS analysis information can be obtained on the bioactivity of individual mixture components with recorded molecular masses.

The drug targets studied in this thesis are the α7-nicotinic acetylcholine receptor (α7-nAChR) and the serotonine type 3 receptor (5-HT_3R). These receptors are known to be involved in many central nervous system (CNS) diseases, such as cognitive deficits, schizophrenia, anxiety, depression, epilepsy, Alzheimer's disease, Parkinson's disease and pain. Therefore, identification of new active compounds interacting with these receptors can be a start for further development of new medicines or lead to their development into pharmacological and diagnostics tools. In this thesis different types of bioassays were developed and applied in HRS systems to identify active compounds interacting with the α7-nAChR and the 5-HT_3R.

Chapter 1 aimed to give a comprehensive overview on the background of this thesis, discussing the lacks and needs in fields of neuroscience related to ligand-gated ion channels, drug discovery and screening of natural extracts and other complex mixtures. Chapter 1 was divided into three topics: targets, sources and approaches. The "Targets" section gives an overview of the relevance of the drug targets studied in this thesis. The structure, localization, function and involvement in different diseases of the α7-nAChR and the 5-HT_3R were discussed in detail. The "Sources" section handled the relevance of compounds from different natural sources for drug discovery. Finally, the "Approaches" section discussed the classical and the new analytical approaches, which are used for the identification of new active compounds targeting ion channels. The venom-based drug discovery workflow was treated and actual examples leading to bioactive compounds were given. Finally, new analytical approaches using hyphenated systems in the endeavor of natural extract screening for bioactive compounds were discussed.

The aim of Chapters 2-4 is to overcome the limitation of using large sample volumes in traditional BGF and HRS systems employing normal bore LC column dimensions. For

this, miniaturized, or microfluidic, on-line HRS systems were developed which require only nanoliters or micrograms of sample, allowing screening of small-size samples. Examples are venom samples of rare species or animals producing only small quantities of venom. Animal venoms turn out to be rich sources of bioactive compounds that are able to activate, inhibit or modulate receptors, ion channels or enzymes. Therefore, venom-based drug discovery is an important tool for finding new leads for new medicines. The microfluidic on-line HRS setups developed employs nano-LC coupled to MS equipped with nano-electrospray ionization (nano-ESI) and in parallel a microfluidic chip-based continuous flow bioassay with an in-house built microfluidic fluorescence detector.

In Chapter 2 an analytical workflow is described in which the acetylcholine binding protein (AChBP) was used as screening target. The AChBP is a soluble binding protein, which is homologous to the extracellular domain of the α7-nAChR. This workflow enabled the bioactivity assessment of peptides and proteins from complex natural mixtures by an initial screening using the miniaturized on-line HRS platform, followed by a subsequent MS-guided purification and chemical identification using proteomics approaches. The workflow was demonstrated with the venom of the African spitting cobra, *Naja Mossambica*. From this venom, medium- and low-affinity ligands of the AChBP were identified. After purification of the bioactives by MS-guided fractionation, the bioactivity of the purified toxins was confirmed by rescreening using the microfluidic on-line HRS system. From in-solution tryptic digestion experiments the amino-acid sequence of the bioactive peptides was confirmed and yielded Cytotoxin 1 and Cytotoxin 2 as the bioactives. These cytolytic and carditoxic peptide toxins were not known to bind to the AChBP.

Chapter 3 describes a follow-up study of the analytical workflow described in Chapter 2. In this study the analytical methodology was demonstrated for the screening of small molecules and peptides from toad skin extracts and cone snail venom, respectively. Next to MS and MS/MS identification of the bioactives found, additional NMR analyses were performed on the bioactives discovered in the toad skin extract in order to achieve their full structural elucidation. A bioactive peptide in the *Conus textile* snail venom was pinpointed amongst >1,000 other peptides. This identification process was rapidly performed employing only two duplicate analytical runs (60 min each). The number of cysteine bridges in the bioactive peptide was subsequently uncovered by reduction experiments using dithiothreitol (DTT). In this experiment, after reduction of the cysteine bridges, the number of cysteine bridge pairs can be determined by MS measuring an increase of the accurate mass of target peptide with *m/z* +2.016 (2x1.008; the total mass of 2 H+ ions arising from reducing an S-S group to SH). The analytical workflow to screen for non-peptide small molecule bioactives (molecular weight of 200 to 1,000 Da) was demonstrated by the screening of *Bufo alvarius* and *Bufo marinus* toad skin extracts. Several tryptamine-like and steroidal binders of the AChBP were detected in the crude skin extracts. After MS-guided purification, the chemical identity of the compounds was assessed by NMR and MS/MS, and their biological activity was tested and confirmed in conventional radioligand binding assays.

Chapter 4 described the development of a fluorescence enhancement assay for the serotonine binding protein (5HTBP) in a microplate reader format followed by the development and application of a microfluidic on-line HRS format. The 5HTBP is an engineered binding protein which has the ligand recognition properties of the 5-HT_3R in the scaffold of the AChBP. This robust and straightforward fluorescence enhancement

assay is a good initial screening method of complex mixtures for finding novel bioactives targeting the 5-HT_3R. Main strengths of this fluorescence-based assay are its cost-effectiveness and ease of operation compared to the radioligand binding variant, and the ability to perform the assay in microfluidic on-line HRS format when analyzing mixtures. Application of this analytical screening format for screening mixtures was demonstrated using *Pseudonaja inframacula*, *Pseudonaja affinis* and *Dendroaspis polylepis* snake venoms. It was shown that the bioactives found could rapidly be correlated to their accurate masses obtained by MS.

A typical characteristic of the microfluidic on-line HRS system developed and applied in Chapters 2-4 is the possibility to perform the analysis with very small sample volumes. This is a great advantage in natural extract screening in cases that only low amounts of sample are available. In this thesis, microfluidic on-line HRS methodologies are described with two different assays: one for AChBP and the other for 5HTBP, both based on the fluorescence enhancement assay principle. The generic microfluidic on-line HRS setup is intrinsically adaptable to many other assay formats. Examples of other microfluidic on-line HRS formats, that are not described in this thesis, focus on screening for activators and/or inhibitors of thrombin and for factor Xa[1]. These assays are based on the enzymatic cleavage of a fluorogenic substrate by thrombin or factor Xa. We foresee other types of assays, mainly fluorescence based binding assays and enzymatic cleavage based homogenous assays, that are applicable to the microfluidic on-line HRS setup for screening of animal venoms for new drug leads. A generic limitation of on-line HRS setups is that only homogenous assays, with short (seconds to minutes range) incubation times, can be implemented in a straightforward manner. Assays that for example require long incubation times, filtration based assays, and/or cellular assays with adherent cells are out of scope for the microfluidic on-line HRS screening.

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The new analytical workflow introduced in this thesis - with the subsequent LC-MS guided purification of the accurate masses of bioactives identified using the microfluidic on-line HRS - was able to efficiently purify bioactives from animal venoms. This workflow is much more straightforward compared to the traditional BGF approaches, since accurate mass is directly obtained by the microfluidic on-line HRS screening. With this workflow the repeated bioassay steps after each purification step in BGF could be circumvented. This is beneficial only small sample quantities are available and also cost-effective, since bioassays are often quite expensive.

In Chapters 2-4 the developed and applied bioassays screen for affinity towards soluble binding proteins, which mimic real receptors. Only ligand binding affinity information is obtained from such assays. For measuring functional information (i.e whether a ligand is an activator or a blocker of an ion channel), cell based assays that include measuring ion channel activity are needed. Coupling cell-based assays in on-line HRS is a complex endeavor due to the technical difficulty of combining living cellular systems with LC eluents. Therefore, as an alternative, the recently introduced at-line nanofractionation methodology can be used when screening complex mixtures with cell based assays. The nanofractionation approach uses automated high-resolution fractionation of LC column effluents on high density well plates. Chapter 5 describes the development of an at-line cell-based screening methodology encompassing LC with nanofractionation followed by a functional fluorescence-based calcium-flux assay of the α7-nAChR. As for the on-line HRS analytics, in the nanofractionation approach, parallel MS detection is carried out in order to simultaneously assess accurate masses

of eluting bioactives. The applied assay uses a human neuroblastoma cell line stably overexpressing the α7-nAChR and was used for screening agonists and allosteric modulators of the α7-nAChR. After method development, the applicability of the new methodology was demonstrated by screening a hallucinogenic mushroom extract (from *Psilocybe mckennaii*)., This study also demonstrated that two orthogonal separations of the crude extract can facilitate the identification of bioactive compounds when extracts are very complex and bioactives co-elute in one dimension. In this orthogonal separation approach the same crude mixture was analyzed two times; the first analysis using reverse phase LC and the second analysis using hydrophilic interaction LC (HILIC). Accurate masses correlated to bioactivity in the first dimension, should also show bioactivity in the second dimension, thereby strongly reducing the bioactive candidates. As follow-up research we envision screening approaches using direct 2D LC separation in combination with on-line or at-line bioassays and parallel MS detection. These systems would be powerful for profiling very complex mixtures.

The cell-based screening approach described in Chapter 5 combines high-resolution nanofractionation with a functional cell-based assay. This at-line nanofractionation methodology has high potential for screening natural extracts, and it improves the traditional BGF screening approach by collecting fractions in the resolution of seconds instead of minutes. The at-line nanofractionation approach can be combined with many different assay formats performed in microplate readers. Recent examples are at-line nanofractionation methodologies with radioligand binding assays[2-3] for screening chemokine receptor ligands, fluorescence-based assays screening for thrombin and factor Xa modulators[4], and cell-based assays for screening G-protein coupled receptor (GPCR) targets[5]. Next to natural extract screening, the nanofractionation approach has also been applied in other fields, such as in metabolic profiling of drugs[3] and in environmental analysis[6]. As future perspective we see the further miniaturization of the nanofractionation approach which would allow performing the bioassay in array format and which would require only minute sample amounts. We furthermore foresee the continuation of developing cell-based assays combined with at-line nanofractionation for various types of drug targets. Looking beyond the cellular level, biological assays at the whole organ level, or even at the animal model level, might be combined with at-line nanofractionation approaches. Assays in organ-on-a-chip microfluidic format using three dimensional (3D) cell cultured tissues, neurons-on-a-chip, liver cells or kidney cells[7] could potentially be combined with at-line nanofractionation. Zebrafish embryo assays in microfluidic HTS format comprise a whole animal model which is widely used for drug lead discovery, drug target discovery, disease modeling and toxicology[8-9], and could be adapted for, and implemented to, nanofractionation screening. Advantages of zebrafish embryo assays are that they are *in vivo* assays that are both ethically sound and cost-effective compared to more conventional animal models, and that they are easy to automate for HTS screening. Combining at-line nanofractionation with organ-on-a-chip assays or zebrafish embryo assays for screening campaigns of natural extracts can give not only cellular readouts, but also functional responses on whole living organisms of individual bioactives in complex natural extracts.

Chapter 6 introduced the concept of a post-column continuous flow cell-based bioactivity screening assay. The system combines LC separation with an on-line mammalian cell-based assay using flow cytometry (FC) as readout with parallel MS. In this system the same functional calcium-flux assay was applied as in Chapter 5 using

human α7-nAChR expressing SH-SY5Y neuroblastoma cells. The advantage of using on-line screening compared to the at-line nanofractionation approach is that on-line assay in continuous flow is much faster and that the bioassay readout obtained has higher resolution. The LC-FC-MS screening system was developed in two assay modes: agonist and mixed antagonist-agonist mode. The latter mode gives the possibility of simultaneous detection of agonists and antagonists. The applicability of the system was demonstrated in two proof-of-principle experiments by the screening of tobacco plant leaf extracts in agonist mode, and snake venoms in mixed antagonist-agonist mode, as we expected to detect agonists from the tobacco extract (i.e. nicotine) and antagonists (i.e. three finger toxins) in snake venoms.

Follow-up research should concentrate on overcoming some shortcomings of the prototype system. The current screening setup in principle is well suited for suspension cell-lines (for example blood cells), which do not need to be mechanically kept in suspension. In the prototype system an adherent cell line had to be kept in suspension using a small stirr bar in a superloop (i.e. a pressurized container) and cells were continuously infused into the flow cytometer. In future setups, the cell viability of adherent cell lines could be improved by using cell-culture microparticles or beads. We also foresee development of advanced superloops with oxygen-pressurized compartments allowing long-time continuous infusion of viable cells into the FC bioassay part of the system. The setup described in Chapter 6 is using an LC system with a normal bore column. However, because of the low compatibility of mammalian cells with organic solvents, the LC effluent had to be highly diluted before mixing with the continuous flow bioassay. The unfavorable dilution could be decreased by using nano-LC or micro-LC columns that use down to nl/min flow rates instead of the 120 µl/min used in the current setup. Other chromatographic separation techniques, such as ion-exchange chromatography (IEC) or size-exclusion chromatography (SEC) could be used instead of reversed-phase LC. For these alternative separation techniques, however, other limitations arise connected to MS and bioassay compatibility. For instance, salt concentrations should still be tolerable for living cells and not cause ion suppression in MS. Flow cytometry can give additional bioassay readout information in HRS screening setups. First of all, responses of individual cells are measured. Secondly, modern flow cytometers can monitor multiple fluorescence and scattering signals, which allows performing different assays simultaneously as well as measurement of cellular morphology and/or clustering changes over time and upon mixing with bioactives eluting from the chromatographic system. As an example, the measurement of calcium fluxes by a green fluorescent dye (detected in one channel of the flow cytometer) in combination with the simultaneous recording of cell viability by a specific fluorescent dye (detected in another channel of the flow cytometer) is demonstrated in Chapter 6. Finally, software algorithms of most FCs allow gating, or selecting and analyzing only a group of cells detected by the FC.

A general concern regarding cell-based assays in any new cell-based screening setup is that cells express many different receptors and ion channels. This means that a signal, such as a calcium flux, can be caused not only by the receptor studied (which is usually over expressed by the cell), but also by other receptors. Therefore, it is crucial to use receptor selective antagonists and allosteric modulators as controls to confirm that signals observed are caused by the activation or inhibition of the specific receptor under study. This might be used in an advantageous manner if the cell line is used for

screening multiple drug targets expressed by the cell at the same time[10-12]. On the other hand, performing assays using target-specific modulators and/or inhibitors allows to selectively modulate one receptor for the screening. Other receptors present in the cells, which are not aimed to be studied in the assay, can be inhibited thereby, ensuring that the signals observed are specifically caused by the receptor under study.

Altogether, the screening methodologies described in this thesis are new analytical tools that are sensitive, fast and straightforward, and as such valuable additions to the field of natural extract screening and venom based drug discovery programs. The analytical methods in this thesis were applied to natural extract based drug discovery for CNS diseases, but these methods show great potential for screening other types of complex mixtures and other drug targets.