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Portable and low-cost biosensor towards on-site detection of diclofenac in wastewater

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ABSTRACT

Wastewater treatment plants are the main release sources of pharmaceutical compounds present in surface waters. Even at low concentrations, many of these substances have long-term adverse effects on the environment. For an efficient control of pharmaceutical removal, a real-time recognition is a prerequisite. Currently, quantification of such compounds is done in special equipped laboratories and is rather time-consuming and expensive. Here, we introduce a novel biosensor for the detection of the pharmaceutical compound diclofenac, which can be produced with low costs, is easy in handling and can be applied directly on-site. Recognition of diclofenac is based on genetically engineered yeast cells which produce green fluorescent protein in a diclofenac concentration-dependent manner. Centerpiece of the sensor is a foil-based microfluidic flow cell, which allows supply with nutrient solution and analyte while preventing loss of reporter cells. Readout of data is accomplished by a newly developed spectrometric detection unit. With this device, we are able to determine diclofenac concentrations in a range from 10 to 50 µM.

1. Introduction

The non-steroidal anti-inflammatory drug diclofenac (2-(2,6-dichloranilino)phenylacetic acid, $C_{14}H_{11}Cl_2NO_2$) is worldwide known as pain killer, and thus, one of the most popular pharmaceutically active compounds [1]. Additionally, diclofenac is used for veterinary purposes. Zhang et al. estimated the globally consumed volumes of diclofenac to be 940 tons per year [2]. Excretion and disposal via wastewater are the main routes of introduction into municipal wastewater, but direct input from the pharmaceutical industry is also reported [3]. Diclofenac is excreted in its native form or as metabolite, and is at the best partially removed in wastewater treatment plants. A removal efficiency of around 21–40% can be achieved by conventional removal methods (activated sludge processes, where microorganism are applied to mineralize the pollutants to water) [2]. Additional removal strategies of diclofenac could be adsorption on activated carbon followed by ozonation [4] and photocatalysis [5], but are routinely not applied. As a consequence, diclofenac is found in surface but also in ground and drinking water at relevant concentrations [1,2]. Therefore, at least a periodic monitoring of diclofenac concentrations in influent and/or effluent of wastewater treatment plants is essential.

Various methods are applied for the detection of diclofenac: UV–vis spectrophotometry [6], fluorimetry [7], chromatography methods [8,9], capillary electrophoresis [10], LC-MS [11], differential scanning calorimetry [12], nuclear magnetic resonance spectroscopy [13] and electrochemical sensors [14]. Furthermore, different groups performed diclofenac detection with chemo/biosensors based on recognition elements such as molecularly imprinted polymers [15–17] or aptamers [18–21]. However, most of these methods require to be performed in special laboratories, and thereby, are time and cost consuming. Thus, there is a need for the development of detection methods that are simple, fast, on-site-applicable and cost-effective.

Several whole-cell biosensors have been engineered for measuring water contaminants using genetically modified microorganism (bioreporter) in the categories of “light off” (constitutive expression of reporter protein hampered by analyte) and “light on” (expression of reporter protein induced by analyte) assays [22]. Because cells inevitably react to compound classes rather than a single chemical, they are

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preferentially attractive for environmental monitoring [22,23]. Lately, there have been attempts to integrate bioreporters into miniaturized devices to allow autonomous on-site detection of analyte [23–26]. On-site detection would be especially important for a routine screening in order to be able to react immediately to critical concentration values.

Recently, we introduced a PDMS-based flow-through device encapsulating genetically modified yeast cells [27]. The yeast cells express a fluorescent protein upon stimulation with diclofenac [28]. We demonstrated determination of diclofenac in the concentration range from 10 to 75 µM. However, the sensitivity of the whole system (diclofenac-sensitive yeast combined with flow cell and spectrometer detection unit) was still too low for the detection of diclofenac concentrations present in wastewater (down to 0.05 µM [29]) or surface water (down to 0.02 µM [30]). For this purpose, engineering of the PDR5 promoter region of the yeast cells [28] and applying of an amplification system [31] are objects of further research in order to lower the detection limit for diclofenac. Irrespective of these attempts to lower the detection limit for diclofenac, here we focus on technical steps of the whole cell sensor development that are necessary to allow for an on-site detection of diclofenac. On the one hand, we will introduce an automated spectrometric detection unit. On the other hand, we report on the design and assembly of a single-use and low-cost foil-based microfluidic flow-through device to encapsulate the genetically modified yeast cells. We will show that by using single-use foil-based flow cells, we are able to detect the induction of fluorescence light upon stimulation of the yeast cells with diclofenac.

2. Material and methods

2.1. Fabrication of PDMS flow cell

The design, manufacturing and assembling of the poly(dimethylsiloxane) (PDMS) flow-cell has been described previously [27]. Briefly, the sensing yeast cells are immobilized in two PDMS chambers, which are confined by a glass slide from the bottom and a porous membrane (Unique-Mem® Track-etched membrane with defined pore diameter of 1 µm, Oxyphen, Switzerland) from the top such that the sensing cells cannot leave the setup. Two further PDMS chambers, which serve as supply channels, are positioned above the membrane and can be perfused with nutrient solution and analyte (Fig. 1(a)), which are pumped through the cannels using a syringe pump (Harvard Apparatus, USA). Connection of the supply chambers to the microfluidic tubes is achieved through a polycarbonate body with fluidic in- and outlets (MicCell™, GeSiM, Germany).

2.2. Fabrication of the single-use foil-based microfluidic flow cell

The single-use foil-based microfluidic flow cell consists of several stacked foil layers. The basic design is the same as for the PDMS flow cell (Fig. 1), but two different polymer foils are replacing the PDMS: one serving as a spacer forming the cell and supply chambers and the other one as closure. Fig. 2 shows (a) a cut-away as well as (b) a top-view drawing to illustrate the setup. Upper and lower closures of the stack are formed by a polycarbonate foil (Lexan 8040, Sabic Innovative Plastics, Saudi Arabia). Lateral boundaries of cell and supply chambers in a polyester tape (#9960, 3 M™, USA) were generated by damage-free microstructuring by ultrashort-pulsed laser using UV wavelength (Time Bandwidth Products Fuego, Switzerland). The enabling technological trick was the depth-selective layout cutting of the respective layer without influencing the underneath layers (Fraunhofer IWS, Dresden). Therefore, height of supply and cell chambers is defined by the thickness of the polyester tape. Increasing the height of the channels is possible by stacking multiple layers of the tape. For our experiments, the height of the supply chambers was always 100 µm. The height of the cell chambers was varied between 100 and 200 µm by using one or two layers of polyester tape. As in the PDMS flow cell, a porous membrane (Unique-Mem® Track-etched membrane, Oxyphen, Switzerland) with a defined pore diameter of 1 µm is used to separate supply and cell chambers. Because the polyester foil forming cell and supply channels is a double-sided tape, cohesion of the stack is given and a roll-to-roll processing is applicable later on. Final thickness of the stack is 0.6 mm. Microfluidic connections are, similar to the PDMS device, achieved through a polycarbonate body (Fig. 2 (a) and (e)) that can be mounted on the microscope but also on the spectrometric detection unit via an adapter plate (Fig. 3).

2.3. Growth media, chemicals and yeast strains

Transformation of the yeast strain and the subsequent cultivation has been performed as described previously [27,28]. In brief, yeast strain *Saccharomyces cerevisiae (S.c.)* BY4741 p426PDR5:TGFP(1000), which produces turbo green fluorescent protein (TGFP) in presence of diclofenac, has been pre-cultured in selective synthetic complete medium without uracil (SC-ura), containing 6.9 g/l yeast nitrogen base with ammonium sulfate (Formedium, UK), 20 g/l D(+)-glucose (Roth, Germany) and 1.962 g/l Kaiser mix SC complete drop-out: ura (Formedium, UK). Diclofenac stimulation in the microfluidic devices occurred in synthetic minimal medium (MM), containing 6.9 g/l yeast nitrogen base with ammonium sulfate, 20 g/l D(+)-glucose, 60 mg/l L-histidine, 80 mg/l L-leucine and 20 mg/l L-methionine (Roth, Germany). Agarose with low melting gelling temperature (Plaque agarose) was purchased from Biozym (Germany). Ultra-pure water with 0.055 µS/cm (Micro Pure UV/UF, TKA, Germany) was used for the preparation of all solutions. Diclofenac sodium salt (Sigma, Germany) was used for preparation of a 20 mM diclofenac stock solution in absolute ethanol (VWR, France). Dilutions of the diclofenac stock solution have been prepared using absolute ethanol.

2.4. Diclofenac stimulation in the flow cells

Diclofenac-sensitive yeast cells were pre-cultured in SC-ura in a shaker at 180 rpm at 30 °C for 20 h, washed twice with fresh MM

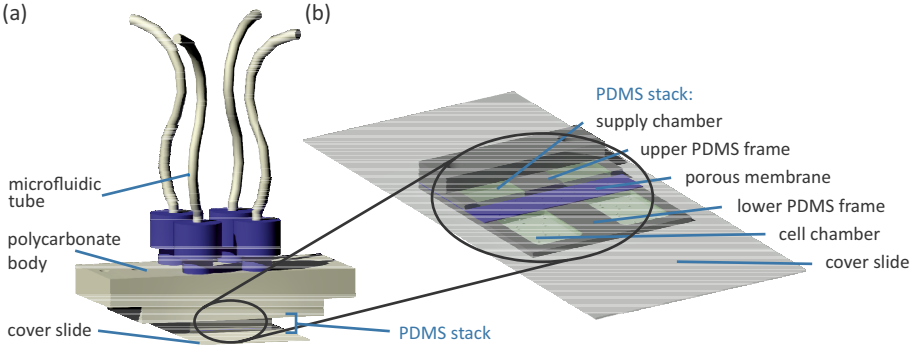

Fig. 1. Design of the PDMS flow through device. (a) Overall view; the sensor chambers are located between the cover slide and the polycarbonate body with the microfluidic tubes. (b) Cut-away drawing of the sensor chambers. The yeast cells are immobilized in the two lower chambers and covered by a porous membrane, which allows diffusion of nutrients and analytes to the cells but prevents them from leaving the cell chambers.

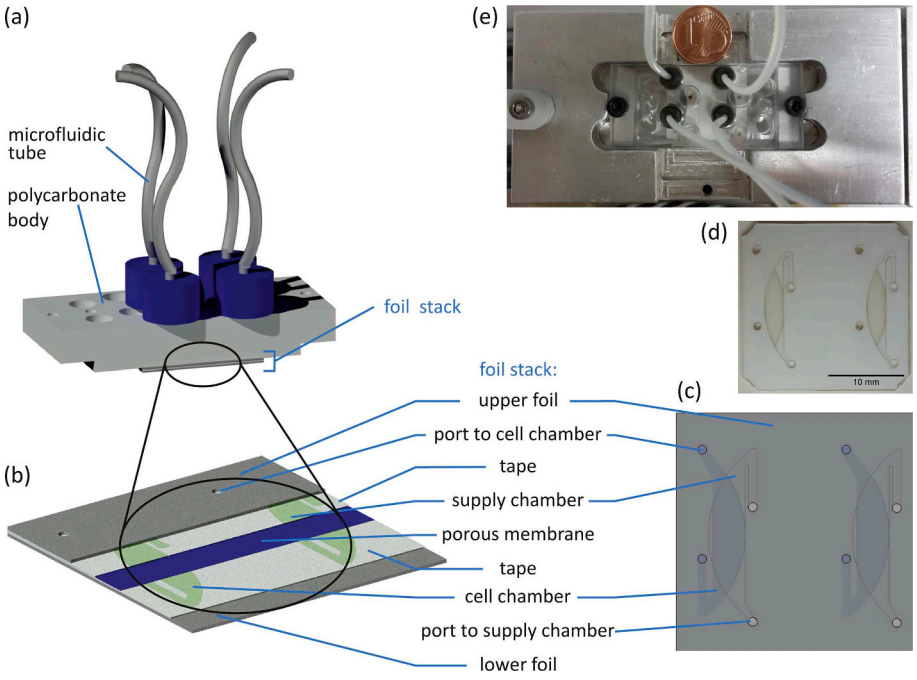

Fig. 2. Single-use foil-based microfluidic flow cell. (a–c) Design of the flow cell: (a) Overall view. Microfluidic connections to the foil-based flow cell are achieved through a polycarbonate body. (b) Cut-away drawing of the foil stack. Cell chambers and supply chambers are formed by tape and separated by the porous membrane. Lower and upper closures of the stack are constituted by a transparent polymer foil. (c) Top-view drawing of the foil stack. All membranes are drawn transparently to illustrate the setup. (d) Photo of the single-use foil-based microfluidic flow cell. (e) Flow cell connected to the tubes via a polycarbonate body that is mounted on the spectrometer unit.

without glucose and adjusted to an OD_{600} (optical density at 600 nm) of 2.0 in MM without glucose containing 0.75% agarose for immobilization of the yeast in the cell chambers. For the PDMS flow cell, 40 µl of the solution were pipetted into each lower chamber and covered with the Oxyphen membrane and the upper PDMS chambers after solidification. The foil-based flow cell was filled with the cell-agarose suspension directly through the port to the cell chamber. Thereafter, the upper channels of the flow cells and the tubes were filled with MM. Medium was pumped with a syringe pump (Harvard Apparatus, USA) with a rate of 20 µl/min (Fig. 3(a) and (b)). Cells were allowed to equilibrate in the glucose containing medium for 1 h after assembly. For stimulation of the yeast cells, MM with different diclofenac concentrations was pumped through the supply chambers for 12 h. All media were tempered to 30 °C in a heating block (Stuart, UK) during the whole experiment.

2.5. Fluorescence detection using microscope and spectrometric detection unit

The measurement with the spectrometer was done with a confocal setup, using an excitation LED and optical filters. The miniature spectrometer AvaSpec-Mini2048L-V25 (Avantes, Netherlands) with a wavelength range of 350–885 nm was used to measure the resulting fluorescence signals of the protein. For the TGFP excitation, a high power LED (APG2C3-470, Roithner Lasertechnik GmbH, Austria) with a center wavelength of 470 nm and a full width at half maximum (FWHM) < 30 nm was used. The TGFP-specific filter set XF411 (Omega Optical, USA) was used for the separation of the excitation and fluorescence signal. The dichroic filter was used to establish the confocal setup with the sample on top (for transmission spectra of the filter set and fluorescence spectra of TGFP refer to SI-1). PDMS as well as foil-based flow through device are mounted via a moveable adapter plate on the spectrometric detection unit, which allows taking measurements at different cell chambers (Fig. 3(c)). A Matlab (Mathworks, USA) program was written to perform the measurements automatically by controlling the spectrometer, the LED (to avoid bleaching) and the position of the sample slide. Measured intensities in wavelength range from 502 to 580 nm were integrated. Since signals were relatively low, they were summed up over a time period of 200 ms. In order to diminish background noise, two signals were averaged ($i_c(t)$).

For the detection of fluorescence by using an optical microscope (Axio observer Z1, Carl Zeiss Microimaging, Germany), in each of the two cell chambers, microscopy images (camera evolve®-EM 512, Photometrics, USA) of five different positions of the middle horizontal plane were taken with 200 × magnification and an exposure time of 30 ms (filter set 10, Carl Zeiss Microimaging, Germany). Readout of optical response was performed using the open source image processing software ImageJ [32] with the bio-formats plugin [33]. At every time point, the mean of the fluorescence intensity $i_c(t)$ of the five xy-positions in each chamber was calculated.

For the analysis of TGFP fluorescence with both spectrometer and microscope, signals were recorded every 20 min over a time period of 12 and 14 h for the PDMS and the foil-based flow cells, respectively. The obtained data have to be normalized with respect to the (1)

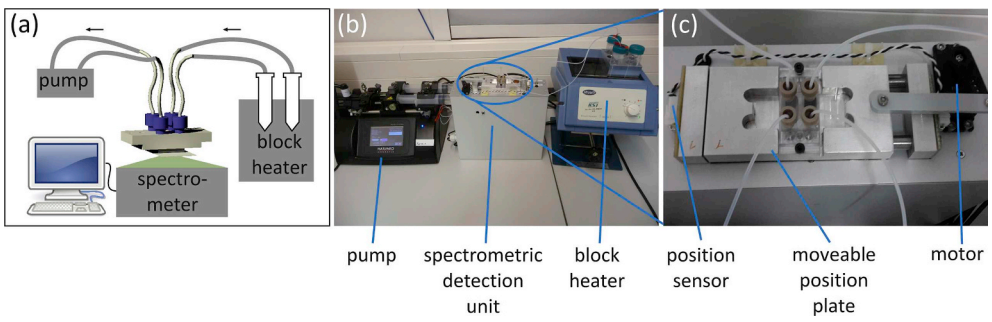

Fig. 3. (a) Schematic drawing of the spectrometric measurement setup. (b) Photograph of the spectrometric measurement setup. The spectrometric detection unit consists of a mini spectrometer, a filter set and an excitation LED. The flow through device (either PDMS based or foil based) is mounted on the spectrometric detection unit using an adapter plate (c), which can be relocated over the spectrometric detection unit to allow measurements in both cell chambers.

different initial values and (2) the bleaching of both the fluorescence proteins and the leakiness of the promoter. As a first step, the first 10 intensity values after diclofenac addition ($0 < t < 3h$) were averaged for each concentration curve ($i_c(0 < t < 3h)$) and subtracted at each point of time: $I(t) = i_c(t) - i_c(0 < t < 3h)$. Secondly, we took the leaking of the promoter and the bleaching of the fluorescent protein into account by subtracting the fluorescence intensity values $I_0(t)$ of the control cells induced with 0 µM diclofenac: $\Delta I_c(t) = I_c(t) - I_0(t)$. For raw data without this normalization, refer to SI-2.

3. Results and discussion

3.1. Fluorescence detection using the PDMS flow cell

Previously, we reported on a novel flow-through PDMS device with immobilized genetically modified yeast cells that produce fluorescent TGFP upon stimulation with diclofenac. Using this PDMS flow cell, mounted on a fluorescence microscope, we have shown that there is a clear dependency of fluorescence intensity on diclofenac concentration in a range from 10 to 75 µM [27]. Since for future applications of the method an on-site use of the device is required, we developed an automated spectrometric detection unit described here. Quantifying the fluorescence intensity values detected with this device, we observed an increase of fluorescence intensity with increasing diclofenac concentration in a range from 5 to 50 µM (Fig. 4(a)). With 75 µM diclofenac, no further enhancement of fluorescence intensity could be monitored compared to 50 µM, which can be explained by the toxic effect exerted by high diclofenac concentrations (Fig. 4(a)) [27,34]. Then, these measurements were compared to the fluorescence development in the PDMS flow cell under the same conditions but detected with the microscope. With this measurement, we could confirm that the fluorescence intensity increases with augmenting diclofenac concentration in a range from 5 to 50 µM, with a signal decrease at 75 µM diclofenac (Fig. 4(b)). However, a direct quantitative comparison of the fluorescence intensity values obtained with both detection units is not possible, because of the different illumination systems and detectors. That is why we calculated an "x-fold induction value" of fluorescence after 12 h of diclofenac stimulation. Therefore, the fluorescence intensity values for each diclofenac concentration obtained with microscope and spectrometric detection unit were divided by the corresponding fluorescence intensities obtained with 5 µM diclofenac. The induction of fluorescence obtained with both detection devices is in a similar range for all concentrations tested (Fig. 4(c)). It reaches from about 2-fold induction for 10 µM to about 5-fold induction for 50 µM diclofenac. In conclusion, these results show that the spectrometric detection unit can replace the microscope as detecting device, which is an important step forward towards diclofenac on-site detection.

3.2. Fluorescence detection using single-use foil-based microfluidic flow cell

Although the detection of fluorescence intensity with the PDMS flow cell works well, preparation and assembly of this cell is quite labor-intensive. In order to simplify the procedure, we developed the described single-use foil-based microfluidic flow cell that can be manufactured in advance and has only to be filled with yeast-agarose-mixture prior to start of diclofenac stimulation. Thereby, preparation time of the flow cell is remarkably reduced. As a proof-of-principle, we stimulated the yeast cells within the sensor chamber with 0 and 50 µM diclofenac and see an increase of fluorescence signal over time measured with the spectrometric detection unit (Fig. 5(a), green graph).

In principle, the setup of both the PDMS and the foil-based flow cell is the same, except for the dimensions of the chambers. The base area of the PDMS flow cell delineates a rectangle. This leads to problems in filling the upper chambers with nutrient solution, because air bubbles easily occur. In order to eliminate this issue for the foil-based flow cell, its shape was made elliptic. Thereby, however, the foil-based flow cell

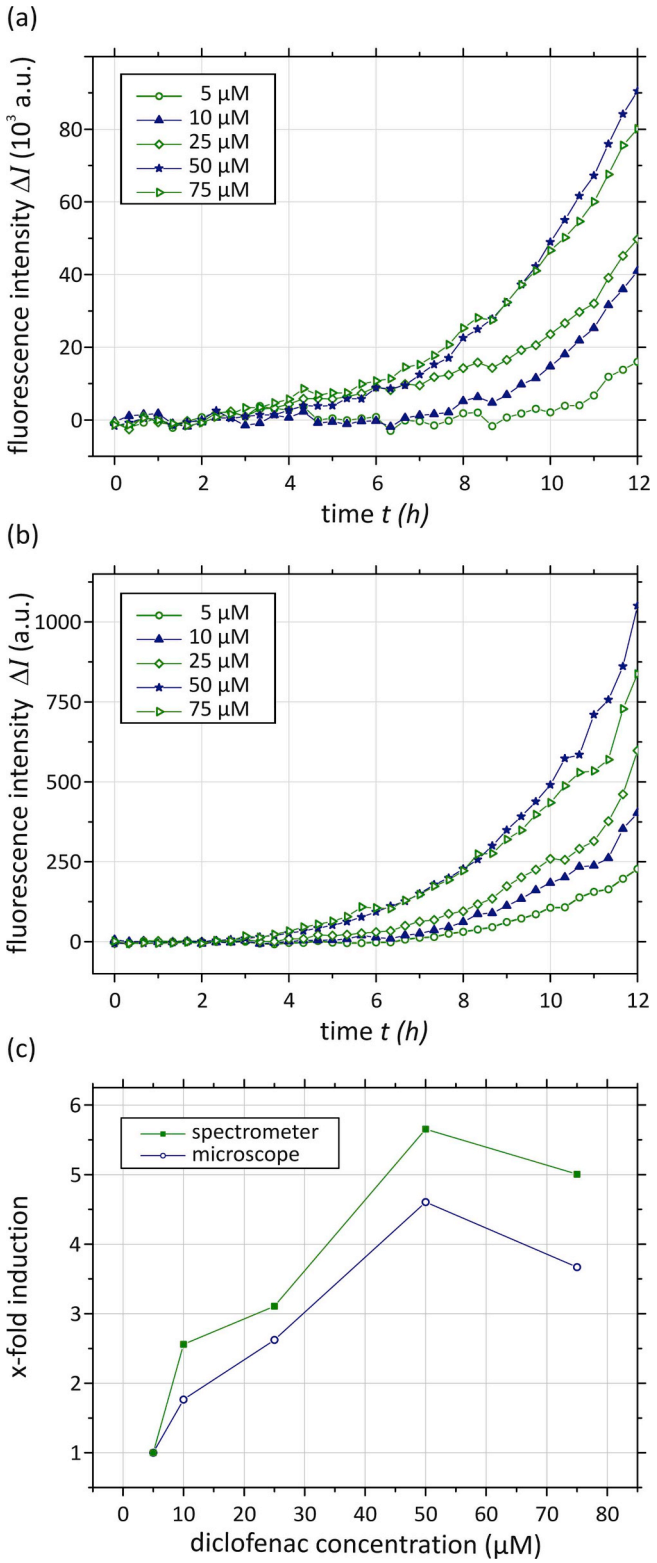

Fig. 4. Diclofenac-sensitive yeast cells were immobilized in the lower sensor chambers of the PDMS flow cell. Temporal development of fluorescence intensity with different diclofenac concentrations (stimulation starting at time point 0 h) was monitored via spectrometric detection unit (a) and the microscope (b). For each diagram, one independent experiment out of three is shown (Raw data of (a) and (b) without normalization to different initial values, bleaching and leaking of the promoter are shown in SI-2). (c) For comparison of spectrometric and microscopic measurements, data are displayed as x-fold induction at 12 h referring to the values obtained with 5 µM diclofenac.

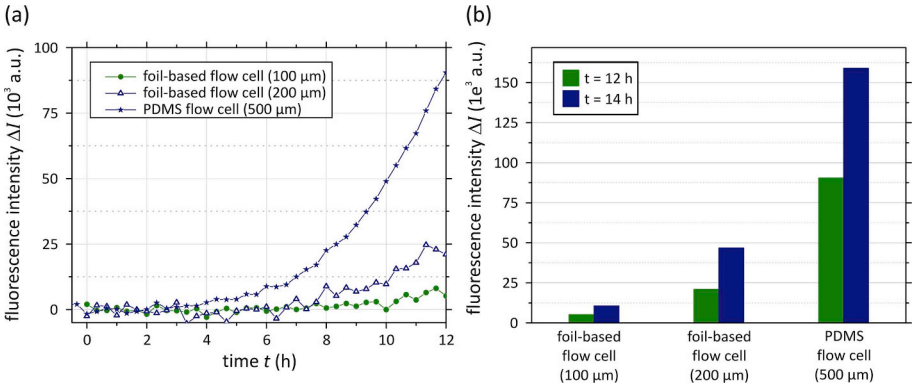

Fig. 5. (a) Diclofenac-sensitive yeast cells were immobilized in the lower sensor chambers of the foil-based flow cell and the PDMS flow cell. Temporal development of fluorescence intensity with 50 µM diclofenac (stimulation starting at time point 0 h) was monitored via the spectrometric detection unit. (b) Comparison of fluorescence intensity values obtained with spectrometric detection unit with the different flow cells 12 and 14 h after start of stimulation with 50 µM diclofenac. Heights of the respective cell chambers are given in parentheses.

exhibits approximately a base area of a half of the PDMS flow cell. Since the foil-based flow cell consists of several stacked foil layers, the height of the cell chambers is given by the thickness of the polyester tape, which is 100 µm, or a multiple of 100 µm. Firstly, we used foil-based flow cells with a cell chamber height of 100 µm. In contrast, the height of the PDMS flow cell is 500 µm. Altogether, the cell chamber volume of the foil-based flow cell is about one tenth of the PDMS flow cell. Comparing the obtained fluorescence intensity in the foil-based flow cell and the PDMS flow cell well reflects this geometric factor. As expected, the height of the fluorescent signal in the 100-µm-foil-based flow cell is roughly by a factor of 10 smaller compared to the PDMS flow cell (Fig. 5 (b)). Consequently, we would expect the fluorescent signal to increase, when the dimensions of the foil-based flow cells are scaled up. In the next step, we increased the volume of the cell chamber by adding an additional polyester tape layer, resulting in a cell chamber that has a height of 200 µm instead of 100 µm. Again, a considerable increase of fluorescence intensity is visible with 50 µM diclofenac (Fig. 5 (b)): As expected, fluorescence intensity with 50 µM diclofenac approximately doubles in the 200-µm-flow cell in comparison to the 100-µm flow cell when measured 12 h after start of stimulation with the spectrometric detection unit (Fig. 5 (b)).

Since the cell chamber volume of the foil-based flow cell is five times smaller than that of the PDMS flow cell, it comprises five times less yeast cells contributing to fluorescent protein production. Thus, we expect the development of a measurable fluorescent signal in the foil-based flow cell to be postponed compared to the PDMS flow cell. In addition to increasing the fluorescent signal by enlarging the cell chambers, another possibility is to examine fluorescence development for a prolonged time scale in order to be able to discriminate between different diclofenac concentrations. Thus, we evaluated the fluorescence development in the foil-based flow cells after 14 h instead of 12 h. As expected, we could see a further increase of fluorescence intensity after stimulation with 50 µM diclofenac in both foil-based flow cells, which in addition reflects the explained volume factors of the respective cell chambers (Fig. 5(b)). A third possibility to obtain higher fluorescent signal intensities is to increase bioreporter density within a given cell chamber volume. Due to the continuous supply of nutrients to the yeast cells by diffusion of fresh medium through the membrane, yeast cells will grow at higher optical densities than in batch culture. Thereby, we can increase the fluorescent signal at a given point of time by increasing the starting OD_{600} (see Figure SI-3). However, increasing of OD_{600} only does work to a certain extent, because prior to GFP production, diffusion of diclofenac into the cell, binding to the promoter, transcription and translation of the fluorescent protein have to take place. In conclusion, since genetically engineered yeast cells can be produced cost-efficiently in large quantities, enlarging of the cell chambers and additionally enhancing of cell density are good alternatives in order to obtain higher fluorescence signals in shorter time.

In future, the foil-based microfluidic flow cells can be automatically produced and prefilled with the diclofenac-detecting yeast cells. After a short equilibration time, diclofenac quantification can be started without time-consuming pretreatment. The flow cells are designated for single-use and must be replaced prior to the next diclofenac detection. Because the flow cells consist of foils, a roll-to-roll processing is possible. Furthermore, an automated dispenser may spot the yeast-agar mixture into the cell channels. Subsequently, the prefilled flow cell may be stored up to final usage. First trials of that showed promising results (data not shown). In summary, all these different contributions allow a fast and low-cost mass production.

PDR5 gene in yeast encodes for a plasma membrane ATP-binding cassette (ABC) transporter, which is involved in cellular detoxification, transporting a huge array of chemically and mechanistically distinct substrates [28,35]. Besides diclofenac, it is known that PDR5 cassette also responds to ketoconazole, cycloheximide, clotrimazole (antifungal agents), rifampicin, chloramphenicol (antibiotics), rhodamine 6G (fluorescent dye), tetrabutyltin (stabilizer for PVC, biocides, fungicides and anti-biofouling agents), tritylimidazole and iodoarylazidoprazosin [35,36]. However, engineering of the PDR5 promoter region took place prior to introducing the PDR5-TGFP gene construct into the yeast cells [28]. That's why further research is needed in order to determine which substrates except from diclofenac induce fluorescence in the genetically engineered yeasts. Furthermore, growth and activity of the yeast cells within our sensor may be influenced by other (toxic) substances present in the wastewater sample. In order to consider these matrix effects during diclofenac quantification, on the one hand, obtained fluorescence intensity can be related to the cell number within the flow cell. This number can be detected by evaluation of transmission spectra [25] or by impedance spectroscopy [37], for example. On the other hand, correction for matrix effects can be carried out applying an internal calibration curve. Therefore, according to the method of standard addition, aliquots of the unknown sample are spiked with diclofenac standard. Evaluation of the fluorescence intensity in each channel will result in a calibration curve. By this means, the signal readout in the unknown wastewater sample is unaffected by matrix effects and we will be able to conclude on the diclofenac concentration within the wastewater sample.

Altogether, the data indicate that our foil-based microfluidic flow cell in combination with the automated spectrometric detection unit allows for the on-site detection of diclofenac concentrations with genetically modified yeast cells. The obtained results are as sensitive as microscopic detection. In this manner, a fast and easy way for on-site diclofenac analysis will be possible without the need for time-consuming transport to laboratories and expensive quantification with mass-spectrometry or chromatography. Furthermore, a change of the specification of the engineered yeast cells allows the detection of a wide panel of substances that can be parallelized for high convenience and efficiency.

Author contributions

C.S. and J.P. performed the experiments and wrote the manuscript. M.S. and M.G. designed and build the spectrometric detection including software. S.H. designed and manufactured the foil-based flow cell. W.S. and M.M. were responsible for conceptualization, supervision and writing.

Declaration of conflicting interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.05.058.

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