Miniaturized bioactivity screening of complex samples

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Summary

Section 1: Introduction

Since natural extracts are of increasing importance as a source for novel medicines, drug discovery needs analytical solutions for the challenges associated with the screening these complex mixtures. This thesis describes the development (and application) of miniaturized integrated bioanalytical setups which aim to contribute to this need.
The setups are based on the principle of high-resolution screening (HRS), which integrates liquid chromatography (LC) with a biochemical assay and (parallel) mass spectrometry (MS). This can be done on-line, where the effluent of the LC is split towards MS and a continuous-flow bioassay (which employs fluorescence detection as assay readout), or at-line, where the effluent is fractionated with high resolution in a well plate (followed by plate-reader readout). By miniaturizing these screening setups, samples with limited sample amount were allowed to be screened. Section 2 describes the development of an on-line miniaturized HRS setup where nano-LC was hyphenated to MS and a chip-based bioassay, which allowed for the screening of (typically) only 5 µg (snake) venom per analysis. In section 3, at-line setups are described which allow for the screening towards target assays which require longer incubation times. Here, micro-LC was hyphenated to MS and a fraction collector (2 s per fraction). An enzyme assay in 384 and 1536-well plate is used to assess bioactivity. Section 4 allows for the at-line screening of highly lipophilic compounds, by high-resolution fractionation (6 s per fraction) of a gas chromatography (GC) run in a 96-well plate, which allowed for the screening of compounds in a cell-based dioxin receptor assay.

Section 2: Miniaturized high-resolution screening

As the output of high-throughput screening (HTS)-based drug discovery steadily decreased over the last decades, natural product screening (NPS) is making its comeback. Traditionally, NPS is performed with off-line setups where animal or plant-derived material is fractionated by LC, followed by a microtiter plate assay. Due to the complexity of the sample, co-eluting compounds usually require several separation/fractionation and bioaffinity steps before the actual bioactive compound is identified by (tandem)MS and/or NMR. This iterative process of (re)fractionation and purification is time consuming and not practical when screening many natural extracts. Alternatively, HRS can be employed, an (on-line) screening approach developed during the last decennia in our lab. Mixing the LC effluent with a constant assay readout (and parallel MS detection) can be particularly useful when analysing complex mixtures. As HRS constantly monitors both biochemical and MS detection from the same LC-run in parallel, peak shapes and elution order in the binding assay directly correlate to MS detection. This also allows for better identification of poorly separated ligands, which normally would be collected in one fraction when screening at lower resolutions (>1 min fractions).
A drawback of HRS is its relatively large sample and assay consumption. To address this, the complete screening setup was miniaturized by implementing nano-LC (400 nL/min) and a chip-based biochemical assay in which half of the nano-LC effluent was mixed and incubated with a fluorescent enhancement assay, infused at 5 µL/min. For sensitive assay monitoring, a confocal LED-induced fluorescence detector was developed, equipped with a bubble-cell (150

µm i.d.) wherein LED light was collimated. Emitted light was detected by a photomultiplier tube. The remaining LC effluent was directly guided to MS, which was equipped with a nano-source. The miniaturized setup was applied to a fluorescent enhancement assay consisting of the acetylcholine binding protein (AChBP) as receptor and the fluorescent tracer ligand DAHBA, which was synthesized in-house. In **Chapter 2**, a number of small molecules was analyzed as a proof of principle study (the 50% effluent split towards the MS was not implemented in this (preliminary) study. Instead, the whole effluent was directed to the bioassay via an UV detector). Adequate sensitivity and good correlation in bioactivity parameters with literature values was achieved.**Chapter 3** describes the application of the setup to neurotoxic snake venom screening. These venom proteomes contain "three-finger toxins" (3TFXs, 60–80 amino acids, ~7 kDa), which are known ligands of ion channels, including nicotinic acetylcholine receptors (nAChRs). AChBP, a water-soluble homologue of the neuronal α7 nAChR, was therefore a suitable target. The implementation of parallel MS detection allowed for (provisional) peptide identification by correlating the nominal mass (adjusted for disulfide bonds) with literature via Uniprot. The miniaturized setup was evaluated using the strong α7 ligand α-bungarotoxin (α-BTX), with and without *Vipera ammodytes* venom, a haemotoxic venom not expected to have nAChR ligands. The LOD of α-BTX was 2 ng (and 50 ng for MS detection). *Vipera ammodytes* analysis also obtained a (weak) AChBP ligand. Next, *Dendroaspis jamesoni kaimosae*, *Naja annulifera*, and *Naja nivea* were profiled. This yielded 19 additional ligands, 11 of which were previously reported as either cytotoxin, cardiotoxin or orphan toxin. This proves the screening power of the miniaturized HRS setup, with only micrograms of venom consumed. The venom samples were not subjected to sample pre-treatment as this might influence the bioaffinity of the toxins. Lyophilized venoms were dissolved in 5% ACN, 0.1% TFA and 40 µM nicotine, which served to align biochemical and MS detection. Typically, 5 µg was injected to identify lower affinity binders, followed by a dilution series (down to 0.2 µg) to analyze high-affinity ligands. **Chapter 4** describes how the miniaturized HRS setup could be applied in a more extensive natural product screening workflow as an efficient and sensitive pre-screening tool. This was shown by isolating *Naja mossambica mossambica* toxins by LC–MS guided fractionation. This fractionation was targeted by the isotope pattern *m/z* of AChBP ligands as detected by the miniaturized HRS. After rescreening of the purified toxins (which were classified in literature as cytotoxins), the identity of the peptides was confirmed by MALDI–MS sequencing (after tryptic digestion). α7 nAChR affinity of the peptides was validated by radioligand binding towards neuroblastoma cell membranes expressing α7 nAChR. This exhibited low affinity binding for one of the isolated (cyto)toxins, which was not reported in literature before. **Chapter 5** shows how the miniaturized screening setup was capable to identify a known nAChR ligand from *Conus textile* venom, a proteome consisting of 1000 (smaller) bioactive peptides (15 to 30 amino acids). Besides, the applicability for the screening of small-molecule nAChR ligands was shown by the analysis of *Bufo marinus* and *Bufo alvarius* toad-skin secretions. Here, NMR spectroscopy enabled structural confirmation. α7 nAChR radioligand binding confirmed the bioactivity of four tryptamine-ligands and two steroidals.

Section 3: Miniaturized at-line high-resolution screening

A post-column continuous-flow bioassay set-up poses limitations in terms of the maximum permitted incubation time (up to ~ 2 minutes), which restricts target selection to fast converting enzymes or receptors with low ligand dissociation rates, thus ruling out cell-based assays and the use of membrane-bound target proteins. These limitations can be circumvented by the use of post-column micro-fractionation in 96, 384 or 1536-well microtiter plates (with a conventional plate-reader readout). By fractionation in highresolution, these at-line setups still allows for efficient screening of complex mixtures and good correlation between bioactivity information from the bioassay and identity information from the parallel MS detection. An additional advantage of this principle is the possibility to evaporate LC solvents from the fractionscontaining a.o. formic acid, methanol or acetonitrile. This allows the use of essays which are sensitive to these organic solvents. **Chapter 7** demonstrates the potential of an at-line bioassay for Protein Kinase A inhibitors spiked to or present in natural extracts, separated by micro-LC and fractionated onto a 1536-well plate. **Chapter 8** describes an in-house built fractionation device (nano-spotter technology) which was developed and applied in high-resolution bioactivity profiling of mixtures using an at-line AChBP bioassay in 1536-well plates.

Section 4: GC-based HRS screening

So far, on-line and at-line HRS has been only performed in combination with LC-based separations. This section describes the implementation of GC-based HRS, which may be important in some application areas, for instance in effect-directed analysis (EDA) of environmental samples containing (highly lipophilic) polycyclic aromatic hydrocarbons (PAHs) and pesticides. In **Chapter 9**, post-column high-resolution fractionation of compounds separated by GC was achieved by hyphenating the GC outlet into a continuous-flow carrier solvent which is subsequently fractionated in a 96-well plate (6 s per fraction). The developed system was characterized in detail, and applied to the analysis of a PAH mixture. After GC separation and post-column microfractionation, a cell based DR-LUC bioassay was seeded onto the GC-fractions, which confirmed binding of the PAH compounds. GC–MS analysis (in parallel) identified bioactive PAHs.

Future developments

Miniaturization is key in future drug discovery which benefits from assay and sample consumption, increased sensitivity and automation. At-line HRS strategies are clearly the most applicable in this miniaturization trend, as it offers a wider assay applicability (especially cell-based assays) and more robustness. Since nHTS is currently miniaturizing assays in 384, 1536 and even 3456-well plates (containing ~ 10.000 down to 200 cells respectively), high-resolution fractionation of nano/micro LC can be conveniently implemented with modern fractionation technologies.
Miniaturized on-line HRS approaches might become as applicable when “lab on a chip” technology is in a higher state of development.