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Highly resolved dose–response functions for drug-modulated bacteria cultivation obtained by fluorometric and photometric flow-through sensing in microsegmented flow

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ABSTRACT

The application of the micro fluid segment technique for the investigation of highly resolved dose–response relationship at the physiological parameters segment–internal pH and growth of the cell culture is introduced. The method was used to study the concentration-dependent response of an *Escherichia coli* culture for the effectors 2,4-dinitrophenol (DNP) and an antibacterial peptide amide (KKVVFKVKFK-NH_2). Large sequences with up to 250 micro fluid segments containing gradually varying concentrations of the effectors were generated using a PTFE micro fluidic arrangement, including a 7-port-manifold and PC-controlled syringe pumps. The response of the cell culture was characterized by a double sensor system allowing for simultaneous read out of metabolism-related changes as well as changes in cell density. A twin arrangement of a micro flow-through photometer and a micro flow-through fluorometer based on LED devices in connection with the application of pH-sensitive polymer sensor particles was applied. This experimental setup allows a detailed determination of drug-related changes in fluorescence intensity by the *E. coli* culture and the polymer particles as a function of time by tracking changes in pH and cell density. The application of the segmented flow technique for multi-parameter drug screenings provides new insights into the biological answer of bacteria cultures cultivated at the nanoliter scale.

1. Introduction

The investigation of biological effects of an increasing number of compounds, in particular the need for toxicological evaluation, demands new miniaturized analytical strategies. This challenge is further enforced by the insight that diverse organisms, tissues, cells and microorganisms show different responses. Moreover, it is known that their response is dependent on other chemical factors and surrounding conditions. Besides the large number of biogenic and synthetic substances which affect the viability and growth of cells, their combination and unknown synergetic and compensatoric factors produce an even larger number of parameters that need to be investigated. Therefore, classical methods of toxicology have to be supplemented by new, highly parallelized and miniaturized techniques and devices to ensure that all relevant parameters can be thoroughly examined in a short period of time.

The fast evaluation of changes in the growth of cell cultures is a key issue for miniaturized and automated toxicity tests. Therefore, fast acting non-invasive optical methods are preferable to other methods. Cell densities can be determined by microscopic imaging – in particular by dark field illumination, by light scattering or UV absorption [1–4]. Higher sensitivities can be achieved by fluorometric, bio- or chemiluminescence techniques, but are restricted to selective test systems such as, for example, GFP-labeled microorganisms [5–7]. Miniaturized optical detectors for micro flow-through measurements are reported for different applications [8,9]. Micro flow-through photometers and fluorometers using LED diodes are applicable for modulation rates up to several kHz [10].

Beside the well-established conventional 96-well micro titerplates (MTP), miniaturized devices as nano titerplates (NTP) were introduced in order to handle smaller volumes and larger series of samples [11,12]. The biochips as a second approach are suited for detection of molecular interactions at high degrees of paralleliza-

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tion [13–15] but are less suited for the application of cell-based tests.

Here, sequences of micro fluid segments represent an interesting alternative strategy. They are well suited for the generation of larger sample series of small volumes. Furthermore, they provide the possibility for future high-throughput screenings (HTS) in toxicological and drug research fields. As a result, micro fluid segments represent an interesting method of artificial compartmentation for complete separation of small reaction or cultivation volumes. They are not only under study for synthetic purposes [16,17] and in analytical chemistry [17,18], but also for biochemistry [19], protein techniques [20] and DNA analysis by micro flow-through PCR [21–23]. In particular, micro fluid segments sequences stored in PTFE capillary tube coils offer considerable advantages for the search for rare organisms [24] and for the cultivation and testing of microorganisms in drug development [25,26]. They are not only suited for tests with prokaryotic and eukaryotic cells, but can also be applied for screenings with multicellular systems like embryos [27]. It was shown in previous investigations that such systems are applicable to fast growth of *Escherichia coli* colonies but also for long-term incubation of slowly growing microorganisms. So, the micro fluid segment technique can be used for a large spectrum of different targets for toxicological studies.

The generation and characterization of a large series of micro fluid segments with single volumes in the nanoliter range allows systematic investigations in large parameter areas with a minimum of applied chemicals [28]. This specific advantage can be used in particular for detailed toxicity tests in dose- and time-dependent analytical procedures. In this paper, we report on the application of fluid segment sequences with stepwise varying concentrations for microtoxicological investigations and the determination of dose–response functions of selected effectors on *E. coli* in nanoliter segments.

2. Experimental

2.1. Chemicals

The medium LB-Lennox consisted of 10 g bacterial peptone, 5 g yeast extract and 5 g NaCl dissolved in 1 L distilled water. The synthetic medium was made up of the following components: 0.56 g K_2HPO_4, 0.3 g KH_2PO_4, 0.012 g $MgSO_4{\cdot}7H_2O$, 0.1 g NH_4Cl in 90 mL distilled water and 10 mL 0.1 g L^{-1} glucose solution. Perfluoromethyldecalene (PP9) was obtained from F2 Chemicals Ltd. (Lancashire, UK), poly(ethylene glycol) (PEG) from Roth GmbH (Karlsruhe, Germany), poly(vinyl pyrrolidone) (PVP) and dimethylsulfoxide (DMSO) from Merck-Schuchardt (Hohenbrunn, Germany), cochenille red A from VWR (Darmstadt, Germany) and 2,4-dinitrophenol (DNP) from Acros (Gel, Belgium).

The peptide amide KKVVFKVKFK-NH_2 [29] was prepared by solid phase peptide synthesis using Fmoc/tBu chemistry and Rink amide MBHA polystyrene resin. For coupling of Fmoc amino acids double couplings and a sevenfold molar excess of amino acids were performed. The peptide amide was cleaved from the resins and purified to >90% as determined by HPLC–MS, 214 nm.

2.2. Organism

For all experiments described in this paper, the *E. coli* strain RV308 (provided by the Hans-Knöll Institute (HKI), Jena, Germany) was used. The *E. coli* was first cultivated in a LB medium for 24 h at 37 °C under stirring. Afterwards it was streaked out of LB agar plates and cultivated at the same conditions as described above. To obtain an almost homogenous stock culture, a single colony was removed from the agar plate and transferred into an Erlenmeyer flask filled with the synthetic medium and recultivated.

2.3. Experimental arrangement

A segmentation module (7-Port-Peek Manifold, Upchurch, Washington) was used for generating micro fluid segments (Fig. 1). The 7-port-manifold was connected through Teflon® tubes (0.5 mm ID, Bohlender GmbH, Grünsfeld, Germany) with a computer-controlled syringe pump equipped with six dosing units (Cetoni GmbH, Korbußen, Germany), including syringes (ILS, Stützerbach, Germany) with volumes of 500 μL and 2500 μL for the samples and the carrier liquid, respectively. The combined application of a micro flow-through photometer and fluorometer allowed the simultaneous measurement of the scattered light and fluorescence intensity directly through the tube. A light emitting diode (LED, Agilent, Santa Clara, CA, United States) with a peak wavelength of 505 nm was used for the scattering measurement. The incident light traversed the tube and was scattered randomly at the tube and the fluid segment content on the way. The intensity of the passed through light was detected with a photodiode (Type SFH 206K, Siemens, München, Germany).

A LED with a peak wavelength of 470 nm (CML, Bayern, Germany) served as the excitation light source for the micro flow-through fluorometer. Its light first passed a short-pass filter (Laser Components GmbH, Olching, Germany) to isolate the light of excitation wavelengths up to 480 nm. An aperture with a split width of 1 mm focused the light beam on the tube. When light hits the sensor particles, their fluorescence is excited to emit green light. A concave mirror was embedded to focus most of the emitted light into the detector.

The light beam was parallelized by a convex lens and sent to the detector through a long-pass filter with an emission wavelength of 510 nm. The emitted photons were counted by a photon counting module (PCM, PerkinElmer). A tube coil was used to store the generated segments between the single measurement periods. The coil consists of a PMMA plate comprised of a rolled transparent PTFE (Teflon®) tube with a length of almost 1 m. The fluid flow was controlled with a LabVIEW™ program (National Instruments). The in situ monitoring of the scattering and the fluorescence signals of microbeads, which had been introduced inside the micro fluid segments with the cell suspension, and microorganism was realized by combining two optical sensors (Fig. 2).

In this manner, it was possible to measure the change in cell density during the growth of the bacteria culture and the metabolic change occurring inside the single micro fluid segments.

2.4. pH-sensitive microbeads

Microbeads with a diameter of 3 μm were introduced in order to analyze the pH change due to the metabolic activity of *E. coli* inside micro fluid segments [30–32]. They consisted of the pH probe HPTS (8-hydroxypyrene-1,3,6-trisulfonate) which was covalently immobilized on the p-HEMA (poly-hydroxyethylmethacrylate) particle [33]. The microbeads were swollen in DMSO and a PVP solution inside an ultrasonic bath before the application to prevent clustering. The HPTS beads and cells were combined in a suspension, which was then injected into the various segments.

2.5. Experimental procedure and data collection

2.5.1. Preparation of the E. coli suspension and effector solution

For the experiment with DNP the sample solution consisted of 51 μL *E. coli* suspension (stock culture 7.8×10^5 cells mL^{-1}), 8 μL HPTS/p-HEMA microbeads (stock suspension 8×10^9 beads mL^{-1}) and 989 μL of synthetic medium.