

A microfluidic-based enzymatic assay for bioactivity screening combined with capillary liquid chromatography and mass spectrometry

Arjen R. de Boer,* Ben Bruyneel, Johannes G. Krabbe, Henk Lingeman, Wilfried M. A. Niessen and Hubertus Irth

Received 10th May 2005, Accepted 26th August 2005

First published as an Advance Article on the web 22nd September 2005

DOI: 10.1039/b506559c

The design and implementation of a continuous-flow microfluidic assay for the screening of (complex) mixtures for bioactive compounds is described. The microfluidic chip featured two microreactors (1.6 and 2.4 μL) in which an enzyme inhibition and a substrate conversion reaction were performed, respectively. Enzyme inhibition was detected by continuously monitoring the products formed in the enzyme-substrate reaction by electrospray ionization mass spectrometry (ESI-MS). In order to enable the screening of mixtures of compounds, the chip-based assay was coupled on-line to capillary reversed-phase high-performance liquid chromatography (HPLC) with the HPLC column being operated either in isocratic or gradient elution mode. In order to improve the detection limits of the current method, sample preconcentration based on a micro on-line solid-phase extraction column was employed. The use of electrospray MS allowed the simultaneous detection of chemical (MS spectra) and biological parameters (enzyme inhibition) of ligands eluting from the HPLC column. The present system was optimized and validated using the protease cathepsin B as enzyme of choice. Inhibition of cathepsin B is detected by monitoring three product traces, obtained by cleavage of the substrate. The two microreactors provided 32 and 36 s reaction time, respectively, which resulted in sufficient assay dynamics to enable the screening of bioactive compounds. The total flow rate was 4 $\mu\text{L min}^{-1}$, which a 25-fold decrease was compared with a macro-scale system described earlier. Detection limits of 0.17–2.6 $\mu\text{mol L}^{-1}$ were obtained for the screening of inhibitors, which is comparable to either microtiter plate assays or continuous-flow assays described in the literature.

Introduction

The discovery of new chemical entities is primarily performed by high-throughput screening (HTS) technologies in microtiter-plate formats. Large libraries are screened against drug targets for their biological activity. A recent trend in HTS is the miniaturization of the screening assay to reduce costs, as less reagents and sample are consumed. This miniaturization leads to chip-based systems that are able to screen up to 1000 samples per squared cm, using only nanoliters of sample per well.^{1–6} Alternatives are the microfluidic screening devices in which homogeneous enzymatic assays are performed. After incubation of the reactants, on-chip electrophoresis is performed in these devices to separate the reaction product(s) from the substrate, thereby enabling the quantitation of the enzyme activity.^{7–12} However, despite their speed and efficiency, these technologies can only be used for the screening of pure compound libraries. In many cases, samples to be screened are mixtures rather than pure compounds. Examples of mixtures are the reaction products of combinatorial synthesis, natural product extracts or mixtures from

active metabolite profiling. Typically, mixtures are deconvoluted by employing fractionation strategies, resulting in time-consuming and laborious screening activities.

Recently, we have described an on-line high-performance liquid chromatography (HPLC) biochemical assay system using electrospray ionization mass spectrometry (ESI-MS) as detection method.¹³ This approach overcomes the necessity of fraction collection allowing the simultaneous chemical and biological characterization of active sample components. So far, on-line systems have been used mainly on micro-HPLC levels with reagent flow rates in the order of 25–50 $\mu\text{L min}^{-1}$. For the screening of expensive protein targets, further miniaturization would be very beneficial. It is demonstrated that mixing enzyme and substrate on a chip in a continuous-flow mode is not problematic.^{14–16} In addition, the use of a microfluidic system would allow the use of a wide variety of materials, dimensions and designs for the biochemical reactors.

Although microfluidic systems have many advantages, the small size also has some drawbacks. Because the amount of sample that can be used decreases proportional to the size of the chip, more powerful detection methods are required. For example, laser-induced fluorescence (LIF) detection technique is mostly used for fluorescence-based assays.^{17–19} However, most lasers are not tunable over a wide wavelength range, which imposes restrictions to the substrate labeling. An alternative detection method used is MS.^{14,17,19} Unlike LIF, no labeling is

Vrije Universiteit Amsterdam, Faculty of Sciences, Department of Chemistry and Pharmaceutical Sciences, Section Analytical Chemistry & Applied Spectroscopy, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. E-mail: ardeboer@gmail.com; Fax: +31 (0)20 5987543; Tel: +31 (0)20 5987536

required for MS. ESI-MS is highly compatible with the low flow rates used in microfluidics. Finally, identification of the molecular mass of bioactive compounds is possible.

The present paper describes the development and optimization of a chip-based screening system comprising on-line preconcentration, HPLC separation of ligands, an on-line enzyme inhibition assay and ESI-MS detection.

Experimental

Reagents

Cathepsin B₁ (EC 3.4.22.1, bovine spleen; activity, 19 units per mg protein; one unit hydrolyses 1 $\mu\text{mol L}^{-1}$ of *N*- α -CBZ-lysine *p*-nitrophenyl ester per min at pH 5.0 at 25 °C; M_r 27 500), *Z*-Phe-Arg-AMC (CBZ-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, M_r 649.2), biotin (SMC1, M_r 244.3), cAMP (SMC2, adenosine 3',5'-cyclic monophosphate, M_r 329.2), antipain hydrochloride (*N*-(*N*- α -carbonyl-Arg-Val-Arg-al)-Phe, HCl content: not more than 1.8 mol mol⁻¹, M_r 604.7 (free base)), CA-074 ((*L*-3-*trans*-(propylcarbonyl)-oxirane-2-carbonyl)-*L*-isoleucyl-*L*-proline, M_r 383.4), E-64 (*L*-*trans*-3-carboxyoxiran-2-carbonyl-*L*-leucylagmatine, M_r 357.4), leupeptin (*N*-acetyl-Leu-Leu-Arg-al, hemisulfate salt, M_r 426.6 (minus hemisulfate)), DTE (1,4-dithioerythritol) and ammonium formate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Methanol (HPLC grade) was from J. T. Baker (Deventer, The Netherlands). Water was from a Milli-Q purification system (Millipore, Amsterdam, The Netherlands). All the chemicals used were of analytical grade or higher. Carrier solution/mobile phase consisted of 20 mmol L⁻¹ ammonium formate in water (pH 7.0). The percentage of methanol in the mobile phase was dependent on the experiment. Enzyme (150 nmol L⁻¹) and substrate (50 $\mu\text{mol L}^{-1}$) were separately dissolved in carrier solution, which contained SMCs (60 $\mu\text{mol L}^{-1}$) and DTE (50 $\mu\text{mol L}^{-1}$). Concentrations were in-syringe concentrations.

Instrumentation

The design of the microfluidic system is shown in Fig. 1. Sample and mobile phase were introduced by an Ultimate

HPLC pump and a Famos autosampler (LC Packings, Amsterdam, The Netherlands). The autosampler was coupled to a trapping column (Inertsil C₁₈, 5 μm particles, 300 μm \times 5 mm, LC-Packings) and to a six-port switching valve. For sample trapping, the valve was positioned to direct the flow to the waste (3A). After 2.5 min sample trapping the valve was switched to a plug (3B), which forced the flow to the analytical reversed-phase column (Biobasic C₁₈, 5 μm particles, 180 μm \times 10 cm, Thermo Electron, Waltham, MA). After conditioning (2.5 min), the amount of methanol in the carrier was increased to 30% for analysis. Alternatively, a gradient was applied to 77% of methanol in 30 min, in combination with homemade PRP-1 (5 μm particles, Hamilton, Bonaduz, Switzerland) columns of 180 μm \times 6 mm (trapping) and 180 μm \times 125 mm (analytical). The microfluidic chip was placed after the column, in which the column effluent (2 $\mu\text{L min}^{-1}$) was subsequently mixed with enzyme and substrate solution (both 1 $\mu\text{L min}^{-1}$). Enzyme and substrate solution were added by pressure-driven flow from syringe pumps (Harvard, Holliston, MA). The chip outlet was connected to an ion-trap mass spectrometer (LCQ Deca, Thermo Electron).

Microfluidic chip

The chips were fabricated by Micronit Microfluidics (Enschede, The Netherlands) out of two layers of borofloat glass 33/D263. Chip dimensions were 45 mm \times 15 mm \times 2.2 mm (Fig. 2), having channels of 70 μm deep \times 125 μm wide. Channels and interconnection holes were created by photolithography and by powder blasting of Al₂O₃-particles on the substrate using a moving nozzle. The average surface roughness of the channels was 2 μm . The diameters of the conical-shaped holes were 600 μm to 1.6 mm. For the enzymatic reactions, two open tubular microreactors were created with volumes of 1.6 and 2.4 μL . The glass layers were joined by direct bonding at high temperatures. For analysis, a chip was placed in a chip holder (Standard 4515, Micronit). Connections to and from the chip were made by fused silica capillaries (id, 50 μm ; od, 360 μm). The chip can withstand pressures up to 80 bar and was therefore fully compatible with the back pressure from the MS interface.

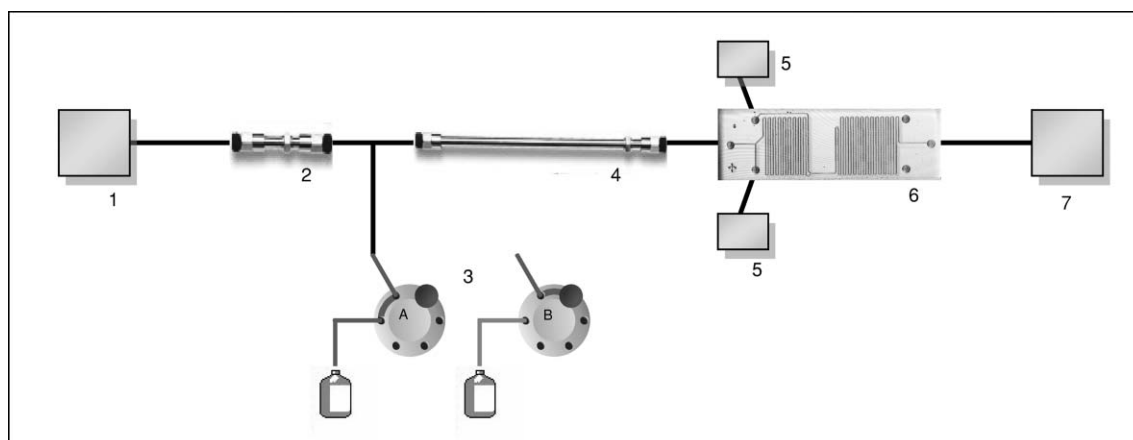


Fig. 1 Schematic overview of the on-line continuous-flow system: 1, pump and autosampler; 2, trapping column; 3, switching valve; 4, analytical column; 5, syringe pumps; 6, microfluidic chip; 7, mass spectrometer.

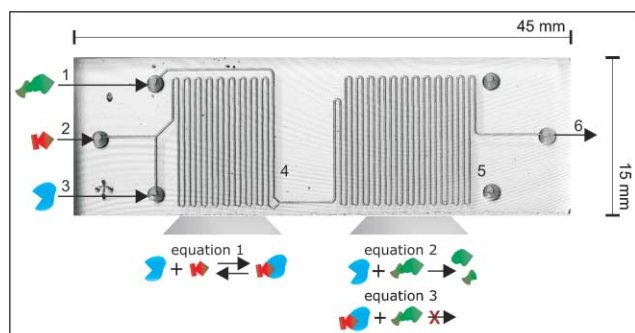


Fig. 2 The microfluidic chip as used for bioactivity screening: 1, substrate solution; 2, LC effluent; 3, enzyme solution; 4, open tubular microreactor with a volume of 1.6 μL ; 5, open tubular microreactor with a volume of 2.4 μL ; 6, flow towards mass spectrometer. The enzyme hydrolyses the substrate into products (equation 2) if no bioactive compound is eluting from the column. Bioactive compounds present in the eluate bind to the enzyme (equation 1), resulting in a decrease of substrate turnover (equation 3).

Enzymatic assay

The cysteine protease cathepsin B²⁰ was used as model enzyme to evaluate the performance of the microfluidic system. In the chip, cathepsin B was assayed with the substrate Z-Phe-Arg-AMC, which was hydrolyzed into Z-Phe-Arg-OH, Z-Phe-Arg-O-CH₃ and AMC. DTE was added to maintain the active state of cathepsin B by preventing the formation of disulfide bonds. Enzymatic reactions were performed at 22 °C.

Mass spectrometry settings

The ion-trap mass spectrometer was equipped with electrospray ionization (ESI), having an in-source low flow metal needle for an improved performance at low flow rates. Instrument control, data acquisition and data processing were performed using Xcalibur 1.2 software (Thermo Electron). The following parameters were used: capillary temperature, 150 °C; sheath gas flow, 5 (AU); source voltage, 4.0 kV; capillary voltage, 34 V; tube lens offset, 0.0 V; quadrupole offset, -3.0 V; octapole offset, -9.0 V; inter-octapole lens voltage, -23 V, and entrance lens, -50 V. For MS/MS experiments, the following settings were used: for product Z-Phe-Arg-OH (m/z 456.6) the MS²-settings were: activation-amplitude, 35%; activation-Q, 0.25, and for AMC (m/z 176.5): activation-amplitude, 40%; activation-Q, 0.40. The fragments monitored in selected MS/MS were m/z 395.2 and m/z 117.2, respectively.

Results and discussion

Design of the microfluidic system

The core of the present analytical screening system is a continuous-flow enzyme assay (Fig. 2). In the dual-reactor set-up, a solution comprising the active enzyme is mixed with the sample, which is introduced either directly (flow-injection mode) or *via* an LC column. In the first microreactor, active ligands present in the sample are allowed to interact with the enzyme (equation 1). The (remaining) enzyme activity is subsequently assayed by the addition of a substrate. The

enzyme–substrate reaction proceeds in the second microreactor (equation 2). Readout of the screening reaction is performed by on-line detection of the substrate conversion products using ESI-MS. Changes in the product signal reveal the presence of bioactive compounds in the sample, caused by the inhibition of the enzyme (equations 1 and 3). In the miniaturized system described in this paper, the dual-reactor set-up is implemented on a glass microchip.

The advantages of chips as microreactor over fused silica capillaries are in their compactness, strength, greater degrees of freedom in design and material, and the presence of hair-pin curves to increase the diffusion rate.²¹ An in-solution enzymatic assay was chosen over an immobilized format^{22,23} in order to avoid immobilization. Thereby, switching between enzymatic assays becomes easier and regeneration after reaction with an irreversible inhibitor is not an issue.

Miniaturizing a conventional-flow screening system¹³ (macro-scale system) to a chip-based system comprises a number of changes such as flow rates, reagent supply and the material. While the conventional system with the open tubular reactors is restricted to polymer reactors, the choice of materials for the chip is much larger, like glass, silicon, plastic, quartz and fused silica. The design of the chip is mainly dictated by the flow rates compatible with electrospray MS. In order to achieve proper mixing on the microchip, flow rates of 2 $\mu\text{L min}^{-1}$ for capillary LC and 1 $\mu\text{L min}^{-1}$ for both enzyme and substrate solutions were chosen. The choice of a total flow rate in the chip of 4 $\mu\text{L min}^{-1}$ resulted in reaction times of 32 and 36 s in the two reactors, respectively. In comparison with the macro-scale system, the flow rates of both enzyme and substrate were reduced by a factor of 25. Employing the optimum concentrations of the macro-scale system did not result in sufficient product formation for screening. For that reason, the enzyme concentration was increased 5-fold, having an overall decrease in enzyme and substrate consumption of 5 and 25 times, respectively.

Chip performance

Extra column band broadening is a key concern when implementing chip-based microfluidics in a capillary LC system. Band broadening negatively influences the sensitivity for bioactivity detection as the sensitivity is dependent on the height of the inhibitor peaks in the product trace(s). Both the design of the microreactors and the connections to the LC column and mass spectrometer are crucial. The band broadening was investigated by flow-injection of the inhibitor E-64 at various flow rates and injection volumes by calculating the peak width at half height ($w_{1/2}$). Data were obtained by experiments using an autosampler connected to a UV-detector in the absence and presence of the chip.

As expected, the lower flow rates and injection volumes resulted in broader peaks when using the microfluidic system (Table 1). At an injection volume of 0.1 μL , 85% of the band broadening can be contributed to the microfluidic chip, independent of the flow-rate. The reason is that the connections and channels of the chip increased the extra column volume and thus the sample dilution. At larger injection volumes (1 μL), the percentage of band broadening that can be

Table 1 The effect of the chip implementation on the band-broadening. Peak widths at half-height ($w_{1/2}$) were calculated by means of the sum of the variances of the individual contributions (σ^2). E-64 (1.1 mmol L⁻¹) was injected at 0.1 and 1 μ L at various flow rates. UV absorbance was performed at 214 nm. No reagent flows were pumped in the chip

Flow rate/ μ L min ⁻¹	$w_{1/2}/s$ at an injection volume of 0.1 μ L		$w_{1/2}/s$ at an injection volume of 1 μ L	
	Total microfluidic system	Contribution of the chip	Total microfluidic system	Contribution of the chip
1	39	33	100	49
2	26	23	54	29
3	21	18	39	23
4	18	15	31	20

contributed to the chip was less (60%). A reason for this difference could be that diffusion at the borders of the sample plug is relatively more problematic for smaller injection volumes.

The band broadening and the analyte dilution resulting from it were compared between the microfluidic chip system and the macro-scale system. The maximum concentration of the bioactive peaks (C_{\max})²⁴ was calculated for E-64 injections from calibration curve measurements. The dilution factor was almost similar, only 10% higher for the microfluidic system. The peak height and shape was adequate for bioactivity screening.

Sensitivity of the chip-based screening system

The sensitivity of the microfluidic system was determined by measuring calibration curves of four cathepsin B inhibitors. The inhibitors caused negative peaks in the product mass chromatograms by inhibiting cathepsin B and thus the substrate turnover. The heights of which were plotted against the concentration (Fig. 3). Fig. 3 shows that similar to conventional screening assays, leupeptin has the highest and CA-074 the lowest affinity for cathepsin B. The affinity for cathepsin B is also expressed in IC₅₀ values (the concentration of inhibitor that inhibits 50% of the enzyme activity), which are derived from Fig. 3 (Table 2). The measured order of affinities of the four inhibitors is in agreement with the affinities determined in microtiter plate assays and the macro-scale system.

Limits of detection (LODs) and IC₅₀ values derived from the calibration measurements were compared with the conventional system (Table 2). The concentration LODs of the microfluidic system were 6 times higher under similar experimental conditions, while the IC₅₀ values were 4 times higher. These differences could be caused by less efficient mixing of sample and reagents in the chip compared with the macro-scale system. In general, the application of microfluidics results in decreased channel diameters, which negatively

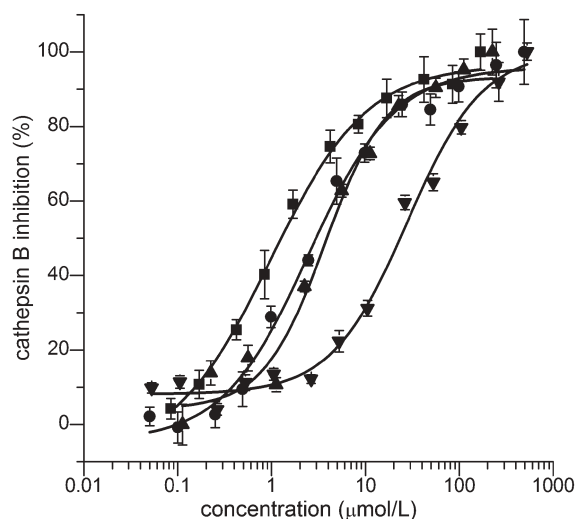


Fig. 3 Calibration curves of four cathepsin B inhibitors. Curves were obtained after plotting of the enzymatic activity (peak height) sigmoidally against the concentration inhibitor. The x -axis has a logarithmic scale to obtain typical concentration–response curves: ■, leupeptin; ●, antipain; ▲, E-64; ▼, CA-074. The error bars represent 1 SD ($n = 6$). No LC column was present in the system. The injection volume was 0.4 μ L. Concentrations plotted were the concentrations present in the autosampler.

influences the fluid mixing. Mixing becomes a slow process as it is controlled by molecular diffusion only, having a strictly laminar flow.^{25,26} Another reason could be wall effects, which means that non-specific binding of enzyme and substrate to the walls changed the overall enzymatic performance. However, with the current system no significant non-specific binding was determined. Despite the more unfavorable detection limit compared to the macro-scale system, the LODs are still in the concentration range for bioactivity screening, while the complete system is miniaturized to a micro-scale level. In addition, the absolute LODs and IC₅₀ values with the chip

Table 2 Comparison of LODs and IC₅₀ values of the chip-based system with the conventional system.¹³ The LODs were determined by the lowest concentration of inhibitor that could be detected using the product mass chromatograms. LODs were calculated from the mean of blank injections assuming a signal-to-noise ratio of 3. IC₅₀ values were derived from Fig. 3. Concentrations in the table are the concentrations that were present in the autosampler

Inhibitor	Concentration LOD chip/ μ mol L ⁻¹	Concentration LOD conventional/ μ mol L ⁻¹	Mass LOD chip/pmole	Mass LOD conventional/pmole	Concentration IC ₅₀ chip/ μ mol L ⁻¹	Concentration IC ₅₀ conventional/ μ mol L ⁻¹
Antipain	0.25	0.060	0.10	0.60	2.6	0.37
CA-074	2.6	0.35	1.0	3.5	28	5.6
E-64	0.20	0.10	0.080	1.0	3.7	2.1
Leupeptin	0.17	0.018	0.068	0.18	0.98	0.11

were 4 times and 6 lower, respectively, which means that less sample is required for screening.

Implementation of MS for detection

The main advantages of MS in comparison to fluorescence detection are the possibility to perform label-free assays, to detect all the products formed, and to identify the molecular mass of bioactive compounds simultaneously. An essential requirement of MS is the use of volatile buffers, *e.g.*, ammonium formate, in order to prevent ion suppression. Coupling the pressure-driven chip to the mass spectrometer was straightforward, generating product traces that were stable for hours.

Screening by MS was performed in the full spectrum MS and MS/MS mode. In the MS/MS mode, the enzymatic product is dissociated and the fragments were monitored for bioactivity detection. While the MS/MS mode provided more selectivity and stability, regarding sensitivity there was no real benefit from using MS/MS instead of full spectrum MS.

The conventional assay was also performed using a Shimadzu LCMS-2010 quadrupole mass spectrometer (Kyoto, Japan). To make sure that differences between the chip-based assay and the conventional assay were not originating from the type of MS used, calibration curves of cathepsin B inhibitors were generated with both instruments. The results showed no significant differences.

In general, the ionization efficiency in MS is enhanced by the use of organic solvent. The enzymatic activity, however, decreases in most cases if organic solvents are used.²⁷ An optimum for LC-MS in the percentage of organic solvent used in the carrier flow was found at 35%. Due to dilution by reagent addition, the actual percentage of organic solvent in the chip was 18–23%.

Screening mixtures

Screening mixtures for bioactivity not only requires an enzymatic assay, but also the separation of the compounds. Microfluidic separation is in most cases performed by capillary electrophoresis, but here LC was selected for its simplicity of combining with a microfluidic chip and MS. The suitability of the microfluidic system for screening mixtures was demonstrated by injecting a sample containing antipain and E-64 (Fig. 4).

The enzymatic reaction products were monitored by MS and reflect the enzymatic activity in the microreactor (A,B,C). At point 1 the valve was switched to force the flow to the analytical column, where at point 2 the methanol content in the mobile phase was increased for analysis. The higher level of methanol in the microreactor increased the involvement of methanol in the substrate hydrolysis and thus the intensity of mass chromatogram C. The negative peaks in the product mass chromatograms at 9.2 and 14.3 min reveal the presence of bioactive compounds, because less product was detected. This is due to a temporary decrease in substrate turnover by the inhibited enzyme. Mass spectra in the negative peaks (D,E) show the *m/z* values that motivates plotting of the extracted ion chromatograms (F,G). The positive peaks in the extracted ion chromatograms of *m/z* 358.7 and 637.3 have, compared to

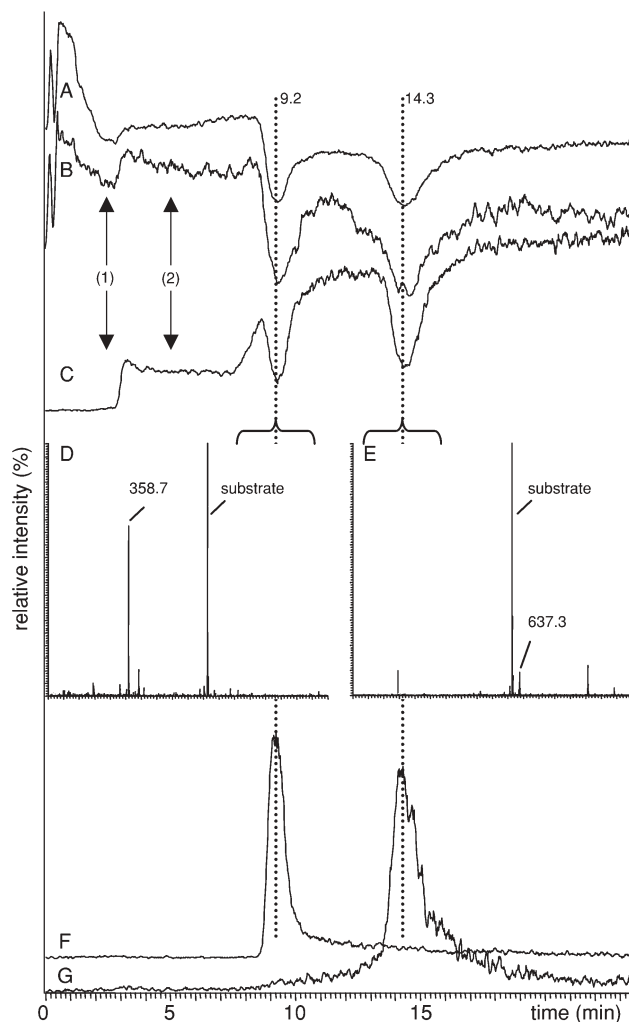


Fig. 4 Screening of a mixture containing two inhibitors by the microfluidic screening system. A, mass chromatogram of product AMC, *m/z* 176.5; B, mass chromatogram of product Z-Phe-Arg-OH, *m/z* 456.5; C, mass chromatogram of product Z-Phe-Arg-O-CH₃, *m/z* 470.4; D, mass spectrum in the negative peak at 9.2 min; E, mass spectrum in the negative peak at 14.3 min; F, extracted-ion chromatogram of *m/z* 358.7; G, extracted-ion chromatogram of *m/z* 637.3.

the negative peaks, a similar retention time and peak shape, which indicate that they correspond to the bioactive compounds. *m/z* 358.1 correlates with inhibitor E-64, where *m/z* 637.3 belongs to the inhibitor antipain. Even though the mobile phase conditions for the separation were not optimal because of assay requirements, bioactive compounds were separated and detected by the microfluidic system.

To prove that the negative peaks were not caused by pumping instabilities or ionization suppression, two system monitoring compounds (SMCs) were present in the enzyme and substrate solutions. Because the MS traces of the SMCs show no negative peaks, the negative peaks in the product traces were from bioactivity (data not shown).

The microfluidic screening system was also applied for the screening of a complex sample for cathepsin B inhibitors (Fig. 5). A 20% ethanolic green tea extract (80% water) was

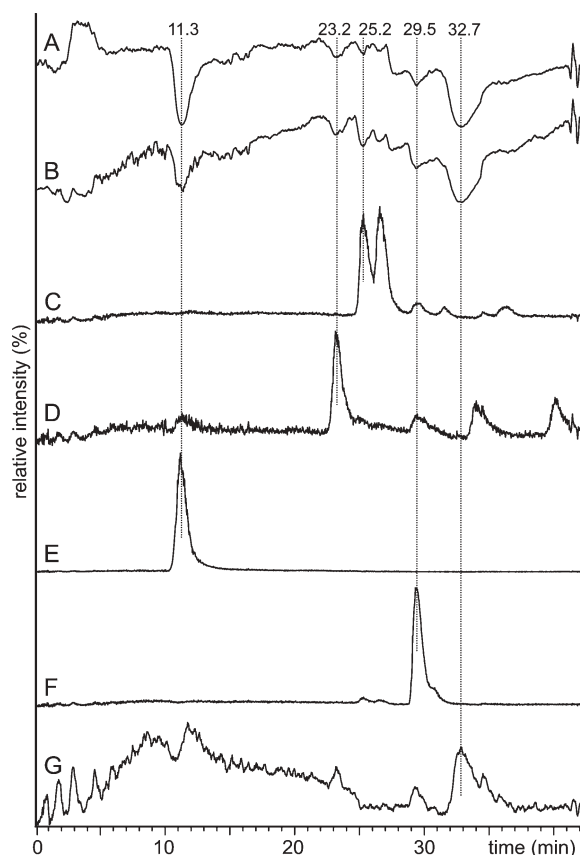


Fig. 5 Screening a green tea extract by means of gradient elution. A, mass chromatogram of product AMC, m/z 176.1; B, mass chromatogram of product Z-Phe-Arg-OH, m/z 456.2; C, extracted-ion chromatogram of the negative peak at 25.2 min; D, extracted-ion chromatogram of the negative peak at 23.2 min; E, extracted-ion chromatogram of the negative peak at 11.3 min; F, extracted-ion chromatogram of the negative peak at 29.5 min; and G, extracted-ion chromatogram of the negative peak at 32.7 min.

spiked with antipain and E-64 and separated by LC gradient elution. An LC gradient was applied to achieve more separation efficiency.

The negative peaks in the product mass chromatograms (A,B) can be correlated to compounds present in the green tea extract, using the same procedure as demonstrated in Fig. 4. The negative peak at 11.3 min is due to E-64 (E) and at 32.7 min to antipain (G). The negative peak at 23.2 min belongs to an unknown compound with an m/z of 313.1 (D). Based on its molecular mass and its natural presence in green tea, it is likely that the peak at 25.2 min (m/z 459.1; C) is (–)-epigallocatechin gallate or its isomer (+)-gallocatechin gallate, while the peak at 29.5 min (m/z 443.1; F) can be (–)-epicatechin gallate. The identity of these compounds is not confirmed. Despite the fact that cathepsin B inhibition by catechins is not reported in the literature, it is not unlikely as catechins inhibit various enzymes (also cathepsin G)²⁸ that are involved in cancer metastasis.^{29,30} SMC traces show no negative peaks, proving that these were from bioactivity (data not shown).

The application of gradient elution increased the power for screening necessary for these kinds of samples. Despite

changes in the mobile phase composition during the run, the assay continues to show sufficient enzymatic activity for detecting bioactivity. Similar to the isocratic-LC screening, the molecular masses of the bioactive compounds were detected.

Conclusions

Miniaturization of a continuous-flow system to a microfluidic format reduces the consumption of sample and reagents and provides a larger flexibility in the choice of reactor materials and designs. Integration of the chip-based assays with LC-MS is straightforward due to the compatibility of ESI-MS with the low flow rates used in the microfluidic assay. The continuous-flow principle also works on chip-level, with an enzyme that continuously cleaves substrate into products.

With respect to band broadening and signal stability, the microfluidic chip system is comparable to macro-scale screening systems. Mixing reagents at the microfluidic level is more difficult and for that reason, the system performance can decrease. However, despite somewhat higher limits of detection, the results show that screening for bioactivity by the chip-based system can be performed in a useful concentration range. Not only sufficient sensitivity is provided, determination of the relative binding constants of the bioactive compounds is possible as well.

The system provides the necessary separation for (complex) mixtures and detects bioactive components present in the sample. Gradient elution makes the microfluidic system complete for bioactivity screening.

References

- B. J. Battersby and M. Trau, *Trends Biotechnol.*, 2002, **20**, 167–173.
- M. Entzeroth, *Curr. Opin. Pharmacol.*, 2003, **3**, 522–529.
- D. N. Gosalia and S. L. Diamond, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 8721–8726.
- R. P. Hertzberg and A. J. Pope, *Curr. Opin. Chem. Biol.*, 2000, **4**, 445–451.
- A. Schober, G. Schlingloff, A. Groß, T. Henkel, J. Albert, G. Mayer, H. Wurziger, D. Döring and H. Tietz, *Microsyst. Technol.*, 2004, **10**, 281–292.
- J. Wölcke and D. Ullman, *Drug Discovery Today*, 2001, **6**, 637–646.
- C. B. Cohen, E. Chin-Dixon, S. Jeong and T. T. Nikiforov, *Anal. Biochem.*, 1999, **273**, 89–97.
- J. Dunne, H. Reardon, V. Trinh, E. Li and J. Farinas, *Assay Drug Dev. Technol.*, 2004, **2**, 121–129.
- A. G. Hadd, S. C. Jacobson and J. M. Ramsey, *Anal. Chem.*, 1999, **71**, 5206–5212.
- S. Lin, A. S. Fischl, X. Bi and W. Parce, *Anal. Biochem.*, 2003, **314**, 97–107.
- S. A. Sundberg, A. Chow, T. Nikiforov and H. G. Wada, *Drug Discovery Today*, 2000, **5**, S92–S103.
- H. Xu and A. G. Ewing, *Anal. Bioanal. Chem.*, 2004, **378**, 1710–1715.
- A. R. de Boer, T. Letzel, D. A. van Elswijk, H. Lingeman, W. M. A. Niessen and H. Irth, *Anal. Chem.*, 2004, **76**, 3155–3161.
- S. Benetton, J. Kameoka, A. Tan, T. Wachs, H. Craighead and J. D. Henion, *Anal. Chem.*, 2003, **75**, 6430–6436.
- K. Kanno, H. Maeda, S. Izumo, M. Ikuno, K. Takeshita, A. Tashiro and M. Fujii, *Lab Chip*, 2002, **2**, 15–18.
- J. Wang, *Electrophoresis*, 2002, **23**, 713–718.
- K. B. Mogensen, H. Klank and J. P. Kutter, *Electrophoresis*, 2004, **25**, 3498–3512.
- M. A. Schwarz and P. C. Hauser, *Lab Chip*, 2001, **1**, 1–6.
- K. Uchiyama, H. Nakajima and T. Hobo, *Anal. Bioanal. Chem.*, 2004, **379**, 375–382.

-
- 20 J. S. Mort and D. J. Buttle, *Int. J. Biochem. Cell Biol.*, 1997, **29**, 715–720.
- 21 Y. Yamaguchi, F. Takagi, K. Yamashita, H. Nakamura, H. Maeda, K. Sotowa, K. Kusakabe, Y. Yamasaki and S. Morooka, *AIChE J.*, 2004, **50**, 1530–1535.
- 22 J. Krenková and F. Foret, *Electrophoresis*, 2004, **25**, 3550–3563.
- 23 S.-S. Park, S. I. Cho, M.-S. Kim, Y.-K. Kim and B.-G. Kim, *Electrophoresis*, 2003, **24**, 200–206.
- 24 B. L. Karger, M. Martin and G. Guiochon, *Anal. Chem.*, 1974, **46**, 1640–1647.
- 25 L. E. Locascio, *Anal. Bioanal. Chem.*, 2004, **379**, 325–327.
- 26 J. M. Ottino and S. Wiggins, *Philos. Trans. R. Soc. London, Ser. A*, 2004, **362**, 923–935.
- 27 A. M. Klibanov, *Trends Biotechnol.*, 1997, **15**, 97–101.
- 28 L. Sartor, E. Pezzato and S. Garbisa, *J. Leukocyte Biol.*, 2002, **71**, 73–79.
- 29 V. Crespy and G. Williamson, *J. Nutr.*, 2004, **134**, 3431S–3440.
- 30 C. Jedeszko and B. F. Sloane, *Biol. Chem.*, 2004, **385**, 1017–1027.