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Short Communication

An integrated on-chip sirtuin assay

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Abbreviations: **HPMC**, hydroxypropylmethylcellulose; **Sir**, silent information regulator; **SIRT**, sirtuins

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A microchip-based assay to monitor the conversion of peptide substrates by human recombinant sirtuin 1 (hSIRT1) is presented. For this purpose a fused-silica microchip consisting of a microfluidic separation structure with an integrated serpentine micromixer has been used. As substrate for the assay we used a 9-fluorenylmethoxycarbonyl (Fmoc)-labeled tetrapeptide derived from the amino acid sequence of p53, a known substrate of hSIRT1. The Fmoc group at the N-terminus resulting from solid phase peptide synthesis enabled deep UV laser-induced fluorescence detection with excitation at 266 nm. The enzymatic reaction of 0.1 U/ μ L hSIRT1 was carried out within the serpentine micromixer using a 400 μ M solution of the peptide in buffer. In order to reduce protein adsorption, the reaction channel was dynamically coated with hydroxypropylmethyl cellulose (HPMC). The substrate and the deacetylated product were separated by microchip electrophoresis on the same chip. The approach was successfully utilized to screen various sirtuin inhibitors.

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6 Sirtuins, also called class III histone deacetylases, represent a family of nicotinamide
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8 adenine dinucleotide (NAD⁺)-dependent enzymes that catalyze the deacetylation of acetyl-
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10 lysine residues of histones, transcription factors and other proteins. The catalytic
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12 mechanism includes a transfer of the acetyl group to the cofactor NAD⁺ yielding
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14 nicotinamide, 2'-O-acetyl-ADP-ribose and deacetylated protein as products [1,2,3]. At
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16 least seven members of this protein family have been identified in humans (hSIRT1-
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18 hSIRT7) [4], which differ in their cellular location and their respective substrates. Class III
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20 deacetylases have been associated with cellular functions that are involved in age-related
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22 diseases, e.g. obesity, type-II diabetes and neurodegenerative disorders like Alzheimer's
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24 and Parkinson's disease [2]. However, the whole complexity of biological pathways and
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26 mechanisms is not completely understood up to now. Therefore, it is important to develop
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28 powerful screening methods to investigate activities, substrate specificities or the
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30 effectiveness of potential activators and inhibitors.
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37 In the literature several assays for sirtuin enzymes have been reported. Early approaches
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39 monitored the release of radioactive products derived from either ³[H]-acetylated
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41 substrates [1,5,6,7] or ¹⁴[C]-NAD⁺ and ³²[P]-NAD⁺, respectively [8,9]. Later developments
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43 mainly utilized fluorescence detection, e.g. the commercially available *Fluor de Lys*TM-
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45 assay. It is based on the deacetylation of acetylated tetrapeptides labeled with 7-amino-4-
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47 methylcoumarin at the C-terminus. After enzymatic conversion the product becomes a
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49 substrate for trypsin, generating an increase in fluorescence by the release of the
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51 fluorophore [10,11,12]. An alternative method using Z-Mal as a substrate was shown by
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53 Heltweg et al. [13,14]. Other approaches include the separation of acetylated peptides and
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55 their respective deacetylated products by high performance liquid chromatography (HPLC)
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57 after enzymatic reaction [1,9,15]. Furthermore, Marcotte et al. reported on a fluorescence
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59 resonance energy transfer (FRET)-based assay using peptides modified with a quencher
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3 at the N-terminus and a fluorophore at the C-terminus [16]. Liu and coworkers used
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5 Caliper's sipper chips to realize a microfluidic mobility shift assay for the determination of
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7 K_m and IC_{50} values [17]. They also employed luciferase in a bioluminescence assay [17]
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9 based upon quantitation of remaining NAD^+ . Moreover, the deacetylation of proteins has
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11 been studied by means of an enzyme-linked immunosorbent assay (ELISA) [18]. Scriba's
12
13 group demonstrated the determination of kinetic and inhibition parameters of hSIRT1 and
14
15 hSIRT2 establishing a validated capillary electrophoresis (CE) method. As substrates
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17 9-fluorenylmethoxycarbonyl (Fmoc)-labeled peptides were synthesized and evaluated
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19 [19,20]. Recently, Denu's group developed a spectroscopic assay capable of determining
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21 steady-state kinetic parameters and continuous product monitoring. Formed nicotinamide
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23 is converted on a microplate by additional enzymatic and oxidative steps yielding $NAD(P)^+$
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25 [21]. Finally, a fluorogenic assay based on the quantification of remaining NAD^+ was
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27 published. In this approach the remaining cofactor is converted to a highly fluorescent
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29 compound with a two-step fluorogenic reaction [22].
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36 Although various analytical approaches are already described in the literature, there is a
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38 need for very fast methods. In this context chip-based microfluidic techniques integrating
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40 chemical reactions and analysis on a single device are very attractive [23,24]. An ideal
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42 technique for high speed on-chip separations is chip electrophoresis enabling separations
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44 within seconds or even subseconds [25, 26, 27] applying very high field strengths. In
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46 previous work we demonstrated a first approach for integrating chemical reactions and
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48 analysis on a single microchip for the screening of enantioselective biocatalysts [28].
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52 In the current work we extended this approach to develop a rapid microfluidic assay to
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54 investigate the deacetylation of acetyl-lysine residues by sirtuin enzymes. The technique
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56 was evaluated for the identification of potential inhibitors.
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59 The peptidic structure of the substrate derives from the amino acid sequence of the
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transcription factor p53 which is a known substrate for the enzyme hSIRT1. The positions

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3 379-382 of this protein are RHKK(Ac), where acetylation and deacetylation appear at
4 lysine 382 [29]. In order to monitor the hSIRT1-catalyzed conversion of acetylated lysine
5 residues a tetrapeptide based on this amino acid sequence and the respective
6 deacetylated peptide have been synthesized as described elsewhere [19]. After synthesis
7 the Fmoc group was not cleaved from the N-terminus providing a fluorophore suitable for
8 deep UV laser excitation at 266 nm for both peptides, the substrate Fmoc-RHKK(Ac)-NH₂
9 and the product Fmoc-RHKK-NH₂. The experimental determination of fluorescence
10 properties ($\lambda(\text{exc.}) = 266 \text{ nm}$) of all compounds involved in the reaction was carried out
11 (data not shown). For the peptide substrate and its deacetylated analog a significant
12 fluorescence emission with $\lambda_{\text{max}}(\text{em.}) = 310 \text{ nm}$ was observed due to the fluorophoric
13 group Fmoc. In contrast, no fluorescence signal was detected for the cofactor NAD⁺ and
14 for nicotinamide.

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31 For on-chip experiments a system based on a commercially available inverted
32 fluorescence microscope IX-71 (Olympus, Hamburg, Germany) has been used that was
33 upgraded to allow laser-induced fluorescence detection with excitation at 266 nm as
34 described earlier [30]. The chip design as well as the working principle of the fused-silica
35 microchip (Micronit, Enschede, Netherlands) is illustrated in Figure 1. The microfluidic
36 structure was manufactured by common isotropic etching with hydrofluoric acid. It mainly
37 consists of two functional parts. A simple cross injector including a 70 mm long separation
38 channel is used for the electrophoretic separation of reaction products. A long meandering
39 channel linked to the separation part serves as microfluidic reactor where enzymatic
40 conversions are performed. At the end of each channel access holes are implemented to
41 access the microfluidic structure. These microvials are labeled sample outlet (SO),
42 alternative sample inlet (SI), buffer inlet (BI), buffer outlet (BO) and sample inlets (A and
43 B).

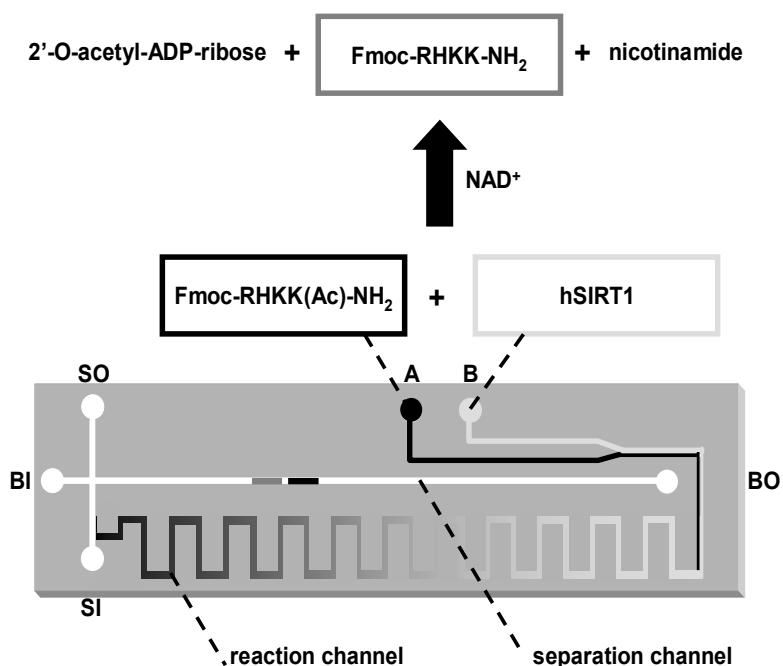


Figure 1. Schematic drawing of the microchip illustrating the working principle. SO: sample outlet, SI: alternative sample inlet, BI: buffer inlet, BO: buffer outlet, A and B: sample inlets.

To prepare the microchip for electrophoretic measurements all channels were initially rinsed with 0.1 M NaOH and deionized water for 4 min. Afterwards, the surface of all microchannels was coated dynamically with HPMC in order to improve the performance and reproducibility of the electrophoretic separation. Therefore, the microfluidic structure was filled with a solution containing 0.025 % HPMC (m/v) in water for 5 min.

The electrophoretic separation of a standard mixture of both peptides containing 200 μ M of each analyte was optimized, especially by evaluating different separation buffers. Baseline separation was achieved utilizing a 200 mM Tris-phosphate buffer at pH 2.7 After optimization of the voltage program for a pinched injection the electropherogram shown in Figure 2 was obtained using the electrical potentials given in the caption.

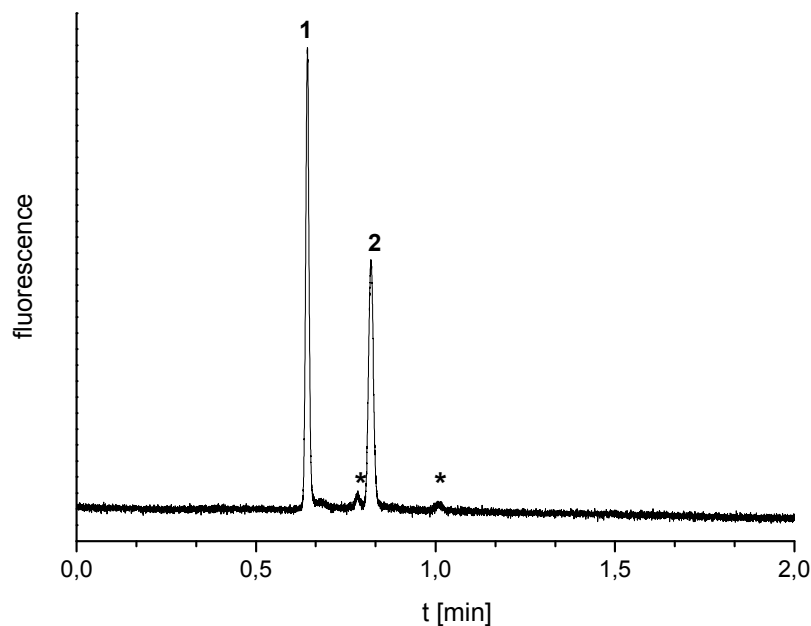


Figure 2. Electropherogram of a mixture of the estimated product Fmoc-RHKK-NH₂ (**1**) and the acetylated substrate Fmoc-RHKK(Ac)-NH₂ (**2**) obtained at a concentration of 200 μ M each. (*) impurities due to solid phase peptide synthesis. Buffer, 200 mM Tris-phosphate, pH 2.7. Effective separation length 2.7 cm. Injection potentials, BI -0.4 BO 0.8 A/B 4.0 SO -0.8 (kV). Separation potentials, BI 2.4 BO 0.0 A/B 1.4 SO 2.0 (kV). Channel surface dynamically coated with HPMC.

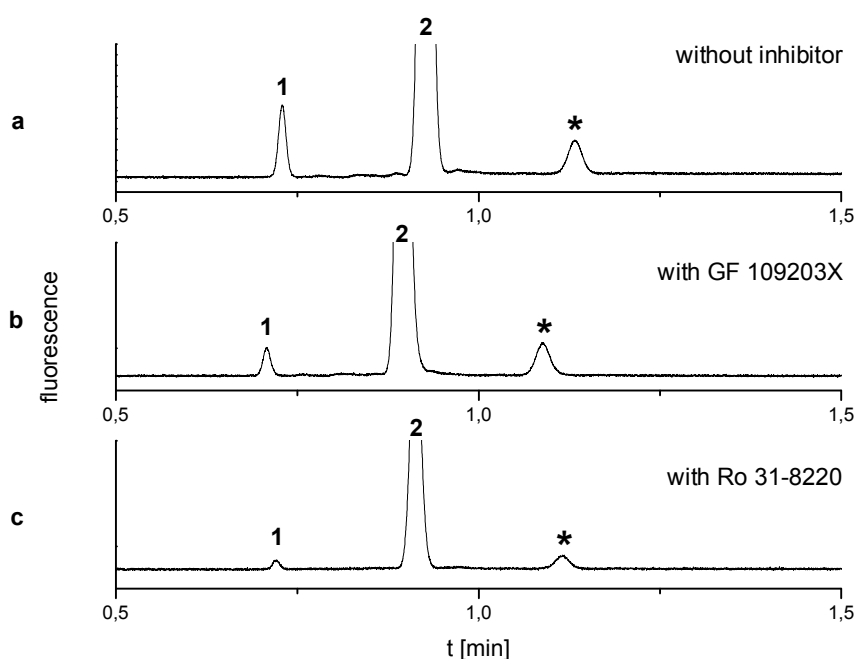
The detection point was set to a separation length of 2.7 cm allowing baseline-separation of both compounds ($R_S = 7.9$) in less than one minute. In order to investigate the reproducibility of the system repetitive separations ($n = 4$) were performed and the peak area ratios of the signal of the starting material to that of the product were calculated. In these experiments we obtained quite acceptable relative standard deviation of 2 % at peak area ratios of about 0.78. Peak assignment was realized by common standard addition. The signal marked with **1** ($t_m = 0.64$ min, $N = 18930$) corresponds to Fmoc-RHKK-NH₂ which has a shorter migration time due to the additional charge and thus higher electrophoretic mobility. Signal **2** ($t_m = 0.82$ min, $N = 15831$) can be attributed to Fmoc-

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3 RHKK(Ac)-NH₂. In all experiments the signal for the respective peptide was accompanied
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5 by a small impurity at longer migration times. These signals are marked with * in Figure 2.
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8 We assume these impurities derive from solid phase peptide synthesis where the purity of
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10 all peptides was greater than 97% as determined by analytical HPLC [19].
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12 Based on these results the separation procedure was combined with the on-chip reaction
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14 i.e. the formation of deacetylated product within the meandering reaction structure. For the
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16 enzymatic reaction it is important to minimize any activity loss of the rather delicate
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18 enzyme hSIRT1. In order to ensure physiological working conditions the reaction solutions
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20 were incubated at a temperature of 37 °C (20 min) before introduction into the chip. In
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22 initial experiments we observed a decrease of enzyme activity using bare fused silica
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24 chips, which was attributed to protein surface interactions. The problem of enzyme
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26 deactivation by adsorption is also apparent in traditional macroscopic methods, where
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28 BSA is often added to the reaction medium at rather high concentrations to prevent
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30 enzyme adsorption to the surface of the reaction vessel. However, this approach is hardly
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32 adaptable for microchip electrophoresis as the addition of BSA at high concentrations
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34 leads to plugged microchannels resulting in non-reproducible results. We therefore
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36 evaluated the common dynamic surface modification procedure applied in chip
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38 electrophoresis [31] by simply rinsing the channels with a solution of a hydrophilic surface
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40 active polymers like hydroxypropylmethylcellulose (HPMC). Applying such a dynamic
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42 HPMC-coating before each experiment improved the reproducibility of the reaction and the
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44 activity of the enzyme significantly. A considerably higher signal of deacetylated peptide
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46 was obtained in comparison to the results using a bare channel surface. For on-chip
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48 experiments a substrate solution with 400 μM Fmoc-RHKK(Ac)-NH₂ and 2 mM NAD⁺ was
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50 employed. Furthermore, the enzyme solution had a concentration of 0.1 U/μL hSIRT1.
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52 Both solutions were prepared in Tris buffer (50 mM, pH 8.0), containing 137 mM NaCl,
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54 2.7 mM KCl and 1 mM MgCl₂. After pipetting the reactant solutions in the respective
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3 microvial A or B (Figure 1), vacuum was applied at SI in order to merge the liquids through
4 the meandering reaction channel. Afterwards the other channels and microvials were filled
5 with separation buffer (200 mM Tris-phosphate buffer, pH 2.7). The reaction products were
6 then transported to the separation channel by applying voltage at the access holes A, B,
7 BI, BO and SO. After 90 s of this pinched injection program [32] the potentials were
8 switched to separation mode. The fraction of the reaction mixture in the cross section was
9 injected into the separation channel followed by subsequent separation in the electric field.
10 In contrast to most assays described in literature the reaction was not stopped by the
11 addition of TFA [16] or equivalent substances. Specific reaction times were determined by
12 switching the voltage program to separation mode and thus initiating the injection of a
13 sample plug. With the current not optimized setup with rather long reaction channels the
14 minimum reaction time monitored was about 10 min.
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56 **Figure 3.** Electropherograms obtained after on-chip deacetylation in the presence of two
57 different inhibitors compared to the result of a reaction without inhibitor:
58 Fmoc-RHKK-NH₂ (1), Fmoc-RHKK(Ac)-NH₂ (2), (*) impurities due to solid phase peptide
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3 synthesis. Conditions are as described in the caption of Figure 2. Zoom factors of all
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5 electropherograms are identical.
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10 An electropherogram obtained after on-chip enzymatic conversion with subsequent
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12 electrophoretic separation using coated microchannels is shown in Figure 3a. Retention
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14 times were 0.73 min (N = 20464) for Fmoc-RHKK-NH₂ and 0.93 min (N = 14164) for
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16 Fmoc-RHKK(Ac)-NH₂ and thus analogous to the results displayed in Figure 2. **The inter-**
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18 **day variations in migration times comparing figure 3 and figure 2 can be explained by**
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20 **variations in temperature and also by a slight variation in the manual alignment of the**
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22 **channel in the ray path of the homebuilt system.**
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27 The setup allowed to monitor the activity of the enzyme quite well. Moreover, we evaluated
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29 the applicability of the system to the screening of inhibitors. For these proof of principle
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31 experiments three known inhibitors for sirtuin enzymes were chosen, namely GF 109203X
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33 [33], Ro 31-8220 [33] and sirtinol [34]. Inhibition studies were carried out by adding 200 μM
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35 of the respective inhibitor to the substrate solution. To guarantee comparable results on-
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37 chip experiments were performed using similar reaction times. Electropherograms
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39 obtained after on-chip deacetylation in presence of GF 109203X and Ro 31-8220 are
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41 shown in Figure 3b and Figure 3c. Resulting migration times and plate numbers were
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43 consistent with the data acquired for reactions without inhibitor. **After calculating the peak**
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45 **area (starting material) to peak area (product) ratio values of 17.8 (without inhibitor), 37.1**
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47 **(with GF 109203X) and 43.1 (with Ro 31-8220) were obtained. Taking into account that**
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49 **the standard deviation is about 2% as shown above the decrease of the product signal in**
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51 **the presence of an inhibitor can be regarded as significant.** Additionally it can be stated
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53 that Ro 31-8220 is a more effective inhibitor for hSIRT1 than GF 109203X which is in
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55 agreement with the literature [20,33]. When the on-chip reaction was performed in
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57 presence of sirtinol no inhibition was observed. In the literature controversial reports on the
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3 inhibitory effect of sirtinol concerning hSIRT1 are published depending on the utilized
4 method. Referring to Grozinger et al. [34] sirtinol is an inhibitor for yeast Sir2p *in vivo* as
5 well as for yeast Sir2p and hSIRT2 *in vitro*. Moreover, sirtinol showed no evidence for
6 inhibition of hSIRT1 as reported by Sauve and coworkers [35]. However, several reports
7 have also been published demonstrating a weak inhibitory effect of sirtinol on hSIRT1
8 [12,14,20].

9
10 Herein we have demonstrated a rapid microfluidic method to monitor the deacetylation of
11 acetyl-lysine residues by sirtuin enzymes. A tetrapeptide based on the amino acid
12 sequence of p53 was synthesized and used as substrate for the sirtuin hSIRT1. For the
13 first time all assay steps, including enzymatic conversion, separation of reaction products
14 and detection were performed within a single microfluidic chip. Three known sirtuin
15 inhibitors, namely GF 109203X, Ro 31-8220 and sirtinol were screened using the
16 presented method, the results were in good agreement with the literature. In our chip-
17 based approach the resulting total analysis times maintained rather short compared with
18 already established and published techniques.

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