Lab-on-a-Chip

DOI: 10.1002/anie.201409663

Chip-Based Free-Flow Electrophoresis with Integrated Nanospray Mass-Spectrometry**

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Abstract: *Free-flow electrophoresis is an ideal tool for preparative separations in continuous microflow. With the approach presented herein for coupling free-flow electrophoresis and mass spectrometry it is now also possible to trace non-fluorescent compounds and identify them by means of mass spectrometry. The functionality of the method and its potential as an integrated separation unit for microflow synthesis is demonstrated by application to a multicomponent [3+2]-cycloannulation.*

Lab-on-a-chip platforms are attractive tools in modern chemistry[1] with high potential to combine different processes, such as chemical synthesis[2] and analytical monitoring[3] on a single device.[4] This approach opens entirely new possibilities to study chemical processes on small time scales. Owing to the tiny amounts synthesized in microreactors, the intended output of the experiments is mainly information.[5] Minimal resource consumption and vastly accelerated reaction rates of diffusion-limited processes[6] facilitate rapid optimization and investigation of chemical synthesis,[7] for example, screening of catalysts.[8] In organic synthesis intermediate purification procedures are often mandatory, which is especially true for multi-step reactions which usually require at least rudimentary clean-up steps. Chemical syntheses on the microscale are mainly implemented as flow-through reactors,[2a–c,6d,9] because the devices are easily manufactured through established methods of micromachining. In contrast, the integration of continuous sample clean-up techniques subsequent to or in-between the synthetic processes is very challenging.[10] While chemical synthesis and subsequent analytical separation have been successfully combined,[7–9] the implementation of micropreparative purification steps, so that the purified compounds can be used in downstream processes, is more complex. Some remarkable approaches on miniaturized systems were already presented, such as on-chip discontinuous distillation,[11] introduction of chemical scavengers,[12,13] and liquid–liquid extraction.[14] For integration into extended lab-on-a-chip platforms micro-free-flow electrophoresis (μFFE) is especially appealing, because it can be combined seamlessly with continuous flow-reactors.[15]

Monitoring of the μFFE separation processes is however challenging. Bar only a few exceptions using Raman spectroscopy[16] and cell sensors,[17] the most prominent detection method used in μFFE is fluorescence.[18] For μFFE to be useful in the intermediate purification of reaction products it is necessary to establish a detection method suitable to identify reagents and products in organic synthesis. In this respect, coupling of microfluidics to mass spectrometry is the most interesting approach, because it allows the unambiguous identification of substances.[3d,19] Combining free-flow electrophoresis and mass spectrometry is more complex than coupling of MS to common separation techniques, because the analyte bands flow side by side instead of eluting sequentially. Chartogne et al.[20] have shown the coupling of a miniaturized free-flow isoelectric-focusing separation of proteins to mass spectrometry. In this approach a conventional micro-FFE-cell was assembled from plates, membranes, and screws. Coupling to the mass spectrometer was achieved by attachment of capillaries.

Herein we describe the first approach of coupling μFFE with MS directly. An electrospray is generated by a monolithic emitter tip machined into the μFFE borosilicate microchip's end. A schematic overview over the instrumental setup to combine micropreparative FFE fractionation and mass spectrometry is presented in Figure 1.

The schematic chip in Figure 1 A shows the separation bed, which is continuously infused with a sample mixture. By application of an electrical field orthogonal to the flow direction the mixture is separated. This separation leads to analyte bands flowing side by side, where the deflection from the flow direction depends on the electrophoretic mobility and is therefore correlated with the charge and size of the solvated ions. At the end of the separation bed, the compounds are guided towards different outlets. The central outlet, titled MS-transfer channel, leads to the electrospray emitter, which is placed in front of the mass spectrometer's inlet. A central aspect to the chip layout are two crossing channels in the middle of the MS-transfer channel (see Figure 2 C). In this cross, additives can be introduced through the make-up flow channel to aid the electrospray process. Additionally, the channels are used to electrically contact the electrospray and serve as flow splitters to establish a flow rate of approximately 1 $\mu L\,min^{-1}$ at the emitter tip. To adjust the

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[**] This publication is supported by LIFE—Leipzig Research Center for Civilization Diseases, Universität Leipzig. This project was funded by the European Social Fund and the Free State of Saxony.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201409663.

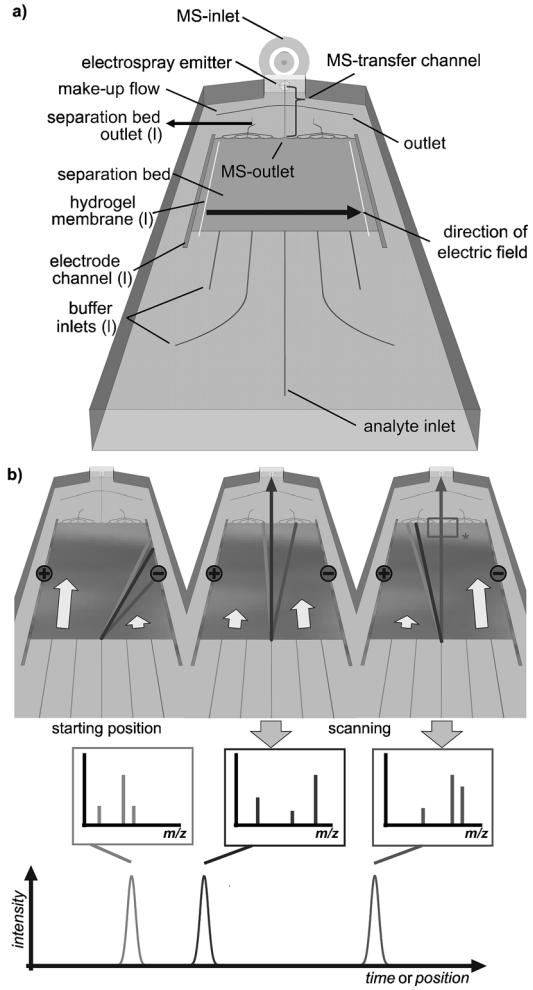

Figure 1. a) Layout of a microfluidic FFE-MS chip (I = left). b) The analysis principle. The separated analytes are directed towards the mass spectrometric outlet by alteration of the buffers' hydrodynamic flow. Arrows indicate relative flow rates and the rectangle (*) labels the area visualized by fluorescence imaging.

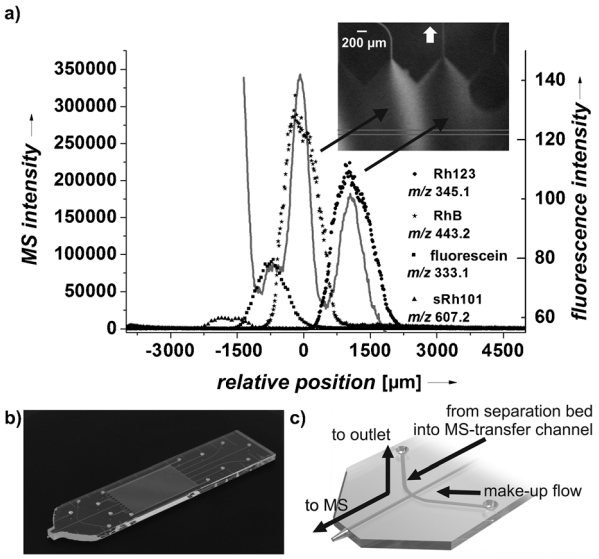

Figure 2. a) Determination of the location of the electrophoretic bands by comparison of MS- and fluorescence data. Rh123, RhB, fluorescein, and sRh101 were introduced into the chip with concentrations of 131, 102, 266, and 160 µmol L^{-1}. Extracted ion currents of protonated molecular ions for the fluorescent dyes as well as fluorescence intensity (in gray) are shown. b) Photograph of the µFFE-MS chip, c) Magnification of make-up intersection. Lengths of arrows are indicating relative flow rates.

flow rate, restriction capillaries of variable length and back pressure are mounted on the outlet channel.

Utilizing the described setup, it is possible to scan the separation bed by means of mass spectrometry. This scanning is achieved by adjusting the flow rates of the buffer streams flanking the analyte bands. While adjusting buffer flow rates a constant electric field is applied to maintain the electrophoretic separation. The separated bands of analytes are sequentially steered to the MS outlet. Since the mass spectra are continuously recorded, the analyte spectra obtained are separated with respect to time. Thereby the spectra also contain positional information about the bands within the separation bed.

The chip layout was initially optimized in polymeric microfluidic chips,[21] and thereafter realized in borosilicate chips, which were equipped with nanospray emitters.[22] To test our strategy, a mixture of fluorescent dyes was used to optimize the approach. To monitor the separation process as well as the transfer of the fluorescent test compounds to the emitter, we placed an epifluorescence microscopic setup underneath the chip. The height of structures within the chips was 20 µm, the channels were 50 µm wide and dimensions of the separation bed were 20 mm × 11.7 mm. For practical reasons the outlets of the separation bed were merged into an anodic and cathodic channel in this prototype. A uniform pressure drop across the outlets was ensured by the attached restriction capillaries. Additionally, the entry of disruptive electrolysis bubbles[18b] into the separation bed was prevented by placement of hydrogel membranes[18a,f] at both sides between the electrode chambers and separation area. For the introduction of those membranes, a silanization step was carried out, which leads to a suppression of the electroosmotic flow as well. Details for setup, modification of the chips, and experiments are included in the Supporting Information.

By separation of a mixture containing rhodamine 123 (Rh123), rhodamine B (RhB), fluorescein, and sulforhodamine 101 (sRh101), the functionality of the system was evaluated. A solution of this mixture was introduced into the separation bed using the analyte inlet. The stream was then directed to the right-hand side by adjusting the individual buffer flow rates (Figure 1b, top). A buffer composition of 2 mM ammonium acetate solution (pH 4) in methanol/water (70:30, v/v) was chosen. Programmable syringe pumps were used to alter the buffer flow rates. The flow rates of the buffers were linearly ramped from 13.2 to 0.4 µL min^{-1} (left) and from 0.4 to 13.2 µL min^{-1} (right). Flow rates of the analyte solution, the electrode stream, and make-up liquid (in this case identical to the buffer) were 0.2, 6.0, and 0.1 µL min^{-1} per channel, respectively. After obtaining stable separation under an applied electric field (−602 V, −33 µA), the flanking buffer

streams were varied as described above to direct the analyte bands from the right to the left side of the separation bed within 150 s. With this procedure each band is positioned toward the mass spectrometric outlet one after another and microscopic fluorescence images (videos) are recorded in parallel to monitor the process. In the displayed fluorescence image of the end of the separation bed, the fluorescein band is located between RhB and sRh101 and obscured by their superior intensity (Figure 2a). Transporting analytes through the MS-transfer channel requires a certain time, which by comparing fluorescence and mass spectrometric data, was determined to be 8 s for this experiment. Since a delay between two arbitrary mass spectra corresponds to a certain distance that the analyte bands moved within the separation bed, a mass spectrometric mapping of the separation is achieved. The extracted ion currents corresponding to the fluorophores are shown in Figure 2a, whereas on the *x*-axis their distance in relation to the RhB band is noted. The split ratio between emitter and make-up outlet was calculated to be 1.4:1.

After successfully demonstrating the concept, we applied the system to study an organic reaction. This is more challenging as most compounds of interest are not fluorescent. To facilitate optical monitoring of the process, a fluorescent dye was added as a reference marker. We chose a multicomponent [3+2]-cycloannulation of anthranilic acid (**1**), benzaldehyde (**2**), and a bis(silyl)dienediolate **3** as a model reaction. This reaction forms a pyrrolobenzoxazinone **4** (Figure 3) and expands our previously described synthesis for pyrrolobenzoxazoles[23] to another group of substances. Preliminary batch synthesis with varying reaction conditions revealed a possible formation of side product **5** by "α-addition".

To a solution consisting of 1.88 μmol **1**; 1.88 μmol **2**; 0.377 μmol $Yb(OTf)_3$ and 30.2 μmol **3**, 24.0 nmol, sRh101 as a reference was added and the diluted sample introduced into the microfluidic chip. In 15 mM ammonium acetate (pH 6.7; 9:1 acetonitrile/water, v/v) this mixture was separated continuously by electrophoresis and in analogy to the above procedure directed across the mass spectrometric outlet within 7.5 min per scan. Consequently a complete mass spectrometric scan of the separation bed was obtained. With the aid of the recorded mass spectrometric traces in the time domain and the position of the fluorescent reference band, the exact location of the electrophoretic bands within the chip can be determined. In these experiments we used a make-up flow consisting of trifluoroacetic acid, acetic acid, water, and methanol in a ratio of 3:1:46:50 (v/v) to improve ionization. In Figure 3 the results of the experiments are summarized. With our approach it was possible to identify and locate the on-chip fractionated compounds. Two distinct bands of product **4** and side product **5** were separated by a distance of (2120 ± 180) μm. The side product **5** loses water during ionization and was detected in MS with an accurate mass of 338.1398 as a constitutional isomer of product **4**. Product **4** was detected with an accurate mass of 338.1440 in comparison. However, the side product **5** exhibits a similar electrophoretic mobility to anthranilic acid **1** so cannot be completely separated from it under these conditions. It is however possible to distinguish

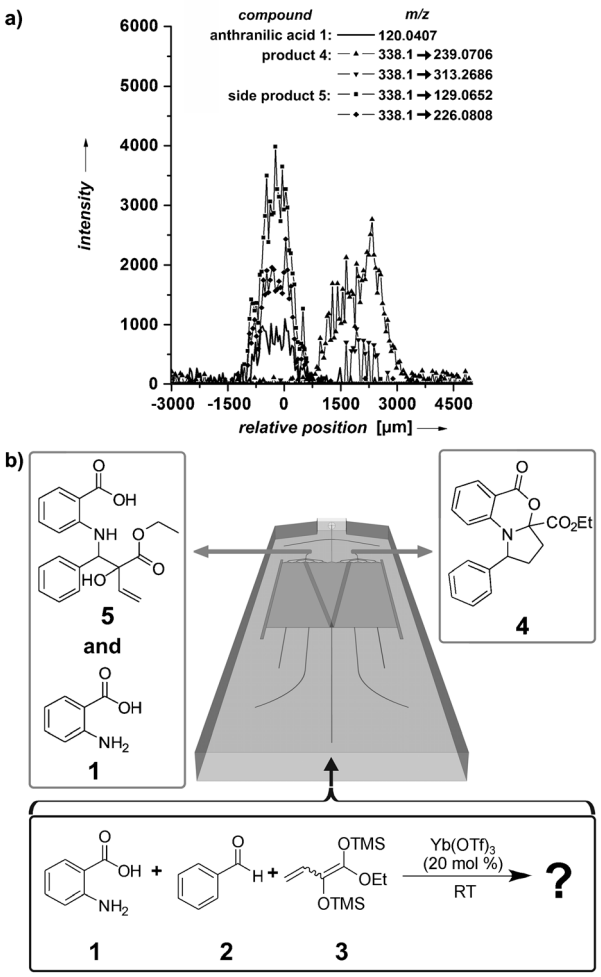

Figure 3. A multicomponent [3+2]-cycloannulation experiment. a) Extracted ion currents of anthranilic acid (**1**) and specific 1st generation fragments of the reaction products **4** and **5**. b) Illustration of obtained fractions.

between the products by using the simultaneously recorded MS^2 data. These results are supported by comparative capillary electrophoretic and stand-alone tandem-MS experiments. In both experiments presented in this work, either analyzing fluorescent dyes or a reaction mixture, transport of the sample thorough the microfluidic chip required less than 2.5 min.

The on-chip coupling of free-flow electrophoresis and mass spectrometry presented herein, enables the monitoring of continuous micropreparative separation, which is especially viable for continuous-flow reactors. A new tool to realize complex chemical circuits is thereby available, which makes the combination of micro-flow-through synthesis, -separation, and analysis possible. This new approach has great potential regarding the implementation of multistep synthesis in highly integrated lab-on-a-chip systems.

Experimental Section

Microfluidic chips were manufactured by iX-factory by etching into borosilicate (BF33) following our layout specifications. Low-pressure

syringe pumps (neMESYS) and an Agilent Technologies G6520 Q-TOF LC/MS were employed. Fluorescence images were recorded using a portable microscope system (TSO Thalheim Spezialoptik) based on our conceptions including LED (Seoul 3.5W, 525 nm, LED-TECH.DE optoelectronics), FITC/Cy3/Cy5-filter set or Cy3-filter set (86016 & U-N41007, Chroma Technology) and a color camera (WAT221S, Watec). A voltage of −218 V (−159 μA) was applied for the separation of the [3+2]-cycloannulation reaction mixture and flow rates for analyte, buffer (left), buffer (right), make-up flow, and electrodes of 0.08; 12.2→0.7, 0.7→12.2; 0.18, and 6.0 μL min^{-1}, respectively, were used. More detailed parameters are included in the Supporting Information. Statistical data are calculated with a confidence level of 0.95 for 3 repetitions.

Received: October 1, 2014
Revised: November 12, 2014
Published online: January 21, 2015

Keywords: cycloaddition · continuous micro flow-through · electrophoresis · mass spectrometry · microfluidics

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