



Single cell analysis in full body quartz glass chips with native UV laser-induced fluorescence detection

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ABSTRACT

In order to investigate the individual and inhomogenous cellular response, e.g. to external stimuli, single cell analysis is mandatory and may provide new cognitions in proteomics as well as in other fields of systems biology in the future. Here, we report on novel chip architectures for single cell analysis based on full body quartz glass microfluidic chips (QG chips) that extend our previous studies in polydimethylsiloxane (PDMS) chips, and enhance the detection sensitivity of native UV laser-induced fluorescence (UV-LIF) detection. Detection of a 10 nM tryptophan solution with an S/N ratio of 11.9, which gives a theoretical limit of detection of 2.5 nM (with S/N = 3), was possible. With these optimizations the three proteins α -chymotrypsinogen A, ovalbumin and catalase each at a concentration of 100 $\mu\text{g}/\text{mL}$ (equal to 4 μM , 0.4 μM and 2.2 μM) were injected electrokinetically and could be separated with nearly baseline resolution. Furthermore, fluorescence spectra (excitation wavelength, $\lambda_{\text{ex}} = 266 \text{ nm}$) clearly demonstrate the favourable properties like the very high UV transparency and the nearly vanishing background fluorescence of the QG chips as compared to PDMS chips and to PDMS quartz window (PQW) chips. Finally we exploit the improved sensitivity for single cell electropherograms of *Spodoptera frugiperda* (Sf9) insect cells.

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1. Introduction

The integration and realization of cell handling and analysis techniques in microfluidic devices has become a field of intense investigations, because of numerous advantages compared to conventional laboratory procedures. Most obvious are the rapid and reproducible measurements on small sample volumes [1]. Moreover, microfluidics allows experiments that cannot be performed simply by miniaturizing and mechanizing conventional laboratory procedures using robotics and microplates. In cell-based studies, for example the transition from 384- to 1536-well plates is challenging, largely because edge effects and uncontrolled evaporation from very small wells result in poorly defined culture conditions [1]. In microfluidic systems, i.e. in cultures on the level of 10^3 cells, cell culturing and subsequent analysis of cell compounds [2,3], as well as mechanical and dielectrophoretic cell manipulation or flow cytometry [4] among other techniques have demonstrated the advantages of microfluidics. Nevertheless, in cell ensembles it remains difficult to address the different and inhomogenous cellular responses to external stimuli and averaging effects from cell-cycle-dependent states, or the introduction of genomic and

proteomic variabilities during cell proliferation [5,6]. Therefore novel and efficient tools and techniques for single cell analysis have the potential to provide new cognitions for systems biology.

Intracellular fluorescent probes have been used to determine the calcium flux [7] or to analyse amino acids from the content of a cell after lysis and electrophoretic separation [8] in microfluidic chips on a single cell level. The extraction of messenger RNA after single cell isolation and lysis [9], has been demonstrated. Recently, the quantification of two distinct proteins, either by fluorescence-antibody labelling or by autofluorescence detection on a single cell level with laser-induced fluorescence (LIF) detection in the visible spectral range was published [10].

First single cell fingerprints with protein staining by a fluorescent dye have been performed via combination of capillary sieving electrophoresis and LIF detection in one dimension [11,12] and later in two-dimensional format [13]. Lately the transfer of separation techniques relevant for proteomics, like electrophoresis, isoelectric focusing and two-dimensional protein separation to microfluidic chips has been shown [14–17]. However, the label-free investigation of the proteome from single cells remains challenging. Mass spectrometry (MS) may provide more information especially about proteins with high copy numbers from single cells and concepts for chip-MS coupling have already been published [18].

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For the detection of low abundant proteins with copy numbers $<10^5$ molecules in single cells, a detection limit less than 100 nM is mandatory, assuming 10^5 protein molecules in a cell with a volume of 1 pL (according to a diameter of $\sim 10 \mu\text{m}$) [19]. A promising approach for the low copy proteins could be the label-free LIF based on the intrinsic fluorescence of the three aromatic amino acids tryptophan, tyrosine and phenylalanine, which has been reported with high detection sensitivity in capillary electrophoresis. Limits of detection in the pM range have been published in 1992 by Yeung and co-workers for a LIF detection method using an Ar^+ -laser ($\lambda_{\text{ex}} = 275 \text{ nm}$) [20]. Later this method was also applied for the separation of haemoglobin variants in red blood cells [21] and for exocytosis monitoring of single mast cells [22]. For the amino acid tryptophan detection limits in the nM range have also been reported with a metal vapour laser [23] or an excimer laser [24]. Also limits of detection in the nM range for peptides [25] or proteins [26] and in the pM range for carbonic anhydrase [27] by the application of solid state lasers have been demonstrated. Label-free UV-LIF in microfluidic devices has first been demonstrated with limits of detection in the μM range [19,28] and after device improvements, like the integration of carbon black particles into PDMS, with detection sensitivity in the nM range [29]. Moreover, after we suggested a method for single cell analysis including selection and navigation of a single cell by optical tweezers, cell lysis on-chip via a high voltage electrical pulse followed by electrophoretic separation and LIF detection in the visible range [19,30], we have been able to combine this method with label-free UV-LIF detection and thus presented the first single cell electropherogram with UV-LIF detection in a PDMS microfluidic chip [29]. Also, the application of PDMS quartz window (PQW) chips for single cell experiments with UV-LIF detection exploiting the longer fluorescence life-time of tryptophan at basic pH [31] yielding further signal enhancement was recently published [32].

In this paper, we focus on the enhancement of UV-LIF detection in microfluidic devices for single cell analysis by the application of novel high-grade fused silica chips with anisotropic channels with a depth of $30 \mu\text{m}$, created by deep reactive ion etching of silicon dioxide (DRIESO). We report the reduced background fluorescence, raised detection sensitivity and separation efficiency with respect to new dynamic surface coatings [33,34] based on our previous studies with PDMS-based microfluidic chips [29,32]. A new detection limit for the aromatic amino acid tryptophan with native UV-LIF detection is demonstrated close to the pM range. Based on these improvements the label-free single cell analysis of Sf9 insect cells and native protein separation of three standard proteins are demonstrated.

2. Experimental

2.1. Chemicals and reagents

Polydimethylsiloxane (PDMS) (Sylgard 184 silicone elastomer and curing agent) was purchased from Dow Corning (Midland, MI, USA). Fused silica wafer ($100 \text{ mm diameter} \times 0.2 \text{ mm thickness}$) and quartz cover slides ($60 \text{ mm} \times 24 \text{ mm} \times 0.2 \text{ mm}$) were from Aachener Quarzglas Technologie Heinrich (Aachen, Germany) and ($100 \text{ mm} \times 0.5 \text{ mm thickness}$) from Schott (Jena, Germany). Pullulan, methylcellulose (MC), tryptophan, the proteins α -chymotrypsinogen A, ovalbumin (from chicken egg) and catalase were obtained from Sigma–Aldrich (Munich, Germany). *Spodoptera frugiperda* (Sf9) insect cells and BacVector Insect Cell Medium were from Novagen (Darmstadt, Germany). Triblock copolymer Pluronic F-108 was a generous gift from BASF (Ludwigshafen, Germany). Dodecyl- β -D-maltoside (DDM), (cyclo-

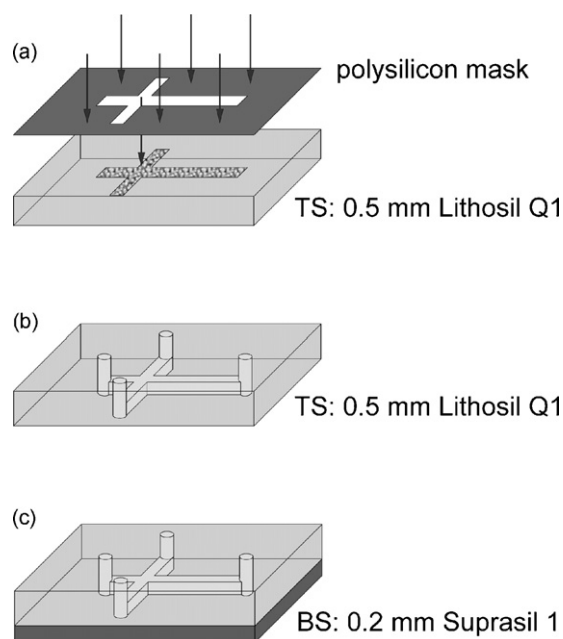


Fig. 1. QG chip fabrication via deep reactive ion etching of silicon dioxide (DRIESO). (a) The structures were formed via deep reactive ion (RIE) etching of silicon dioxide into the top substrate (TS). (b) Masked sand blasting was used for producing the reservoir holes in the TS before (c) the TS and the bottom substrate (BS) are fused together by thermal bonding ($\sim 1100^\circ\text{C}$ to 1200°C).

hexylamino)ethanesulfonic acid (CHES), di-sodium hydrogenphosphate dehydrate, tris(hydroxymethyl)aminomethane (Tris) and phosphate-buffered saline (PBS) were purchased from Fluka (Deisenhofen, Germany). Sodium hydroxide (NaOH) was purchased from Carl Roth (Karlsruhe, Germany). For all solutions deionized water from a Milli-Q biocel (Millipore, Billerica, MA, USA) was used.

2.2. Chip fabrication

For the fabrication of the QG chips the layout of the microfluidic structures was designed with the CAD program Clewin (Phoenix, Enschede, The Netherlands). The etching of the top substrate (TS) (fused silica wafer, $100 \text{ mm} \times 0.5 \text{ mm}$, Schott, Jena, Germany) was performed via deep reactive ion etching (RIE) of silicon dioxide (DRIESO) (Fig. 1) [35]. First the structures were etched with a deep RIE ICP (AMS100SE, Adixen, Annecy, France) into a polysilicon mask, which served as mask for the DRIESO process with another deep RIE ICP (AMS100DE, Adixen, Annecy, France). This way, anisotropic channels ($30 \mu\text{m}$ width, $30 \mu\text{m}$ depth) were etched into the TS by the usage of fluorine gas (C_4F_8). Masked sand blasting was used for producing the reservoir holes in the TS. By thermal bonding the TS and the bottom substrate (BS) (fused silica wafer, $100 \text{ mm} \times 0.2 \text{ mm}$, Aachener Quarzglas Technologie Heinrich, Aachen, Germany) were fused together before sand blasting was used for dicing the single microfluidic devices. For all experiments a simple cross layout was used with 3 cm length of the separation channel and 0.5 cm length of the three other channels, whereas the intersection contained additional obstacles, which act as physical traps for the single cell experiments. The DRIESO process, as well as the bonding and dicing of the QG chips were carried out by Capilix (Enschede, The Netherlands).

The chip design and production of the PDMS chips was adapted from [19,30]. For the fabrication of the PQW chips, copper weights were used to push down small fused silica windows ($5 \text{ mm} \times 5 \text{ mm}$) onto the microchannel at the point of detection before the PDMS was cured, as recently published [32].

2.3. Fluorescence detection

For sensitive LIF detection in the UV range (UV-LIF), an inverted microscope (Axiovert 100, Zeiss, Jena, Germany) served as a technical platform. For excitation the wavelength of a frequency quadrupled Nd:YAG laser (266 nm, 5 mW, Nanolase, Grenoble, France) was coupled via two mirrors (New Focus, San Jose, CA, USA) into the rear port of the microscope. Optional transmission filters (AHF Analysentechnik, Tübingen, Germany) reduced the intensity to 10% or 3% of the incoming beam installed at the filter cube of the microscope. The transmitted excitation light was reflected by a UV dichroic mirror (Laseroptik, Garbsen, Germany) and focused by a UV-transparent objective (40 \times , Optics for Research, Verona, NJ, USA) into the microchannel. A motorised x/y -stage (Märzhäuser, Wetzlar–Steindorf, 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, Tübingen, Germany). A high transmission UV tube lens (Zeiss, Jena, Germany) focused the light through a x/y adjustable pinhole (1000 μm) as spatial filter for confocal detection, onto the commercial photomultiplier built-in module (H6240, Hamamatsu, Shimokanzo, Japan), supplied with 5 V from an external power supply. For all experiments the counts were collected for 100 ms except the determination of the detection limit, where the counts were collected for 200 ms. Optionally a mirror (New Focus, San Jose, CA, USA) replacing the pinhole and the photomultiplier led the beam of emitted fluorescence light into an UV/vis spectrometer (Triax 190, Horiba Jobin Yvon, Edison, NJ, USA) to be monochromatised before detection by the above mentioned photomultiplier.

2.4. Cell culture

Sf9 insect cells were grown to confluence of the first layer in BacVector Insect Cell Medium (Novagen, Darmstadt, Germany) at 27 °C and 90% humidity. The cells were washed three times with PBS to remove secreted proteins and residual substrate from the medium. Then, the cells were resuspended in the separation buffer immediately before single cell analysis.

2.5. Chip operations

The microchannels were initially filled either by capillary action or by applying vacuum to the reservoirs. For the experiments concerning the detection limit of tryptophan solutions (10 mM Tris buffer, pH 8.2), the microchannels were coated with a 500- μm F108 solution (10 mM phosphate buffer, pH 8.6) by an incubation of 20 h followed by thorough washing. For protein separation the separation buffer (10 mM Tris buffer, pH 8.2) contained 0.1% (w/w) DDM as dynamic coating. In the single cell experiments a separation buffer (100 mM Tris, 100 mM CHES, 4% (w/w) Pullulan, pH 11.0) with 0.25% (w/w) DDM and 0.03% (w/w) MC as dynamic coating was used. For an increased reservoir volume a 3 mm thick PDMS block with punched 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, Rosenheim, Germany). Instrumental control and data acquisition were performed by software programmed in LabView (National Instruments, Austin, TX, USA). For all experiments, except the single cell analysis, pinched injection was used. For the single cell analysis the experiments with individual Sf9 cells were performed as previously published [19,30]. First, the cell was navigated and steered along the microdevice by optical tweezers. Then the cell was electrically lysed by a short pulse of high electric field

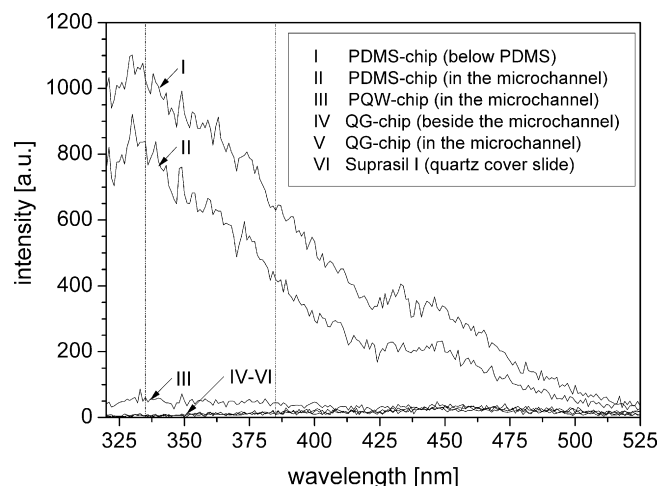


Fig. 2. Fluorescence spectra ($\lambda_{\text{ex}} = 266 \text{ nm}$) of a PDMS chip, a PQW chip [31], a QG chip and a high-grade quartz cover slide (Suprasil I, 60 mm \times 24 mm \times 0.2 mm). The microchannels (30 μm \times 30 μm) were filled with 10 mM Tris buffer at pH 8.2. The excitation was performed at 500 μW laser power.

strength (2000 V/cm for 50 ms) and the cell content was injected into the separation channel, followed by the separation of cell compounds with an electric field strength of 200 V/cm in a Pullulan sieving matrix and UV-LIF detection at a separation distance of 5 mm.

3. Results and discussion

3.1. Fluorescence background

In our previous work, firstly, we used PDMS microfluidic devices on quartz cover slides [29] for single cell analysis. Next we applied PQW chips to yield a reduction of the fluorescence background. Those PQW chips exhibited small fused silica windows integrated into the PDMS microfluidic devices at the point of detection [32]. Here, we extend our studies further reducing the background fluorescence by the application of new full body quartz glass (QG) chips. The benefits are higher signal intensity, lower fluorescence background and thus higher detection sensitivity in UV-LIF detection. Fluorescence spectra ($\lambda_{\text{ex}} = 266 \text{ nm}$) at 500 μW laser power demonstrate the significant lower fluorescence background of the PQW chips and the QG chips as compared to PDMS chips (Fig. 2). Moreover fluorescence ($\lambda_{\text{ex}} = 266 \text{ nm}$) spectra taken at 5 mW laser power demonstrate that the UV-LIF of the QG chips is even lower as compared to the PQW chips and is nearly not distinguishable from highest grade quartz cover slides (60 mm \times 26 mm \times 0.2 mm) (Fig. 3a–d). Unfortunately it is not possible to obtain reliable spectra from the PDMS chips or the PQW chips below the quartz detection window outside the separation channel, where a 30 μm PDMS layer remains (for detailed chip production see [32]), at 5 mW laser power because the PDMS is carbonised due to the high application of energy. To accent the spectral detection region in Figs. 2 and 3a–d the upper and the lower limit of the excitation filter (360/50), used for single cell experiments as well as for the other injections, are marked by lines.

3.2. Detection sensitivity

For the determination of the detection limit tryptophan solutions at different concentrations were injected. Exemplarily Fig. 4 shows an electropherogram of a 10 nM tryptophan solution injected in a QG chip statically coated with the triblock copolymer F108 followed by native UV-LIF detection. The S/N ratio is 11.9, which

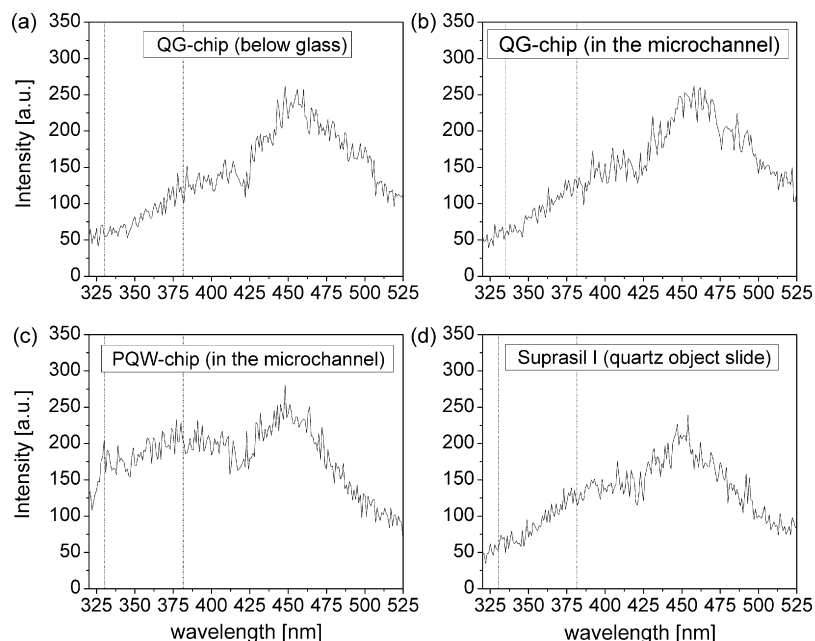


Fig. 3. Fluorescence spectra ($\lambda_{\text{ex}} = 266 \text{ nm}$) of (a and b) a QG chip, (c) PQW chip and (d) high-grade quartz cover slide (Suprasil I, 60 mm \times 24 mm \times 0.2 mm). The microchannels (30 $\mu\text{m} \times$ 30 μm) were filled with 10 mM Tris buffer at pH 8.2. The excitation was performed at 5 mW laser power.

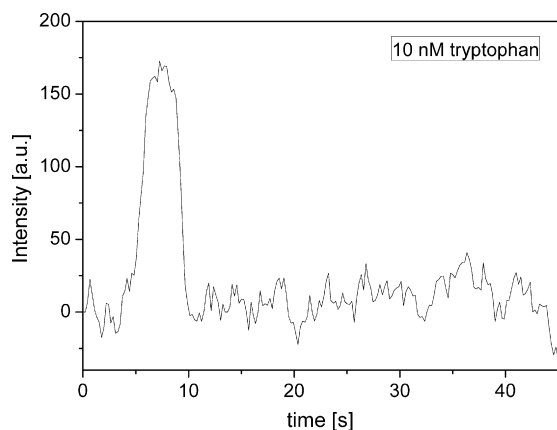


Fig. 4. Native UV-LIF detection ($\lambda_{\text{ex}} = 266 \text{ nm}$) of a 10 nM tryptophan solution injected with an electric field strength of 228 V/cm (pinched injection; detector at 5 mm). Channels in the QG chip were coated statically with the triblock copolymer F108 and UV-LIF was performed at 500 μW laser power with a 1000- μm pinhole. The original data is baseline corrected and smoothed with a five point binning.

results in a theoretical detection limit of 2.5 nM tryptophan (with $S/N = 3$). This is one order of magnitude below the theoretical detection limit of our previous work, where a composition of PDMS and carbon black particles was used to yield improved detection sensitivity [29] and a theoretical detection limit of 25 nM tryptophan was calculated (Fig. 4). According to the change in the signal intensity (120 counts for the 100 nM tryptophan solution measured with a black PDMS chip and 170 counts for the 10 nM tryptophan solution measured with a QG chip) with respect to the concentration of the tryptophan, the detection sensitivity is raised by a factor of 14.2 for our setup. Moreover the detection limit for tryptophan in the dry etched QG chips with perpendicular sidewalls of the microchannels is significantly lower than formerly shown in other fused silica chips made by wet etching, where only a 2- μM tryptophan solution was detectable with native UV-LIF detection [28]. Thereby, to our best knowledge, we reached the lowest detection limit reported for tryptophan with native UV-LIF detection in microfluidic channels.

3.3. Separation efficiency

In response to developments of dynamic coatings for the reduction of the electroosmotic flow (EOF) and reduced unspecific protein adsorption [33] we augment our studies of protein separations with static coatings [29,36] to separations with such dynamic coatings. The separation of three standard proteins was performed with an electric field strength of 286 V/cm and a separation buffer (10 mM Tris, pH 8.2) containing 0.1% (w/w) DDM. The proteins α -chymotrypsinogen A, catalase and ovalbumin each at a concentration of 100 $\mu\text{g}/\text{mL}$ (corresponding to a concentration of 4 μM , 0.4 μM and 2.2 μM) were injected, and could be separated within 2 min with nearly baseline resolution (Fig. 5). The actual plate height H for the first Peak is 98 μm , 68 μm for

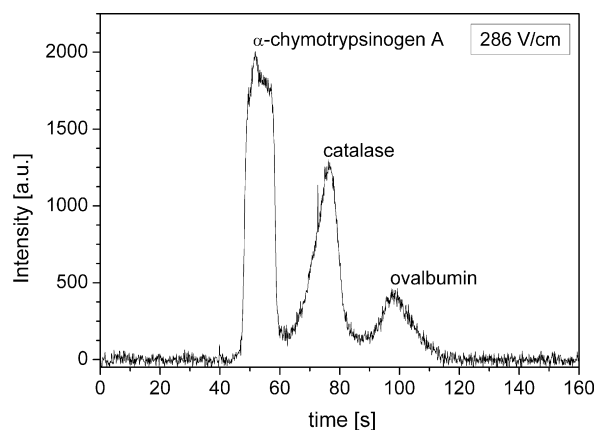


Fig. 5. Baseline corrected electropherogram of the separation of native proteins with UV-LIF detection ($\lambda_{\text{ex}} = 266 \text{ nm}$). The three proteins (α -chymotrypsinogen A, catalase and ovalbumin), each at a concentration of 100 $\mu\text{g}/\text{mL}$ (respectively 4 μM , 0.4 μM and 2.2 μM) were injected in a mixture with an electric field strength of 286 V/cm (pinched injection; detector at 19.5 mm). Channels in the QG chip were dynamically coated with DDM and UV-LIF was performed at 500 μW laser power with a 1000- μm pinhole.

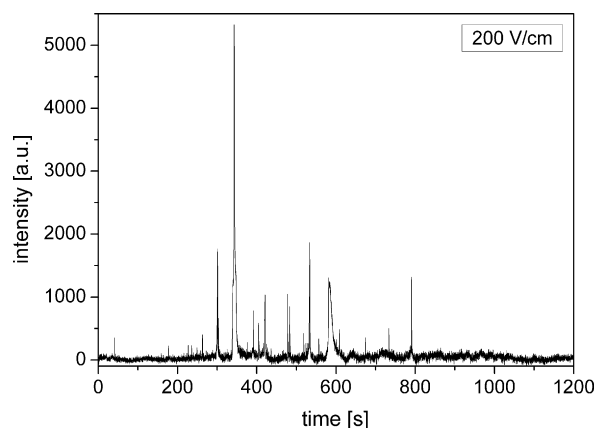


Fig. 6. Baseline corrected single cell electropherogram from a Sf9 cell with native UV-LIF detection ($\lambda_{\text{ex}} = 266 \text{ nm}$). Channels in the QG chip were coated dynamically with DDM and MC at pH 11 of the separation buffer. UV-LIF was performed at $150 \mu\text{W}$ laser power and with a $1000\text{-}\mu\text{m}$ pinhole at an electric field strength of 200 V/cm for separation (detector at 5 mm). Exact chip design and operations are described in Section 2.

the second peak and $74 \mu\text{m}$ for the third peak. The resolution of the neighbouring peaks is greater than one (1.36 for the second and the first and 1.12 for the third and the second one), indicating the distinct separation of the three proteins. The S/N ratio of the first peak from the electropherogram in Fig. 5 was 82.5, which gives a theoretical detection limit of $3.6 \mu\text{g/mL}$ for α -chymotrypsinogen A (equivalent to 145 nM α -chymotrypsinogen A). In contrast to this in other wet etched fused silica chips only $12.5 \mu\text{g/mL}$ (equal to 500 nM) of the same protein could be detected [28].

3.4. Single cell analysis

Since we also strive for label-free protein fingerprinting of single cells in microfluidic devices, the first single cell electropherogram from a single Sf9 cell with label-free UV-LIF detection has already been published [29]. We continued our work by enhancement of the UV-LIF detection for single cell analysis by using PQW chips and adapted the pH of the separation buffer to higher pH in order to yield higher signal intensity in single cell separations [32,31] and applied the new QG chips with dynamic coatings (Fig. 6). Briefly, single cell electropherograms were achieved in four steps [19,30]. First, the isolation of an individual cell of interest by optical tweezers was performed. Then the cell was guided to the intersection position of the microdevice, where obstacles act as physical trap. At this position the cell was lysed by a high voltage electrical pulse (2000 V/cm for 50 ms) and its components were separated via electrophoresis in the separation channel on chip followed by LIF detection [30]. This single cell experiment was performed in a QG chip dynamically coated with DDM and MC to reduce the EOF and the unspecific protein adsorption as well as to raise the viscosity of the separation buffer [34]. The electropherogram in Fig. 6 recorded by native UV-LIF detection from an individual Sf9 cell exhibits ~ 20 distinct peaks with higher fluorescence intensity due to raised pH of the separation buffer (see Section 2) and lowered background fluorescence in the QG chip as compared to our previous work in PDMS chips [29]. Furthermore the peaks exhibit fluorescence intensity comparable to single cell electropherograms obtained with PQW chips at the same pH of the separation buffer but with higher laser power (namely $500 \mu\text{W}$) used for the excitation [32].

4. Conclusions

By the application of new QG chips with anisotropic channels created by DRIESO it was possible to demonstrate a further reduction of the background fluorescence in native UV-LIF detection. This resulted in higher detection sensitivity for our setup whereby the detection of a 10 nM tryptophan solution became possible and gives a theoretical detection limit of 2.5 nM . Moreover this is to our best knowledge the lowest amount of this amino acid detected in a microfluidic chip by native UV-LIF. Furthermore, with the use of DDM as dynamic coating the separation efficiency was demonstrated by the separation of the three proteins α -chymotrypsinogen A, catalase and ovalbumin at low concentrations with nearly baseline resolution in 2 min . Finally, the combination of a higher pH of the separation buffer using DDM and MC as dynamic coating in the QG chips resulted in higher detection sensitivity and separation efficiency for single cell analysis with native UV-LIF detection in microfluidic devices.

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