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Improved native UV laser induced fluorescence detection for single cell analysis in poly(dimethylsiloxane) microfluidic devices

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Abstract

Single cell analytics is a key method in the framework of proteom research allowing analyses, which are not subjected to ensemble-averaging, cell-cycle or heterogeneous cell-population effects. Our previous studies on single cell analysis in poly(dimethylsiloxane) microfluidic devices with native label-free laser induced fluorescence detection [W. Hellmich, C. Pelargus, K. Leffhalm, A. Ros, D. Anselmetti, Electrophoresis 26 (2005) 3689] were extended in order to improve separation efficiency and detection sensitivity. Here, we particularly focus on the influence of poly(oxyethylene) based coatings on the separation performance. In addition, the influence on background fluorescence is studied by the variation of the incident laser power as well as the adaptation of the confocal volume to the microfluidic channel dimensions. Last but not least, the use of carbon black particles further enhanced the detection limit to 25 nM, thereby reaching the relevant concentration ranges necessary for the label-free detection of low abundant proteins in single cells. On the basis of these results, we demonstrate the first electropherogram from an individual S*podoptera frugiperda* (Sf9) cell with native label-free UV-LIF detection in a microfluidic chip.

Keywords: Native laser induced fluorescence; Protein; Microfluidic; Single cell analysis

1. Introduction

For proteom research, effective, sensitive, reproducible and rapid separation and detection techniques are essential and thus a subject of intense investigations. Microfluidic devices have the potential to fulfil these requirements, which is impressively demonstrated by the transfer of proteom relevant separation techniques to the microfluidic format such as protein electrophoresis, isoelectric focusing and two-dimensional protein separation techniques [1–4]. The coupling to mass spectrometers is also feasible, while state-of-the-art concepts for chip-MS coupling have been published recently [5]. This high potential for proteom research also evoked interest for cell analysis in microfluidic chips. Several studies have focused on subjects such as mechanical or dielectrophoretical cell manipulation or flow cytometry [6] as well as cell culturing and subsequent analysis of cell compounds [7,8], thereby handling cell cultures on the level of 10^3 cells. However, averaging effects from cell-cycle dependent states, the different and inhomogeneous cellular response to external stimuli, or the introduction of genomic and proteomic variabilities during cell proliferation are difficult to address upon studying small cell ensembles. Novel and efficient single cell analysis tools are therefore mandatory and have the potential to provide considerable insights for proteom research as well as systems nanobiology.

For single cell analysis, microfluidic devices with characteristic length scales of 10–100 μm are predestined due to typical cell dimensions of several micrometers. In order to significantly contribute to proteomic research, low abundant proteins with copy numbers $<10^5$ molecules in a single cell must be detectable. This would require detection with a sensitivity in the range less than 100 nM considering 10^5 molecules in a cell of 10 μm diameter (corresponding to the volume of one pL) [9]. Thus, single cell analysis in microfluidic format demands new techniques for efficient and sensitive separation and detection.

In capillary format, such as capillary electrophoresis, label-free laser induced fluorescence (LIF) detectors for proteins based on the UV fluorescence of the three amino acids tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) have been reported with ultimate sensitivity. In 1992, Yeung and co-workers pioneered a LIF detection method with 275 nm excitation light provided by an Ar^+-laser with pM detection limits [10]. This method has further served for the exocytose monitoring of single mast cells [11] as well as for the separation of hemoglobin

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variants in red blood cells [12]. Exploiting alternative laser systems, nM detection limits for Trp could be achieved with a metal vapor laser [13] or an excimer laser [14]. With solid state lasers, nM detection limits of peptides [15] and proteins [16], as well as in the pM range for carbonic anhydrase [17] were reported.

With LIF in the visible range, first single cell fingerprinting with capillary sieving electrophoresis in one-dimensional [18,19] and two-dimensional format [20] using a fluorescent protein stain was pioneered by the group of N. Dovichi. Recently, microfluidic devices have been explored for separation and detection of fluorescent dyes [21,22] and a specific small peptide [23] or vitamin [24] from single cells.

More recently, we have proposed a method for single cell analysis combining navigation and steering of single cells with optical tweezers, on-chip cell lysis and electrophoretic separation of proteins with subsequent detection in the visible range by LIF [9,25]. While LIF in the visible range requires adequate on-chip or off-chip labelling steps for the cell component of interest, LIF detection in the UV spectral range allows a direct label-free detection of proteins. However, label-free UV-LIF has only recently been demonstrated in microfluidic devices [9,26] and the detection sensitivity in the μM range has to be improved, in order to address relevant low abundant protein variability in a single cell.

In this paper, we focus on the improvement of LIF detection in the UV spectral range in microfluidic device format with special emphasis on surface design, detection efficiency and background reduction in poly(dimethylsiloxane) (PDMS) microfluidic devices based on our previous experiments [9,25]. We report on improved characteristics that enable protein fingerprinting from single cells in the future and, present the first single cell electropherogram with UV-LIF detection in a microfluidic device.

2. Experimental

2.1. Chemicals and reagents

PDMS (Sylgard 184), was purchased from Dow Corning (USA). Quartz cover slides (60 mm × 26 mm × 0.2 mm) were from Aachener Quarzglas Technologie Heinrich (Germany). Pullulan, tryptophan, the proteins lysozyme C and trypsinogen were obtained from Sigma–Aldrich (Germany). Spodoptera frugiperda (Sf9) insect cells were from Novagen (Germany). Triblock copolymer Pluronics F-108 was a generous gift from BASF (Germany). Disodium hydrogenphosphate dehydrate, (cyclohexylamino)ethanesulfonic acid (CHES) and (hydroxymethyl)aminomethane (Tris) were purchased from Fluka (Germany). SU-8 (25) negative photoresist and developer propyleneglycolmethylether acetate were obtained from Microresist (Germany). Si-wafer (P-Type 100, doped with boron) were from CrysTec (Germany). Tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorsilane (TTTS) and Black Pigment (Conductive) were from ABCR (Germany). For all solutions deionized water from a Milli-Q biocel (Millipore, USA) was used.

2.2. Fabrication of the PDMS devices

The fabrication process can be divided into (i) the production of the master wafer with the desired microstructures and (ii) the fabrication of the PDMS/quartz microfluidic chip itself.

The detailed procedure for the production of the masterwafer was recently published by Duong et al. [27], therefore, only the main steps are briefly depicted. First of all, a silicone wafer was spincoated with a negative photoresist SU-8 (25) before UV exposure through a chromium mask with the desired layout. After development in a developer bath and the hard baking the SU-8 master wafer is silanized with TTTS in a vacuum exsiccator for 30 min to enable repetitive casting of PDMS. For the PDMS mould Sylgard 184 and its curing agent were mixed in a ratio of 10:1 and poured over the wafer. After curing at a temperature of 85 °C for 4.5 h the thin PDMS layer was easily peeled off the wafer and reservoir holes were punched through the structured side for fluid access. Before assembly the structured PDMS slab and the quartz cover slide were exposed to an oxygen plasma for 30 s in a home-built glow discharge unit [28]. Once the two exposed surfaces are brought into contact an irreversible seal was formed. The microchannel cross-sections were typically 20 μm × 20 μm for protein and 30 μm × 30 μm for cell analysis, respectively.

2.3. Fluorescence detection

For sensitive LIF detection in the UV range (UV-LIF), an inverted microscope (Axiovert 100, Zeiss, Germany) served as a platform. For excitation the wavelength of a frequency quadrupled Nd:YAG laser (266 nm, 5 mW, Nanolase, France) was coupled via two mirrors (New Focus, USA) into the rear port of the microscope. A transmission filter (AHF Analysentechnik, Germany) reduced the laser intensity to 10% of the incoming beam installed at the filter cube of the microscope. Optionally, further decrease of the laser intensity was achieved with various grey filters (50% and 3% from AHF (Germany) and 30% and 50% from Melles Griot (France)) installed before laser passage into the rear port. The transmitted excitation light was reflected by a dichroic mirror (Laseroptik, Germany) and focused by a ×40 UV-transparent objective (Optics for Research; USA) into the microchannel. A motorised *x*/*y*-stage (Märzhäuser, Germany) allowed exact positioning of excitation and detection window at various distances from the injector (usually several mm) along the separation channel. The emitted fluorescence was collected by the same objective, passed through the dichroic mirror and spectrally filtered by an interference filter (360/50, AHF, Germany). A high transmission UV tube lens (Zeiss, Germany) focused the light through a *x*/*y* adjustable pinhole onto the commercial photomultiplier built-in module (H6240, Hamamatsu, Japan), supplied with 5 V from an external power supply. Pinholes of various diameters (50–1000 μm) were used as spatial filters for confocal detection.

2.4. Chip operations

Initial filling of the microchannels was performed either by capillary action or by applying vacuum to the reservoirs. For

protein separation, the microchannels were primarily filled with a 500 μM F108 solution (10 mM phosphate buffer pH 8.6). After an incubation of 20 h the channels were thoroughly washed. For all injections, 10 mM phosphate buffer (pH = 8.1) was used.

For an increased reservoir volume a 5 mm thick PMMA block with corresponding access holes was put on top of the microfluidic chip. Voltage was applied via four platinum electrodes dipped into the reservoirs using power suppliers from FUG (model HCN 14-12500 and HCN 7E-12500, Germany). Instrumental control and data acquisition were performed by software programmed in LabView (National Instruments, USA). For investigation of the optimal pinhole diameter a 250 nM Trp solution was injected into a single microchannel. A voltage of 1500 V was applied to the 20 mm long channel. The fluorescence signal was recorded by pumping the Trp solution electrokinetically along the detection window. For all other experiments the pinched injection was used.

Single experiments with individual Sf9 cells were performed as recently published [9,25]. Briefly, a cell is trapped, navigated and steered along the microdevice by optical tweezers. One or four cells are electrically lysed by a short pulse of high electric field strength (50 ms at 1900 V/cm) and thus injected into the separation channel. Separation of cell compounds is performed in separation buffer (100 mM Tris, 100 mM CHES, 4% Pullulan, pH 8.4) at a field strength of 240 V/cm for one cell and at 360 V/cm in the case of four cells at a distance of 2.5 mm from the cross injector and detected by UV-LIF.

3. Results and discussion

3.1. Optimisation with microchannel coating

On the basis of recently developed coatings with triblock copolymers on PDMS surfaces [28] we extend our studies and investigate the performance of electrophoretic separations with such coatings. Our data revealed good surface coverage and stable electroosmotic flow over a week for the triblock copolymer F108. More precisely, the benefit of F108 coatings on the PDMS/quartz surfaces is investigated here under electrophoretic separation conditions.

Electropherograms of the fluorescent amino acid Trp were performed at a 10 μM concentration with an electric field strength of 440 V/cm. The migration time extends to 28.6 s in the case of F108 coating compared to 16.4 s in non-coated channels. This is in agreement with our previous study [28], which indicated a reduction to 50% of the electroosmotic mobility in coated PDMS/quartz microfluidic channels.

Furthermore, we investigated the separation of a model protein sample with native UV-LIF detection of lysozyme C and trypsinogen in a coated PDMS device (Fig. 1). A baseline resolved separation of the two proteins at an electric field strength of 463 V/cm and an analyte concentration of 50 μM was achieved. Injections of the two individual protein samples verified the migration order (data not shown) and served as a control. For lysocyme C, a signal-to-noise ratio of 123 is obtained, so that the estimated detection limit with a signal-to-noise ratio of three results in 1 μM. This result, obtained in a PDMS/quartz microfluidic chip, is in very good agreement with a previously reported detection limit of 0.9 μM lysocyme C in a full-body quartz chip [26]. Furthermore, the separation efficiency in an uncoated PDMS/quartz chip as previously reported [9] could be enhanced by a factor of three. The actual plate height H as calculated from the lysozyme C signal for the present separation is 46 μm (corresponding to a plate number of 730). This is in reasonable agreement with previously reported data for the injection of fluorescently labelled bovine serum albumin (BSA) in a PDMS microfluidic device [29] at equal separation distance (plate height of 30 μm).

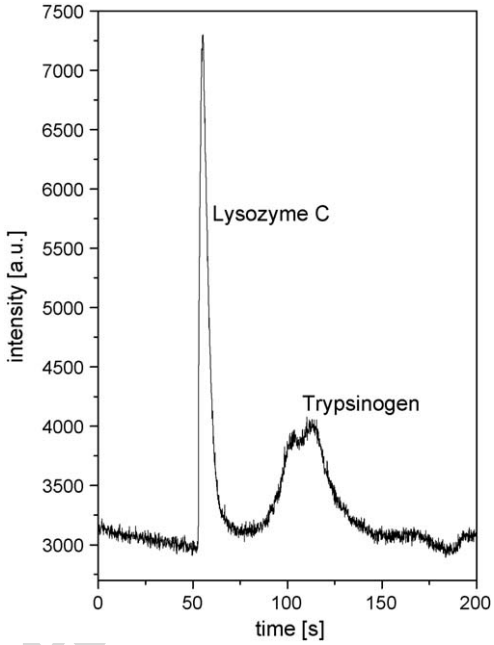

Fig. 1. Separation of lysozyme C (50 μM) and trypsinogen (50 μM) in a 20 μm × 20 μm PDMS microchannel (pinched injection; separation electric field strength 463 V/cm, detection distance 3.5 cm).

3.2. Optimisation of detection sensitivity

Background fluorescence from PDMS is expected to be one major source of limited detection sensitivity in PDMS/quartz chips. In order to corroborate this hypothesis we reduced the incident laser power from 5 mW to 1.1 μW compared to our previous studies on UV-LIF detection in PDMS devices [9]. Fig. 2 demonstrates a calibration curve for Trp injections in a concentration range from 100 nM to 10 μM with a Trp electropherogram at a concentration of 1 μM in the inset. The linear regression with a regression factor of 0.997 shows a very good linearity within this concentration range. As expected the background could be reduced to 605 ± 81 counts which corresponds to a reduction by a factor of 6.4 compared to our previous study [9].

Further, we investigated the influence of spatial filtering on the detection sensitivity in our confocal setup by varying the pinhole diameter. Fig. 3 demonstrates the background corrected signal against the pinhole diameter obtained with electrokinetic injections of 250 nM tryptophan solutions into a single

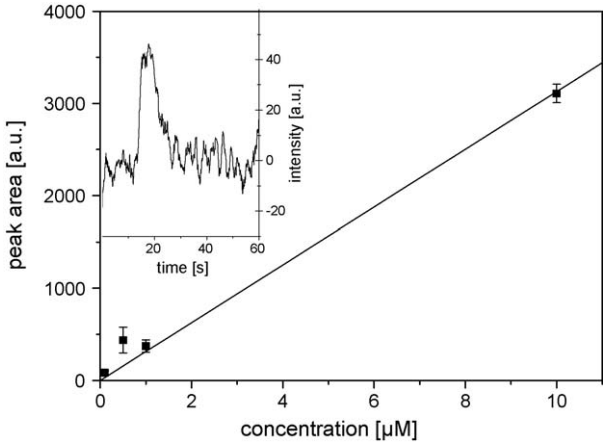

Fig. 2. Peak area vs. tryptophan (Trp) concentration for injections in a PDMS microchannel with linear regression ($r = 0.997$). Inset: Electropherogram of 1 μM injected Trp (detector at 20 mm; pinched injection; electric field strength for separation 440 V/cm, laser power 1.1 μW). Original data is baseline corrected and smoothed with 10 point binning.

PDMS/quartz microchannel. An optimized pinhole diameter for clear PDMS (rectangular symbol) results in 400 μm corresponding to a calculated confocal volume with dimensions of 34 μm and 12 μm, respectively, according to reference [30]. This is in agreement with the experimentally verified laser spot size of 12.7 ± 1.0 μm, which is measured by recording the fluorescence signal of a 1 mM tryptophan solution between two quartz slides for different intensities and exposure times of the charge-coupled device camera (CCD). Although the calculated depth of the confocal volume is slightly larger than the channel depth other pinhole sizes did not result in enhanced fluorescence signals.

Additionally, the sensitivity of the LIF system was further investigated by incorporating carbon black particles into PDMS resulting in black microchip PDMS bodies. Fig. 3 illustrates the background corrected fluorescence intensity versus pinhole diameter for black PDMS (triangle symbol) with a carbon black to PDMS ratio of 1:100. In contrast to clear PDMS, the signal

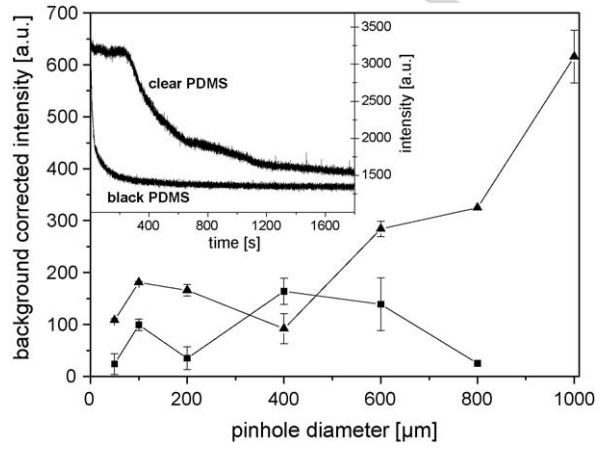

Fig. 3. Background corrected fluorescence signal vs. pinhole diameter: Injections of 250 nM tryptophan solution in a clear (squares) and a carbon black (triangle) PDMS microdevice. Inset: Fluorescence background decay vs. time for clear and black PDMS.

increases steadily with larger pinhole diameter to a maximum at 1000 μm. Compared to clear PDMS with a 400 μm pinhole, the background corrected fluorescence intensity increases by a factor of nearly four for black PDMS with a pinhole of 1000 μm. This difference is attributed to a lower background signal due to light absorption of the incorporated carbon black which is in good agreement with data previously reported by Johnson-White and Golden [31], where a 66% reduction of the background signal when introducing carbon black at a ratio of 1:25 into uncured PDMS was reported.

Significant fluorescence background bleaching was observed for clear PDMS, which only stabilised to constant values after as much as 30 min (Fig. 4 inset). In contrast, PDMS with carbon black showed a rapid decline in fluorescence background which stabilised to constant values at around 400 s and the noise is reduced to approximately 20%. The incorporation of carbon black into PDMS thus represents a significant improvement for UV fluorescence detection.

Finally, the detection sensitivity was investigated with the use of optimized pinhole sizes for 100 nM tryptophan solutions. Fig. 4 demonstrates Trp injections with a pinhole size of 400 μm for clear PDMS and 1000 μm for PDMS with carbon black, respectively. Clearly, 100 nM Trp can be detected in the case of clear PDMS with a S/N ratio of three. Compared to the previously reported detection limit of 17 μM [9], this result indicates an increase in detection sensitivity by approximately two orders of magnitude. Moreover, the electropherogram of injected Trp in black PDMS results in a signal well above the detection limit (see Fig. 4b). Here, a signal-to-noise ratio of 12 is achieved resulting in a theoretical detection limit of 25 nM tryptophan (for S/N = 3). This represents an enhanced detection sensitivity for Trp of at least an order of magnitude compared to previously reported data by Schulze et al. on a full-body quartz microchip [26], thus comprising to our best knowledge, the lowest Trp concentration electrokinetically injected and detected in a microfluidic device.

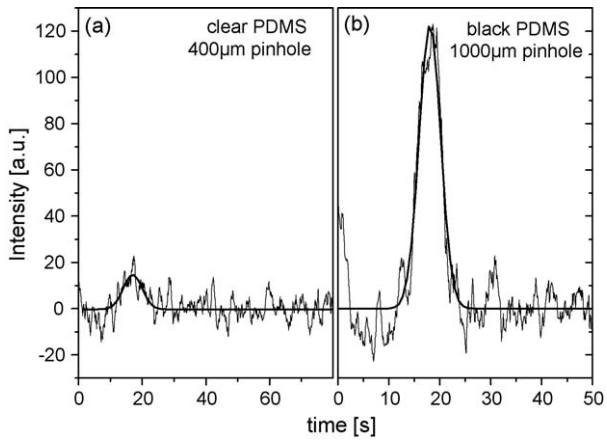

Fig. 4. Native LIF detection of 100 nM Trp injected with an electric field strength of 400 V/cm (pinched injection; detector at 20 mm). The original data is baseline corrected, background subtracted and smoothed with a 10 point binning. (a) Clear PDMS microfluidic device with a laser power of 1.1 μW and a pinhole diameter of 400 μm and (b) Black PDMS (ratio 1:100) with laser power 500 μW and a pinhole diameter of 1000 μm.

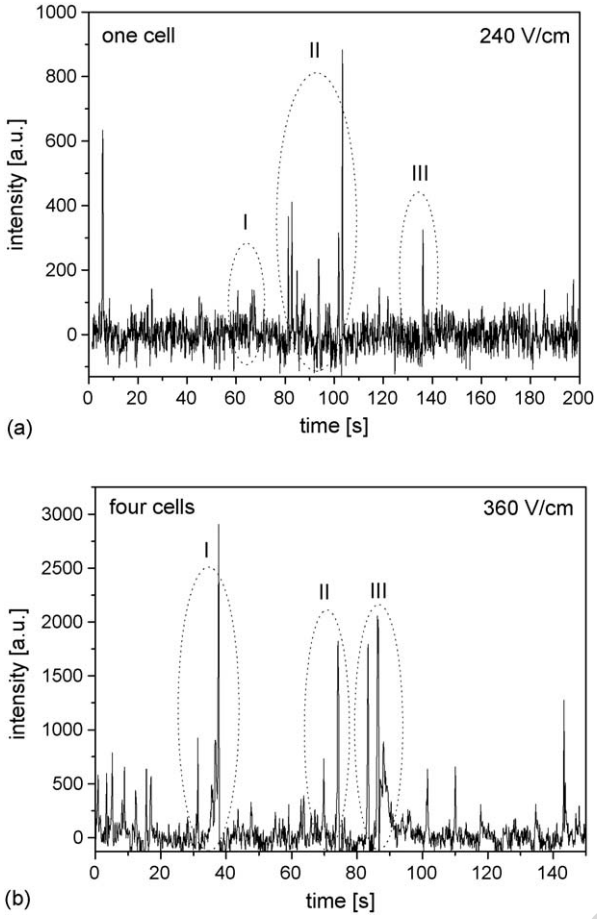

Fig. 5. (a) Baseline corrected electropherogram from a single Sf9 cell with native UV-LIF detection. Channels in the clear PDMS were coated with F108 and UV-LIF was performed at 500 μW and with a 400 μm pinhole at a separation voltage of 240 V/cm. Peak groups I, II and III (dotted ellipses) correspond to 65 s, 90 s, and 130 s, respectively. Detailed chip design and operations are described in the materials section and in references [9,25]. (b) Electropherogram of four Sf9 cells at a separation voltage of 360 V/cm. All other conditions are as in (a). Peak groups I, II and III (dotted ellipses) correspond to 37 s, 74 s, and 87 s, respectively.

As an ultimate goal in the future, label-free protein fingerprinting of a single cell in a microfluidic device is envisioned. Therefore, we attempted to record first single cell electropherograms from single Sf9 cells with label-free UV-LIF detection. The single cell electropherograms were obtained by selecting single Sf9 cells in a corresponding sample reservoir on the chip, which were subsequently navigated to a lysis position on the microdevice with optical tweezers. The cell was electrically lysed and its components were transported towards the UV-LIF detector as recently demonstrated with LIF in the visible range for green-fluorescent protein mutants [9,25].

Fig. 5a demonstrates an electropherogram of an individual Sf9 cell as obtained by native UV-LIF detection. This electropherogram shows ∼10 distinct peaks and was recorded in an F108 coated PDMS device. Additionally and for comparison, we recorded an electropherogram of four cells. As demonstrated in Fig. 5b, ∼20 peaks with increased intensities can be resolved in an electropherogram of four cells. In general, the four-cell electropherogram yielded increased peak intensities that are expected due to an enhanced protein amount compared to one cell. Although protein expression levels in various cells are expected to vary, thus making the interpretation of these electropherograms difficult, we could identify three groups of peaks in both electropherograms labelled I–III in Fig. 5 a and b. The grouping was achieved by reconciling the peak positions with the corresponding migration time taking into account a recalibration factor of 1.5 from the different separation voltages (360 V/cm versus 240 V/cm). Peaks beyond 100 s in the four-cell electropherogram could not be found in the single cell electropherogram, which is attributed to protein concentrations below the detection limit and to the reduced separation voltage.

Although the peak number of detected peaks and the separation efficiency are rather low, we strongly believe that the performance of single cell electropherograms can be improved in the future by more appropriate and elaborate separation and detection concepts. The use of grafted and cross-linked coatings on PDMS as proposed by Allbritton's group [32] could probably improve the separation efficiency. Nevertheless, our data represent to our best knowledge the first electropherogram from a single cell with native UV-LIF detection in a microfluidic device.

4. Conclusion

The reduction of background fluorescence by decreasing the incident laser power and correct adjustment of pinhole size in the confocal setup has successfully enhanced the detection sensitivity by at least two orders of magnitude for native label-free UV-LIF detection of amino acids and proteins compared to our previous studies in PDMS/quartz microdevices [9]. Additional incorporation of absorbing carbon black particles into the PDMS device has further reduced the theoretical detection limit to 25 nM, which is to our best knowledge the lowest amino acid concentration detected by native UV-LIF in a microfluidic device. The use of poly(oxyethylene) based triblock copolymers as coating agents has further improved the separation efficiency for proteins resulting in baseline resolved separations for two model proteins (trypsinogen and lysozyme C). Further, we were able to demonstrate the first single cell electropherogram with native UV-LIF detection in a microfluidic device opening promising perspectives for label-free protein fingerprinting from single cells in the future.

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