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Encapsulating genetically modified *Saccharomyces cerevisiae* cells in a flow-through device towards the detection of diclofenac in wastewater

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ABSTRACT

Recently it has been proposed to use sensors based on genetically engineered reporter cells to perform continuous online water monitoring. Here we describe the design, assembly and performance of a novel flow-through device with immobilized genetically modified yeast cells that produce a fluorescent protein upon stimulation with diclofenac whose intensity is then detected by fluorescence microscopy. Although other devices employing immobilized cells for the detection of various analytes have already been described before, as novelty our system allows safe enclosure of the sensor cells, and thus, to obtain fluorescent signals that are not falsified by a loss of cells. Furthermore, the yeast cells are prevented from being released into the environment. Despite the safe containment, the immobilized reporter cells are accessible to nutrients and analytes. They thus have both the ability to grow and respond to the analyte. Both in cell culture medium and standardized synthetic wastewater, we are able to differentiate between diclofenac concentrations in a range from 10 to 100 µM. As particularly interesting feature, we show that only the biologically active fraction of diclofenac is detected. Nowadays, contamination of wastewater with diclofenac and other pharmaceutical residues is becoming a severe problem. Our investigations may pave the way for an easy-to-use and cost-efficient wastewater monitoring method.

1. Introduction

Environmental monitoring of chemicals can be achieved by traditional laboratory analytical methods. While these methods are very precise and sensitive, they require special laboratories, transport of the samples into the laboratory and especially trained staff to perform the analysis. Therefore, such an analysis is usually very expensive, time consuming and has the risk of transport-mediated damage. Whole-cell biosensors employing living, genetically engineered microbial cells (bioreporters) as recognition element could be used to monitor chemicals directly in the environment (Belkin, 2003; Close et al., 2009; Daunert et al., 2000; He et al., 2016; Park et al., 2013; van der Meer and Belkin, 2010). Compared to enzymes, antibodies, receptor proteins and nucleic acids as recognition elements, microbial cells are easy to manipulate and can be cost efficiently produced in large quantities (Park et al., 2013). Furthermore, they offer a higher stability compared to enzymes. Most importantly, genetically engineered microbial whole-cell biosensors allow to indicate the bioavailability of the analyte (Close et al., 2009).

The concept of whole-cell arrays for parallel detection of a variety of different analytes was introduced by van Dyk et al. (2001) and is reviewed by Elad et al. (2008). So far, most of the presented studies in the field of genetically engineered whole-cell biosensors are laboratory proof-of-principle assays, where the whole-cell bioreporters are incubated with the target chemical and the expression of the quantifiable reporter protein is detected by standard laboratory equipment. Yet, only a few platforms provide a solid support for the bioreporter containment, offer the possibility to supply nutrients and analytes to the

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bioreporters by means of microfluidics and include a sensing device (Roggo and van der Meer, 2017). Elad et al. described a flow-through biosensor for online continuous water toxicity monitoring based on poly(dimethylsiloxane) (PDMS) microfluidics incorporating agar-immobilized bioluminescent recombinant reporter bacteria (Elad et al., 2011). Yagur-Kroll et al. presented a flow-cell biosensor for online continuous water monitoring based on a disposable porous aluminum oxide chip (Yagur-Kroll et al., 2015). Buffi et al. trapped agarose beads containing genetically engineered microorganism in a microfluidic PDMS device (Buffi et al., 2011). Cheol Gil and coworkers developed a toxicity sensor based on agarose immobilized *Escherichia coli* (Cheol Gil et al., 2000). In all of these studies, an escape of genetically engineered microorganisms from the microfluidic device cannot be excluded and potentially provokes harmful consequences for the environment. Furthermore, a variation of signal intensity due to a loss of reporter organisms may occur. Hence, the goal of the present work was to design a microfluidic device based on commercially available microfluidic components that (i) serves as a compartment for the genetically engineered bioreporters, (ii) offers microfluidic supply of the bioreporter cells with nutrients and analyte, and (iii) provides the possibility of signal readout by fluorescence microscopy.

In our studies, diclofenac serves as the analyte to be detected by genetically modified yeast cells. The nonsteroidal anti-inflammatory drug (NSAID) diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid, $C_{14}H_{11}C_{12}NO_2$) is administered as oral tablets or as topical gel. Upon oral administration between 60% and 70% of the dose are excreted in the urine and 20–30% in feces as the parent drug or as metabolites (Vieno and Sillanpää, 2014). Measured mean concentrations in municipal waste water vary between 0.11 µg/l and 2.3 µg/l, but can rise up to 203 µg/l in pharmaceutical manufacturer's waste water in South Korea (Vieno and Sillanpää, 2014). Harmful effects to different organisms have been demonstrated at relevant environmental concentration levels (Stülten et al., 2008), for example renal lesions and alteration of the gills in fish at 5 µg/l diclofenac (Pérez-Estrada et al., 2005). Thus, presence of diclofenac in municipal wastewater treatment plants is a severe problem. To date, for the detection of diclofenac concentration different methods have been described. Besides classical analytical methods, such as UV–vis spectrophotometry (Agatonović-Kuštrin et al., 1991), fluorimetry (Damiani et al., 1999), high performance liquid chromatography (Sane et al., 1987), liquid chromatography (Beaulieu et al., 1990), capillary electrophoresis (Jin and Zhang, 2000), LC–MS (Abdel-Hamid et al., 2001), differential scanning calorimetry (Bucci et al., 2000) and nuclear magnetic resonance spectroscopy (Fattah et al., 1988), different electrochemical sensors (Arvand et al., 2012) have been exploited for the determination of diclofenac. Recently, Schuller et al. reported the engineering of *Saccharomyces* (*S.*) *cerevisiae* yeast cells which produce a fluorescent protein upon exposition to diclofenac (Schuller et al., 2017). Here we describe the design of a microfluidic flow-through device with entrapped reporter yeast cells allowing fluorescence protein detection upon addition of an analyte. The device exhibits two separate chambers, enabling simultaneous analysis of analyte and reference, thus offering the possibility to control for matrix effects. The special design of the device includes a cell-impermeable membrane, which allows employing genetically modified reporter cells that should not escape from the sensor into the environment. Furthermore, we demonstrate that loss of reporter cells would lead to diminished fluorescent signals, thus leading to misinterpretation of the analyte concentration. To prove functionality of the flow-through device, the genetically modified diclofenac-sensitive yeast cells designed by Schuller et al. are used (Schuller et al., 2017). We show that the yeast cells cannot escape from the microfluidic device - they are literally inclosed, but can still be supplied with nutrients through a porous membrane. Additionally, we prove that the analyte passes to the yeast cells and induces the production of a fluorescent reporter protein. The development of the relative fluorescence intensity, detected for the cells in the microfluidic device by fluorescence microscopy, is in line with the results of control experiments using flow cytometry.

2. Material and methods

2.1. Growth media, chemicals and genetically modified yeast strains

Transformation of *S. cerevisiae* strain BY4741 [*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*] was carried out as described previously (Schuller et al., 2017). Resulting transformants were: (1) *S.c.* BY4741 p426PDR5::TGFP(1000), which produces turbo green fluorescent protein (TGFP) in the presence of diclofenac, (2) *S.c.* BY4741 p426GPD::TGFP, constitutively expressing TGFP in the presence of glucose, was used as a positive control, and (3) *S.c.* BY4741 p426GPD, which is not able to produce TGFP, served as negative control. Both diclofenac-sensitive and control cells were 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). For plating of yeast cells, 2.0% (w/v) agar (Fluka, Germany) has been added to the selective synthetic complete medium. Diclofenac stimulation in the microfluidic device 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 were made with absolute ethanol. Synthetic wastewater was prepared on the basis of DIN 38412. For preparation of twofold concentrated synthetic wastewater, 500 mg skimmed milk powder (Gabler-Saliter, Germany), 100 mg peptone (Roth, Germany), 54 mg Na_2HPO_4 (Riedel-de-Häen, Germany), 178.3 mg NH_4Cl (Roth, Germany), 41.7 mg $C_2H_3NaO_2$ (Roth, Germany), 58.4 mg NaCl (VWR, Germany), 14.7 mg $CaCl_2{\cdot}2H_20$ (Fluka, Germany), 20.3 mg $MgCl_2{\cdot}6H_20$ (Roth, Germany), 7.4 mg KCl (Fluka, Germany) were filled to 500 ml with ddH_2O and autoclaved.

2.2. Design and manufacturing of the microfluidic flow-through device

The design of the device is based on a commercially available microscopy cell (MicCell™; GeSiM, Germany) (Gast et al., 2006) with an additional subjacent PDMS layer and an intermediate porous membrane (Fig. 1), as described in the following. A glass coverslip constitutes the bottom of the device, on which a 500 µm thick PDMS-layer frame forming two rectangular chambers (17 mm × 5 mm × 0.5 mm) is placed (Fig. 1a). These "cell" chambers contain the genetically modified yeast cells (Fig. 1b) which are immobilized in agarose. One of the chambers is used for TGFP expressing cells, while the other harbours non-fluorescent control cells. A hydrophilic track-etched membrane with a pore diameter of 1 µm and a thickness of 17 ± 3 µm (Oxyphen, Switzerland) is used to cover the two lower chambers (Fig. 1c). The membrane with an exactly defined pore size and a pore orientation in vertical direction to the membrane surface is permeable for nutrients and the analyte, but impermeable for the yeast cells (average diameter 5 µm). Thus, the genetically modified yeast cells are enclosed in all three dimensions: at the bottom by the glass coverslip, at the sides by the PDMS frame and at the top by the Oxyphen membrane. Supply with medium and analyte is performed from two upper PDMS "supply" chambers with a height of 500 µm, which are placed congruently on the lower chambers (Figs. 1d and SI-1).Fluidic in- and outlets are realized as in the commercially available MicCell™-System.

The masters for manufacturing of both the lower and the upper PDMS chambers were milled from poly(methyl methacrylate) (PMMA). The negative master for molding the upper PDMS chambers can directly