Deep UV laser-induced fluorescence detection of unlabeled drugs and proteins in microchip electrophoresis

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Deep UV fluorescence detection at 266-nm excitation wavelength has been realized for sensitive detection in microchip electrophoresis. For this purpose, an epifluorescence setup was developed enabling the coupling of a deep UV laser into a commercial fluorescence microscope. Deep UV laser excitation utilizing a frequency quadrupled pulsed laser operating at 266 nm shows an impressive performance for native fluorescence detection of various compounds in fused-silica microfluidic devices. Aromatic low molecular weight compounds such as serotonin, propranolol, a diol, and tryptophan could be detected at low-micromolar concentrations. Deep UV fluorescence detection was also successfully employed for the detection of unlabeled basic proteins. For this purpose, fused-silica chips dynamically coated with hydroxypropylmethyl cellulose were employed to suppress analyte adsorption. Utilizing fused-silica chips permanently coated with poly(vinyl alcohol), it was also possible to separate and detect egg white chicken proteins. These data show that deep UV fluorescence detection significantly widens the application range of fluorescence detection in chip-based analysis techniques.

Microfluidic devices offer a great potential in separation science, which is especially true for microchip electrophoresis (MCE) as the currently most successful chip-based separation technique. In MCE, which can be regarded as a higher miniaturized version of classical capillary electrophoresis (CE), it is possible to realize very fast separations with the possibility of integrating complex structures such as for on-chip PCR. This makes the technique very attractive for the realization of so-called micro total analysis systems or lab-on-a-chip devices. While the highly miniaturized and flat format of such chip-based separation devices has several advantages with regard to separation performance, instrument size, and functionality, this compact format is often disadvantageous for the implementation of powerful detection methods.

The most dominant detection technique in conventional CE is UV absorbance detection due to its versatility and ease of operation. However, UV absorbance detection has only rarely been applied to MCE due to geometrical constraints and especially because of the limited optical path lengths. The most commonly used detection technique in MCE is fluorescence detection, which provides high sensitivity. Another reason for the popularity of fluorescence detection in microchip electrophoresis is that this technique can easily be implemented in the experimental configuration using an epifluorescence setup. A drawback of common fluorescence detection utilizing light sources at wavelengths above 300 nm is the necessity to derivatize most analytes prior to analysis. This is not only inconvenient but can also be very troublesome, especially at low analyte concentrations and for compounds lacking appropriate functional groups. For multifunctional compounds such as proteins, it is very difficult to ensure stoichiometrical reactions that can result in complex mixtures after derivatization.

It is well known that the applicability of fluorescence detection can be broadened considerably by using deep UV light sources. This allows the excitation of natural fluorescence of a wide variety of compounds especially proteins containing tryptophan or tyrosine.

There has been numerous reports on native fluorescence detection in classical capillary electrophoresis, most utilizing deep UV laser but also with lamp-based systems. Deep UV fluorescence detection has become a rather mature status in classical CE which is reflected by the availability of several commercial solutions. Recently Sluszny and Yeung demonstrated that native fluorescence detection can also be utilized to detect proteins after miniaturized slab gel electrophoresis. The realization...
of on-chip deep UV fluorescence detection in microchip electrophoresis is however challenging and has to our knowledge not yet been reported. Utilizing deep UV light sources such as frequency quadrupled Nd:YAG laser in common epifluorescence setups is not trivial as the used optical components such as objectives, filters, and condensers have to provide high UV transmittance and low autofluorescence at the excitation wavelength. For this purpose, optical components of high-quality quartz/fused silica are required as common glass objectives transmit only down to 320 nm. Furthermore, the chip material also has to exhibit transmittance and low autofluorescence in the deep UV spectral region. Currently only high-grade fused silica meets this requirements and was chosen in our work as the chip material.

Herein we report on native fluorescence detection in MCE by coupling a deep UV Nd:YAG laser operating at 266 nm to a fluorescence microscope. Such a native fluorescence microscope setup not only enables native detection of proteins and low molecular weight compounds in microfluidic devices but can also be used for imaging applications.

**EXPERIMENTAL SECTION**

To enable microchip electrophoresis with deep UV laser-induced fluorescence detection, we used a similar setup as previously described.\(^{14}\) The optical part is based on the commercial IX-71 fluorescence microscope by Olympus (Hamburg, Germany). A device working as modular filter cube and objective holder was manufactured according to our specifications by TSO GmbH (Pulsnitz, Germany). This combined device can replace standard objectives in the microscope setup and contains components for an epifluorescence setup: a dichroic mirror, an excitation light barrier filter, and an SMA connector for attaching the light fiber (200 µm) for the excitation light. A replaceable 40 x 0.8 fused-silica objective manufactured by Partec GmbH (Münster, Germany) is situated above the cube. The dichroic mirror reflects light with wavelength of <275 nm, whereas light of >280 nm is transmitted. An additional 290-nm barrier filter is situated below the dichroic mirror as shown in Figure 1A. A fiber coupled pulsed, frequency quadrupled Nd:YAG laser (type FQSS266-Q; 4 mW) purchased from Crystal GmbH (Berlin, Germany) was used as excitation light source. A special UV band-pass filter (model DUG-11x) from ITOS GmbH (Mainz, Germany) reducing the background radiation was placed in the ray path below the filter cube. The fluorescence light was detected by a H 7711–03 photomultiplier-tube (PMT) obtained from Hamamatsu, which is mounted at the upper back port of the microscope behind a cylindrical fused-silica collector lens. A Sentronic USB-2000 spectrometer (Dortmund, Germany) can alternatively be installed at the upper back port enabling the collection of fluorescence spectra. Imaging of the microfluidic structures and the fluidic processes can be realized either with a JVC GR-DV 3000 charged

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coupled device camera (Friedberg, Germany) or with the binocular of the microscope setup. The camera was coupled to the microscope by an optical CASC 5210 adapter from Micro-TechLAB (Graz, Austria). A bipolar four-channel power supply of the type HCV 40M-10000 was purchased by FUG Elektronik GmbH (Rosenheim, Germany). This customer-specific power supply allows fast alteration between the injection and the separation potentials.

Fused-silica microchips with a simple cross layout were from Micronit (Einschede, The Netherlands). All channels were 20 μm deep and 50 μm wide at the top. Other system components such as the PMMA electrode plate, the low electric conductive chip carrier plate, or the PMT control box were homemade. To compare the sensitivity of our system with commercially available systems with UV absorbance detection, a P/ACE MDQ CE-system by Beckmann Coulter Inc. (Fullerton, CA) and a Shimadzu MCE-2010 system (Kyoto, Japan) were utilized.

Except for the poly(vinyl alcohol) (PVA)-coated chips the FS surfaces of the microfluidic devices were initially rinsed with water, methanol, and a 1 mol L⁻¹ aqueous NaOH solution. The vials were filled with 2 μL of separation buffer, which flushed the channels by applying vacuum. In the next step, the vials were emptied and refilled with 2 μL of buffer again. Before switching the four-channel power supply in separation mode, the potentials for the injection process focused the analytes in the cross section for 10 s. After each run, the vials were emptied, replenished, and rinsed with buffer for 15 s. Numerical values of the applied potentials are given in the caption below the electropherograms. The beginning of the separation channel is labeled buffer inlet (BI) which leads to the buffer outlet (BO). The injection channel crossing the separation channel has a sample inlet (SI) vial, that ends in sample-outlet (SO). The PVA coatings for microchannels were produced as described by Ludwig and Belder,¹⁵ by thermal immobilization of PVA, except that the solution contained 3% (w/w) of PVA in water.

SAFETY CONSIDERATIONS

The instrumentation uses high voltages and should be handled carefully. Exposure to the UV radiation of the laser is harmful to eyes and skin. Wearing laser protection glasses for deep UV light is absolutely recommended. Caution: sodium tetraborate as well as its resolvable dusts are toxic.

RESULTS AND DISCUSSION

In a previous report, we used an instrumental setup with lamp-based fluorescence detection in MCE that was based on an inverted fluorescence microscope.¹⁴ The instrument provided flexible wavelength excitation and detection as well as imaging at rather high sensitivities. In that instrumental setup, we were however limited to excitation wavelengths above 330 nm. The excitation light had to pass the common optical components of the microscope such as condensor, lenses, filter, and objectives with low UV transmittance. To make native fluorescence detection of proteins and also of aromatic low molecular weight compounds possible, a device was developed to enable the coupling of a deep UV light source for fluorescence detection and imaging to a commercial microscope. This was realized by coupling a 266-nm Nd:YAG laser to a miniaturized filter cube below a fused-silica objective by-passing all common optical parts of the microscope. The excitation light was focused on a fused-silica microchip, and the fluorescence light can be guided to a PMT or to the binocular tube for imaging applications; see Figure 1. As the fluorescence light has longer wavelengths, it is less problematic to use common optical components of the microscope for imaging applications.

Although deep UV excitation light was only exposed to fused-silica optical components, we observed a rather high fluorescence background that could in part be attributed to the microchips. As this interfering background fluorescence appeared mainly at wavelengths of >450 nm, we used an additional band-pass filter (DUG-11x) in the emission light pass (transmission between 280 and 390 nm) that considerably improved the signal-to-noise ratio for the fluorescent compounds of interest. We used a rather wide emission filter for gaining maximum sensitivity while a narrow emission filter would be beneficial to improve selectivity.

Using the described setup, we were able to detect a mixture of unlabeled small molecular weight compounds with native fluorescence detection in microchip electrophoresis. The test mixture contained the biogenic amine serotonin, the β-blocker propranolol, a racemic diol, and the amino acid tryptophan. Baseline separation of all components could be realized in less than 9 s. At analyte concentrations of 40 μg/mL, a good signal-to-noise-ratio of 510 was obtained for peak 1 (Figure 2A). The difference in detection sensitivity is due to differences in fluorescence quantum yields and absorption spectra. By using a tunable light source rather than a single line laser, this could be utilized for enhancing selectivity. An electropherogram well above the detection limit (signal-to-noise ratios of ~8 for compounds 2–4) of the test mixture is shown in Figure 2B. The concentrations of the compounds were at 400 ng/mL (1.9 μM) for serotonin, 1.6 μg/mL (9.5 μM) for the diol (compound 3 in Figure 2B), 400 ng/mL (2.0 μM) for tryptophan, and 400 ng/mL (1.4 μM) for the β-blocker propranolol. These data show that it is possible to

perform deep UV fluorescence detection in microchip electrophoresis with high sensitivity. Accordingly deep UV fluorescence detection in MCE appears to be very attractive as it widens the applicability of MCE.

It is often stated that detection is one of the weak points in MCE as most applications in MCE can also realized in CE but with much higher sensitivity using standard UV absorbance detection for untagged compounds. To compare the detection sensitivity of our system with that obtainable at typical conditions in classical CE, we performed separations of the test compounds on a classical CE instrument (Beckmann P/ACE MDQ CE system) with UV absorbance detection.

As shown in Figure 3A and B, it turned out that the detection sensitivity achieved with our MCE system with deep UV fluorescence detection is comparable to that obtainable in classical CE with UV absorbance detection although the utilized optical path length in MCE is with ~20 µm much shorter compared to a 50-µm i.d. FS capillary. However, the analysis time in MCE is considerably faster, which makes it an attractive alternative to CE, especially if fast separations are required.

For comparison, Figure 3C shows the same separation using the only commercial microchip electrophoresis instrument with UV absorbance detection (type MCE-2010 from Shimadzu, Duisburg, Germany). The detection sensitivity achieved with the Shimadzu instrument is significantly lower compared to that obtained with our system with deep UV fluorescence detection.

These results show that on-chip deep UV fluorescence detection is a valuable detection technique, which can compete with UV absorbance detection in classical CE especially while UV absorbance detection on chips appears to be rather insensitive at its current state.

As shown in Figure 2B, native fluorescence detection of tryptophan (peak 4) could be realized with good sensitivity. This should enable native fluorescence detection of proteins, which is of great importance for realizing microfluidic chip-based analysis systems as for proteomics. The detection of proteins at native conditions after zone electrophoretic separation is challenging and was rarely reported due to the lack of appropriate detection techniques. One of the very few examples of free zone electrophoresis of unlabelled proteins on chip was demonstrated by Zhang et al., who used mass spectrometry for detection of model proteins separated in a coated glass chip.

The separation of basic proteins with deep UV fluorescence detection on a microchip is shown in Figure 4. To suppress analyte adsorption to the surface of the fused-silica chips, 0.05% (w/w) HPMC was added to the running buffer for dynamic coating. As shown in Figure 4, lysozyme, trypsinogen, and chymotrypsinogen are well resolved within 3 min. The analyte concentration in Figure 4A was 300 μg/mL, which enabled an excellent signal-to-noise ratio of 150. Separation of the proteins above the detection limit is shown in Figure 4B. It was possible to detect proteins down to a concentration of 12.5 μg/mL (0.9, 0.5, and 0.5 μM for lysozyme, trypsinogen, and chymotrypsinogen). While on chip CZE separations of model proteins with deep UV fluorescence detection could be realized successfully, we also applied the technique to separation of chicken egg white as a less artificial application. For this purpose we used a fused-silica chip, which was permanently coated with PVA, as dynamic coating with HPMC did not allow us to get a sufficient resolution of the signals. A separation of a 10-fold diluted egg white sample is shown in Figure 5. A sufficient resolution of the major signals corresponding to lysozyme (1), conalbumin (2), and ovalbumin (3) could be obtained in less than 2 min with a signal-to-noise ratio of 45 for peak 1. These results

show that microchip electrophoresis has great potential for fast separation of protein mixtures at native conditions.

**CONCLUSIONS**

Deep UV fluorescence detection is a powerful tool for sensitive and rather versatile detection in MCE. By coupling of a small and rather economic 266-nm Nd:YAG laser to a common epifluorescence microscope, it was possible to detect small aromatic compounds and especially proteins without any labeling. This allows detection of proteins at native conditions, which is important not only for realizing on-chip CZE separation of proteins but also for investigations aimed at protein interactions for diagnostics. Deep UV fluorescence detection schemes should also be applicable to other chip-based analytical techniques such as on-chip chromatography, reactions and also for imaging applications. Due to current limitations in UV absorbance detection, deep UV fluorescence appears to be the most versatile detection technique for chip-based analyses at present. A current drawback is the need for expensive fused-silica chips. A broader application of on-chip deep UV fluorescence detection in future with the consequence of mass fabrication of FS chips could, however, significantly reduce these expenses. With the availability of economic deep UV lasers of even lower wavelengths, the versatility of deep UV fluorescence detection could even be expanded, especially in the case of tuneable lasers.

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