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Investigation of mixture toxicity of widely used drugs caffeine and ampicillin in the presence of an ACE inhibitor on bacterial growth using droplet-based microfluidic technique

Abstract: Droplet-based microfluidic technique is very suitable for a variety of screening processes. The cultivation within nanoliter segments and multidimensional microtoxicological screening of the Gram-negative bacterium *Escherichia coli* were studied under droplet-based microfluidic conditions. In order to evaluate the toxicity of the binary and ternary mixtures of antibiotic ampicillin and caffeine in the presence of the angiotensin-converting enzyme inhibitor captopril, a time-resolved optical double endpoint detection unit was applied. It included a microflow-through fluorimeter and photometer, which can simultaneously analyze changes in the endogenous autofluorescence signal and the cell density of *E. coli* cultivated inside 450-nl microfluid segments. As a result, strong nonlinear combination effects and a concentration-dependent antagonistic effect, as well as formation of activity summits on bolographic maps, were found. Our findings confirm the importance of multiparameter investigations for toxicological studies and could be taken into account in medical practice.

Keywords: ACE inhibitor captopril; antibiotic ampicillin; combinatorial effects; droplet-based microfluidic technique; mixture toxicity.

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1 Introduction

Protection of organisms from the damaging effects of toxic substances is one of the elementary goals of health care and sustainable management of the environment. The effects of chemicals on living beings are dependent on the applied doses. In addition, the sensitivity for toxic substances depends on the type of organism. Different species and their phases of development, as well as diverse types of organs, cells, and tissues, show significant differences in response to a certain effector. As a result, we are confronted with a large spectrum of toxicological problems when we have to judge the toxicity of a compound. The recent strategy of treating this problem is to focus on some standard organisms in toxicological tests. These organisms are used for the determination of dose-response functions, which can help to identify lethal doses and thresholds of toxicity and to evaluate risk potential [1, 2].

Despite the restriction on selected test organisms, there is a second crucial problem of risk assessment: In the real world, organisms are exposed not only to a single substance but also to substance mixtures. A second substance can have no effect on the first regarded substance, but it can also enhance or reduce their toxicity. In many cases, a change in the dose-response function is observed [3, 4]. A reliable analysis of toxic effects in dependence of concentrations of two or more substances demands for a toxicological screening in a two- or higher-dimensional concentration space. The number of required tests increases exponentially with the number of regarded substances. The increase in the number of samples that have to be treated in such a screening requires a reduction in the amounts of needed chemicals and sample volumes for an individual test. In addition, it is impossible to investigate all variants of a complex environment involving many different parameters. Consequently, it is necessary not only to reduce the complexity inside a single test experiment but also to evaluate the interdependence of different effectors on one type of organism by larger series of well-separated single experiments. This problem can obviously not be solved in the frame of conventional toxicological methods. It provides a challenge for a new approach for making an investigation with larger sets of samples and in multidimensional parameter spaces feasible.

Principally, this strategy could be implemented with parallel processing systems like microtiter plates, which allow the definition of many well-separated small test volumes. Microtiter plates have become a standard tool with different analytical, synthetical, and screening applications and have been widely used in clinical diagnostic laboratories [5–9]. Further miniaturization became possible by cell cultivation in nanotiter plates. Automated pipetting techniques are available but demand rather expensive equipment. Another difficulty is the transport of all liquids through the gas phase and the related problems of solvent evaporation, wetting, and pipetting behavior [10]. Furthermore, it has to be considered that surface phenomena are dominating over gravity effects if the sample volume is reduced down to the microliter range. The surface state-dependent wetting, dispensing, and flow behavior completely determine the liquid behavior in the lower microliter and nanoliter range. Namely, the sensitivity of pipetting processes against changes in the liquid composition is a serious problem for the application of microtiter and nanotiter plates.

An alternative to parallel processing systems like microtiter and nanotiter plates is droplet-based microfluidic technique, which is able to form separated small test volumes very quickly and conveniently. This technique is based on the spontaneous and regular formation of droplets when one liquid is injected into a stream of an immiscible second liquid inside a microchannel [11].

In comparison with the conventional and microtiter plate technique, the droplet-based microfluidic technique offers many advantages for cellular screenings. The first aspect is that small liquid compartments with typical volumes in the sub-microliter and the nanoliter range are embedded in a hydrophobic separation carrier liquid and lead to an efficient separation and decoupling of segments. A sequence of about 10,000 segments with an individual volume of 100 nl each means that only a total of 1 ml of cultivation liquid is required.

The second important aspect is the partial or complete decoupling of the cultivation liquid from the channel walls. The problem of interaction of biomolecules and cells with the walls is particularly serious in microfluidic systems because the ratio of surface to volume is dramatically enhanced in comparison with conventional cultivation systems. In microsegmented flow, biofouling can be significantly reduced under conditions with a high wetting of the walls by the carrier solution and a low wetting by the cultivation liquid. In general, perfluorinated tubes and device materials are combined frequently with liquid perfluorcarbons used for the carrier phase. Thus, the carrier solution always has a low contact angle with the wall surface and the cultivation liquid forms a high contact angle corresponding to low wetting. During transport, a liquid film of carrier solution is formed in a capillary slit between the wall and the segment, leading to a complete separation of the segment content from the wall. As result, the adsorption of biomolecules and the adhesion of cells at the inner walls of microchannels are considerably suppressed.

A third important aspect is the reduction in volume in comparison with conventional and microtiter plate cultivation conditions and leads to a reduction in diffusion paths, which results in a faster transport by diffusion. The application of tubes with lower wall thickness consisting of highly gas permeable perfluorinated materials supports the gas exchange between the fluid segments and the environment.

The fourth aspect is the fact that axial transport of segments induces a segment-internal circular convection. It is considerably different from homogeneous microfluidic systems in which a laminar flow is observed in general due to low Reynolds numbers, *Re* (which compares inertial forces with viscous forces). In segmented flow, the interface tension between the aqueous cultivation phase and the lipophilic carrier phase keeps the segments stable during the transport and induces a radial component in the liquid streaming. This circular convection is very efficient for the fast transport of particles and molecules and is of particular importance for mixing processes with vesicles, sensor particles, and cells, which are too large to show a significant diffusive contribution to their transport. The segment-internal convection supports the mixing after addition of components into segments (mixing, dosing) as well as homogenization during further procedures [12].

Furthermore, the incorporation of cultivation volumes inside microchannels provides a safe processing of segments and a low loss of solvent and other volatile components. Although the typical volumes of microfluid segments are about two orders of magnitude lower than the typical liquid volumes in microtiter plates, the evaporation rates are much lower than in open microtiter plates. Thereby, guidance of segments by the tubes or channel walls allows a safe and reproducible serial processing of complete segment sequences without any pipetting steps.

Finally, the fixed order of segments inside the microchannel allows an addressing of individual segments [13]. Therefore, the position of each segment is defined by a predetermined starting composition and an individual process history. Every segment can be related to a fixed point in the space of investigated parameters. Thus, one can easily assign data from measurements on single

segments to a composition and process data. This is an important precondition for using microsegmented flow for investigation of highly resolved dose-response functions and on toxicological screenings in two- and higher-dimensional concentration spaces.

Meanwhile, there exists a consensus that the droplet-based microfluidic technique is a powerful tool for various applications such as analytics [14, 15], organic and nanoparticle syntheses [16, 17], polymerizations [18, 19], protein crystallization [20], DNA analysis [21], drug discovery [22, 23], genomics [24], cell cultivation [25, 26], and embryonic development studies [27]. In the present study, the suitability of this technique was applied for the investigation of the effects of captopril in combination with the application of antibiotic ampicillin and caffeine on *Escherichia coli*.

E. coli bacteria are widely distributed in the environment and within human intestines. Caffeine (1,3,7-trimethylxanthine) is the most widely consumed psychoactive substance in the world. It exerts a wide variety of physiological effects on different organisms and has already been investigated regarding its antibacterial activity [28]. In addition, caffeine can influence multiple pathways involved in the cellular response to DNA damage [29]. It has already been reported that sublethal doses of caffeine can stimulate metabolic activity and may lead to an increase in cell growth [30, 31]. Our previous study has demonstrated a complex response on growing bacterial culture when combining caffeine with phenolic uncoupler 2,4-dinitrophenol and chloramphenicol [31]. The impressive insights on combination with caffeine taken from the previous results motivated us to further investigate combined effects with widely used medications.

At first, for this study, we decided to investigate the effects in the case of the frequently used angiotensin-converting enzyme (ACE) inhibitor captopril. ACE (synonym kininase II) is an exopeptidase found circulating in the bloodstream. It catalyzes the conversion of angiotensin I to angiotensin II, a powerful vasoconstrictor that acts to raise blood pressure. Therefore, captopril is a drug that has found widespread use in the treatment of hypertension and heart failure [32, 33].

Broad-spectrum antibiotic ampicillin was chosen as another drug in this work because it is a traditional beta-lactam antibiotic with various applications in medical therapies. It inhibits the formation of cell walls and therefore possesses a bacteriostatic activity.

Whereas the effect of the individual substance is already known, the interactions among these different substances are rarely studied. Therefore, the aim of this study was to determine the combined effect of the drugs caffeine and captopril with antibiotic ampicillin on bacteria *E. coli*. Our findings could be taken into account in medical practice.

2 Materials and methods

2.1 Organism and chemicals

The *E. coli* strain RV308 (Hans-Knöll Institute, Jena, Germany) was used for the investigation of highly resolved dose-response of single effector and ternary mixtures of ampicillin, caffeine, and captopril. For precultivation, a single colony of *E. coli* was removed from the LB (Lysogeny broth) agar plate (10 g/l bacterial peptone, 5 g/l yeast extract, 5 g/l NaCl, and 13 g/l agar), transferred into an Erlenmeyer flask filled with 10 ml synthetic medium (0.56 g K_2HPO_4, 0.3 g KH_2PO_4, 0.012 g $MgSO_4 \cdot 7H_2O$, and 0.1 g NH_4Cl in 90 ml distilled water and 10 ml 0.1 g/l glucose solution sterilized by filtration), and finally cultivated for 24 h at 37°C while being stirred. Bacteria cultures in the lag phase and the early exponential phase of growth were used for inoculation of separated droplets in the microfluidic system.

In all experiments, perfluoromethyldecaline (PP9, F2 Chemicals Ltd, Lancashire, UK) was used as carrier liquid. Ampicillin was obtained from Sigma-Aldrich, Munich, Germany; caffeine, from Applichem GmbH, Darmstadt, Germany; and captopril, from Molekula GmbH, Munich, Germany.

The final concentrations and stock solutions of all investigated effectors for the different experiments are listed in Table 1. The stock solution with a twofold final concentration was used for investigation of the highly resolved dose-response relationship for individual effector. For the two- and three-dimensional concentration spaces, the stock solutions were set at a fourfold and sixfold final concentration.

2.2 Experimental setup and online measurements

Details on the fluidic experimental setup, the optical microdevices, and the applied methods for the realization and analysis of three-dimensional concentration spaces have been described in an earlier publication [4]. Hereby, a similar experimental setup was used for one-up to three-dimensional screening of effectors based on a syringe pump system with six dosing units (Cetoni GmbH, Korbußen, Germany) (Figure 1).

Table 1 Final concentration and stock solution for the one- up to three-dimensional concentration spaces for all investigated substances.

	Ampicillin (mg/l)	Caffeine (g/l)	Captopril (g/l)
Final concentration	1.5	3.9	0.4
Stock solution for single effector screening	3.0	7.8	0.8
Stock solution for two-dimensional concentration space	6.0	15.6	1.6
Stock solution for three-dimensional concentration space	9.0	23.3	2.4

The generation of the microfluidic segments was realized through a Peek seven-port manifold (Upchurch Scientific, Oak Harbor, USA) by controlled dosing of effectors, cultivation medium, and cell suspension into a flow of a carrier liquid. To accomplish this, the manifold was connected via polytetrafluoroethylene (PTFE) tubes (0.5 mm ID, 1.6 mm OD; Bohlender GmbH, Grünsfeld, Germany) with a computer-controlled, nearly pulsation-free syringe pump, including syringes (ILS, Stützerbach, Germany) with volumes of 0.5 ml for three effector solutions, 1 ml for cultivation medium, 2.5 ml for cell suspension, and 5 ml for carrier liquid. To ensure constant concentrations of cells over a long period of time, a syringe stirrer (neMIX, Cetoni GmbH, Korbußen, Germany) was added for the high-precision syringe pump system.

Generated segments were transported at a constant flow rate through an optical detector unit containing a flow-through photometer and fluorimeter, which measured the segment directly through the transparent fluorinated ethylene propylene (FEP) tubing (0.5 mm ID, 1.6 mm OD; Upchurch Scientific, Oak Harbor, USA). The flow-through photometer with a LED (Agilent, USA) with a peak wavelength of 505 nm has two functions: on the one hand, it was used for the light scattering measurement during the bacterial growth inside the segments; on the other hand, it was used for monitoring the segment size, the segment distance between two neighbor segments, and the segment number. Thereby, determination of segment loss or fusion due to the long cultivation time or remeasurement processes can be executed for error correction. The detection of the bacterial autofluorescence was measured with *in situ* flow-through fluorimetry. Therefore, a LED with a peak wavelength of 470 nm with a combination of a shortpass (480 nm) and longpass (510 nm) filters was used (Laser Components, Olching, Germany). The emitted photons were counted by photomultiplier modules (Hamamatsu, Herrsching am Ammersee, Germany). The autofluorescence intensity value I_n is given by the ratio of the intensity of the measurement value I_{fluo} to the reference fluorescence I_{ref} of the FEP tube filled with carrier liquid subtracted by 1.

$$I_n = \frac{I_{\text{fluo}}}{I_{\text{ref}}} - 1 \tag{1}$$

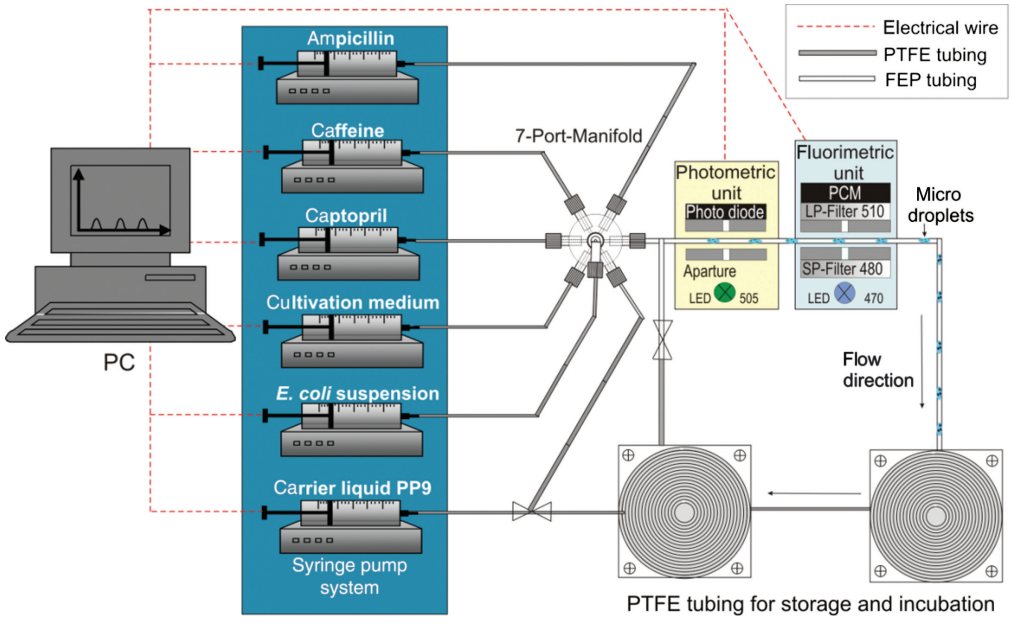

Figure 1 Experimental setup and principle of double endpoint detection for the screening of a three-dimensional concentration space using microfluid segment technique.

The tube coils consisted of a polymethylmethacrylat (PMMA) plate with rolled PTFE tubes with a length of 7 m (0.5 mm ID, 1.6 mm OD), which were used to store and to incubate the generated segment sequences. This closed system is able to prevent undesired evaporation compared with the conventional method. To investigate the dose-response effect, all segments were moved inside one tube coil through the detector unit to a second storage tube coil after a certain "static" incubation time. This technique allows multiple runs without regenerating segments.

2.3 Multidimensional concentration spaces

The strategy for the investigation of two- and three-dimensional concentration spaces was based on the easy possibility of varying the composition of microfluid segments by a continuous shifting of flow rate ratios, which has been described in detail in a previous work [4]. The most important condition for the realization of reproducible concentration programs is the strict control of size and distance of the generated segments. For the two-dimensional screening as well as for three-dimensional screening, the flow rates of the carrier liquid and the cell suspension were kept constant at 40 µl/min and 5 µl/min, respectively. The flow rates of the effectors and cultivation medium were varied depending on the concentration combination but were adjusted to a final flow rate of 5 µl/min. Therefore, the total flow rate for the generation process was set to 50 µl/min.

In Figure 2, the automated flow rate run utilized for the two-dimensional combinatorial variation of the effector concentration is shown. During the automated combinatorial experiment, a complete two-dimensional parameter space was covered in steps of 10% by a set of flow rate changes, which were controlled by the data to the flow rate program. Two effectors in 11 different concentration steps were combined in microfluid segments, resulting in 11^2=121 different combinations. The individual flow rates of the solution stream for each single combinatorial step can be directly taken from Figure 2. For the presented experiments, a stepwise linear variation of the reactant concentrations was chosen.

For the microtoxicological three-dimensional screening experiments with a concentration resolution of 20%, the automated flow rate program in Figure 3 was applied. Three effectors in six different concentration steps (0%, 20%, 40%, 60%, 80%, and 100% of maximum effector concentration) were combined in microfluid segments, resulting in 216 different combinations. In this program, ampicillin was injected over the whole segment sequence with a slowly increasing flow rate ramp in six steps. The flow rate of caffeine was either increased or decreased in a total of six steps (0%–100%), but the entirety of all these steps covered one single concentration step of the former one. The flow rate of the captopril solution was varied in the same manner, covering one single concentration step of both caffeine- and ampicillin-containing concentration steps. The cultivation medium was adapted to the total of the flow rates of all effector solutions, resulting in a constant total flow rate of 5 µl/min.

3 Results and discussion

3.1 Evaluation of dose-related effects of individual substance

Following earlier cultivation experiments with *E. coli* bacteria in microfluid segment [34], fluid segments of 400–450 nl were generated and inoculated with about 100 cells per segment. A strong growth was evaluated after 23 h by measuring the autofluorescence intensity at 470 nm and light scattering at 505 nm. The dose-response curves were fitted to the dose-response data points by using the

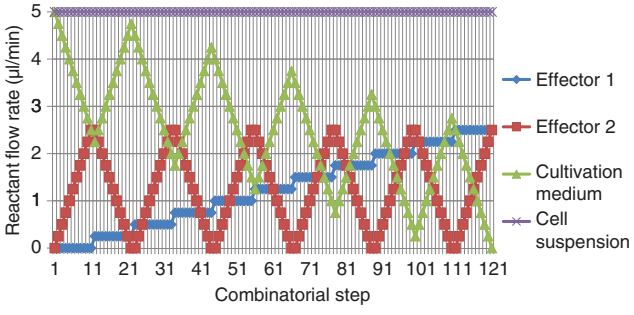

Figure 2 LabVIEW control program for the generation of two-dimensional concentration spaces in 11 combinatorial steps, resulting in 121 concentration points.

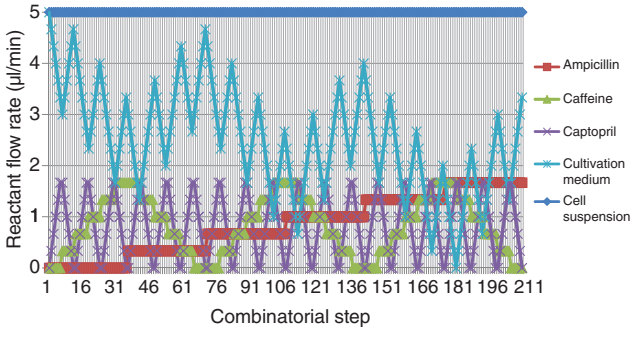

Figure 3 Automated flow rate variation for the realization of the coverage of a three-dimensional parameter space in steps of 20% resolution.

standard sigmoidal function for the relation of cell viability of tested effector concentrations. The response of cell density and endogenous autofluorescence were normalized with positive controls to the interval from 100% (without effector) to 0% (total inhibition).

In a first set of experiments, the EC_{50} values (50% reduction in light scattering and autofluorescence signal of *E. coli* compared with the controls) of the three investigated substances were determined under microfluidic conditions. Large sequences each comprising 200 individual segments with gradually varying concentrations of single effectors were generated within 15 min. In case of ampicillin, the EC_{50} value was determined to be about 0.94 mg/l of cell density (Figure 4, squares) and 0.90 mg/l of autofluorescence intensity (Figure 4, circles), in good agreement with the 1.25 mg/l tolerance observed for *E. coli* using conventional cultivation methods earlier [35]. In this experiment, a sharp transition between full growth and reduced growth was found at 0.75 mg/l ampicillin after a cultivation time of 23 h. A complete suppression of growth occurred above 1.25 mg/l ampicillin.

A much higher tolerance was found with *E. coli* when caffeine was applied. The highly resolved dose-response experiments using the microsegmented flow resulted in EC_{50} values of about 3.09 g/l for the endpoint autofluorescence and 3.32 g/l for the growth after 23 h of cultivation (Figure 5). Both values are in good agreement. The difference in the EC_{50} values by light scattering and autofluorescence intensity taken from a sigmoidal fit can be explained by the strong difference in the slope of both response functions. The high resolution revealed a strict transition between strong growth and complete suppression at a caffeine concentration of 3.9 g/l. These EC_{50} values are similar compared with our earlier report (EC_{50}=3.22 g/l) [31] and the work of Sandlie et al. [36] using a conventional method (EC_{50}=3.8 g/l). In case of captopril,

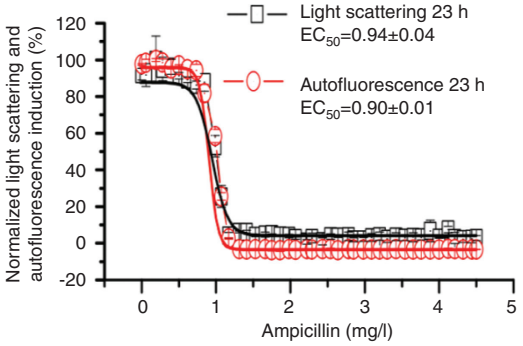

Figure 4 Highly resolved dose-response curves of *E. coli* against ampicillin obtained in microfluid segment sequences with continuously varied effector concentrations.
Circles, autofluorescence measured by using microfluorimetry; squares, light scattering measured by using microphotometry.

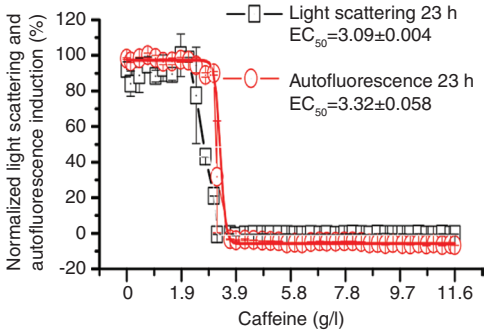

Figure 5 Highly resolved dose-response curves of *E. coli* against caffeine obtained in microfluid segment sequences with continuously varied effector concentrations.
Circles, autofluorescence intensity; squares, intensity loss by light scattering.

the investigations showed no inhibitory effect on *E. coli* cultivated inside microsegment up to a concentration of 1 g/l (Figure 6).

In all cases, both optical methods provided similar values and the results show small deviations from the mean value (Figures 4–6). Thus, all experiments reflect a high reproducibility for the determination of cell growth in segments at a given composition. The low deviation of obtained values of optical density and autofluorescence for single concentrations and the sharp transition demonstrate the applicability of the microfluidic method for a precise determination of the EC_{50} values.

3.2 Combinatorial effect of caffeine, ampicillin and captopril on the growth behavior of *E. coli*

For binary mixtures, a stepwise change in effector concentration was applied for a resolution of 10%. Two effectors

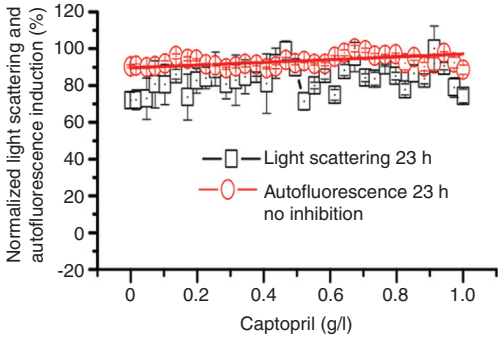

Figure 6 Highly resolved dose-response curves of *E. coli* against captopril determined in one-dimensional screening regarding the endpoints on the light scattering (squares) and autofluorescence intensity (circles).

in 11 different concentration steps were combined in microfluid segments, resulting in 121 different combinations. For this purpose, a microfluid segment sequence containing about 650 microdroplets with a size of 400–450 nl was generated. Therefore, the total consumed test liquid was <300 µl. For each concentration mixture, five repeats were realized. This redundancy was applied in order to prove the reliability of measurements and to exclude or identify stochastic effects caused by the small reaction volume and the small number of inoculum cell density for incubation. The response behavior of *E. coli* against combinations of two effectors can be well characterized by map-like representations. In these bolographic maps, the growth-related autofluorescence intensity is shown as the third dimension using a color code ranging from blue (total inhibition) to red (maximum growth) vs. both effector concentrations displayed on x and y axis.

The transition between concentration-dependent stimulation and subadditive effects between caffeine and ampicillin on the growth of *E. coli* is well illustrated by the isobolographic maps in Figure 7A. For ampicillin, a final concentration of 1.5 mg/l was selected, which was located prior to the total inhibition value of 1.25 mg/l in single effector screening experiment. The stimulation of bacterial growth by sublethal doses of caffeine was not impaired until a concentration of 0.45 mg/l ampicillin was reached. Here, presumably, the ampicillin-induced inhibition of cell wall synthesis overcame the growth stimulation effect of caffeine and represented a typical compensating effect. A nearly independent effect of growth by caffeine took place at sublethal concentrations of ampicillin (0.3–1.0 mg/l). Furthermore, this diagram shows the additive effects of both effectors with ampicillin concentrations between 0 and 0.3 mg/l.

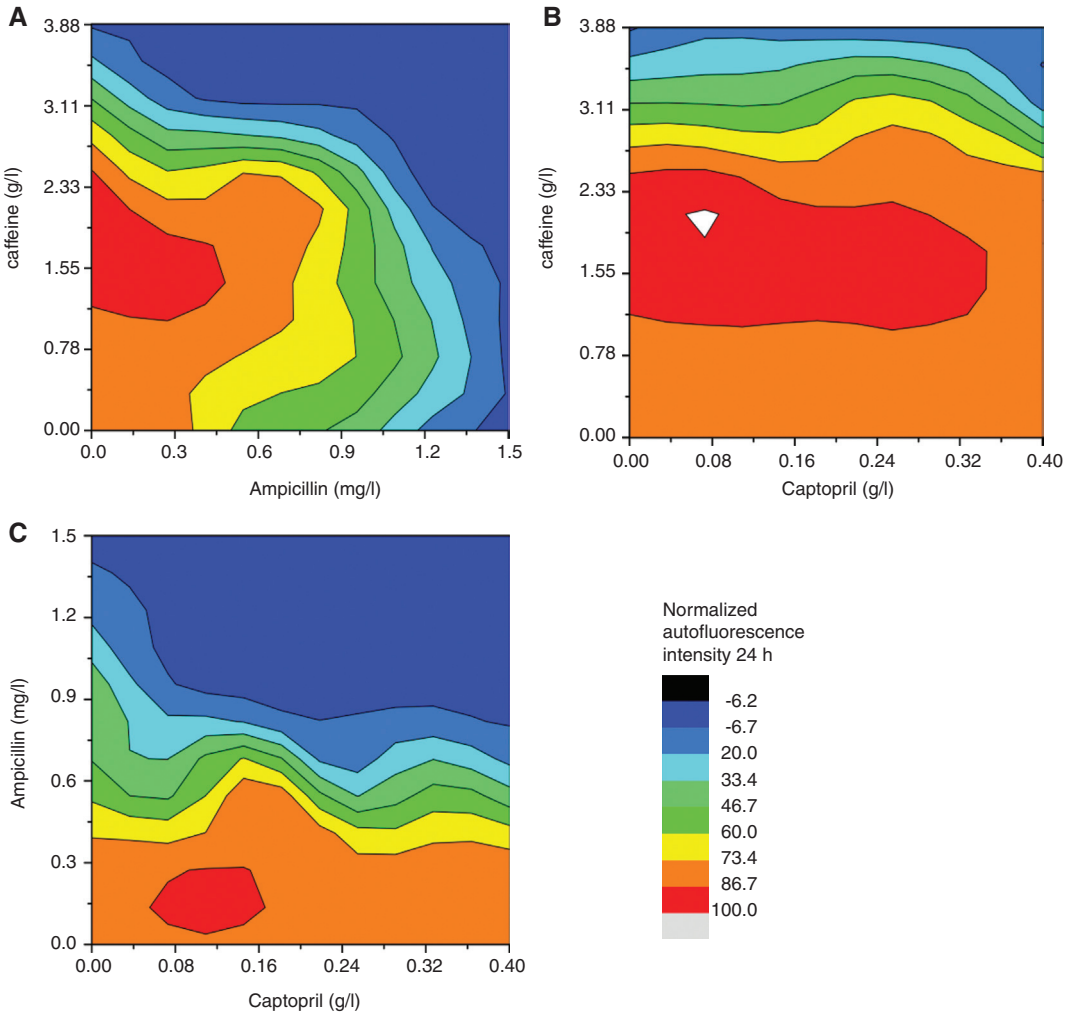

Figure 7 Isobolographic maps show the different responses on the normalized autofluorescence of *E. coli* to the different binary mixtures after 24 h of cultivation inside microdroplets.
(A) Binary mixture of caffeine and ampicillin; (B) binary mixture of caffeine and captopril; and (C) binary mixture of ampicillin and captopril.

The combined effects of caffeine and captopril on the growth of *E. coli* are shown in Figure 7B. A strong autofluorescence was measured in all segments containing between 1.1 and 2.2 g/l caffeine. With increased captopril concentration, the effect of caffeine was not influenced. This strong increase in bacterial growth, indicated by high autofluorescence, reflects the strong stimulation of growth at a sublethal dose of caffeine in the presence of captopril.

Concentrations between 0 and 1.5 mg/l ampicillin were investigated for their combinatorial effect with captopril in the concentration range between 0 and 0.4 g/l (Figure 7C). A nearly independent activity of two effectors was observed for the combination of captopril and ampicillin on *E. coli*. The corresponding presentation in the bolographic map nearly plateaued in the lower part. The observed behavior hints to different mechanisms for the effect of both substances.

3.3 Microtoxicological screening of the ternary mixture of caffeine, ampicillin, and captopril inside microdroplets

Here, the ternary mixture of the effectors ampicillin, captopril, and caffeine was investigated. For the three-dimensional screening, the concentrations of all three effectors were varied in six steps (0%, 20%, 40%, 60%, 80%, and 100% of the maximal concentrations). The different combinations of concentrations were organized in 216 groups of microfluidic segments, each containing about five to six single microfluidic segments. Therefore, we were able to analyze the combined effects within a segment sequence of 1150 segments within a short of time.

A detailed image of the cellular response on the variation of the concentrations by different mixtures in a complete three-dimensional concentration space was given with a set of bolographic maps, as shown in Figure 8. The concentration-independent relationship between caffeine and captopril without any impact of ampicillin is illustrated in Figure 7B. The effect of the supplementation of five different ampicillin concentrations in addition to caffeine and captopril on the growth of *E. coli* is shown in Figure 8A–E. With 0.3 mg/l ampicillin, the caffeine/captopril bolographic map showed a minimal decrease in the EC_{50} of caffeine and the stimulation effect by caffeine was not impaired (Figure 8A). With 0.6 mg/l ampicillin, moderate growth was observed at a low caffeine concentration of 0–0.4 g/l (Figure 8B). Furthermore, Figure 8B showed a continued strong stimulating effect by caffeine between 1 and 2 g/l. With 0.9–1.5 mg/l ampicillin, an enhanced inhibitory effect of ampicillin was observed at a low caffeine concentration of 0–0.4 g/l and low captopril concentration between 0 and 0.8 g/l (Figure 8C–E).

An antagonistic behavior takes place when one effector stimulates the growth of the target organisms above the lethal dose of the other effectors. Such a stimulation effect was observed in case of the combination of caffeine with the captopril in the presence of ampicillin (Figure 8C–E). In a highly resolved dose-response experiment with ampicillin, the EC_{50} value was determined to be 0.90 mg/l and no growth of *E. coli* was observed at a concentration of 1.2 mg/l (Figure 4, circles). But the addition of caffeine led to a strong stimulation of bacterial growth. This stimulation was enforced by the addition of captopril. As a result, a hill-like response pattern was found on the bolographic maps.

The nonmonotoneity and the formation of activity hills of the response of bacteria on caffeine and captopril at elevated ampicillin concentrations between 0.6 and 1.5 mg/l stand for principle changes in chemical interactions or physiological mechanisms inside the *E. coli* bacteria. Therefore, the result implies the sensitivity of critical doses on combinatorial effects.

4 Conclusion

In conclusion, this study confirms that the droplet-based microfluidic technique is well suited for the investigation of microorganisms toward single and combinatorial effects. These investigations become possible without an excessive consumption of chemicals and biological material and at reasonable costs and time through the application of single test volumes in the nanoliter range.

In analogy to DNP combined with caffeine and chloramphenicol, also in case of captopril, nonlinear combinatorial effects in the presence of caffeine and ampicillin are shown. The effects of single substances were studied in detail with high concentration resolutions. The obtained dose-response functions not only show the determination of critical concentrations but also similarities as well as characteristic differences in the reaction of *E. coli* in response to caffeine, ampicillin, and captopril. In addition, this technique enables for a detailed study of nonlinear combinatorial effects. In two-dimensional combinatorial experiments, a concentration-dependent stimulatory effect of caffeine was observed in the presence of ampicillin in sublethal concentration ranges. In the three-dimensional

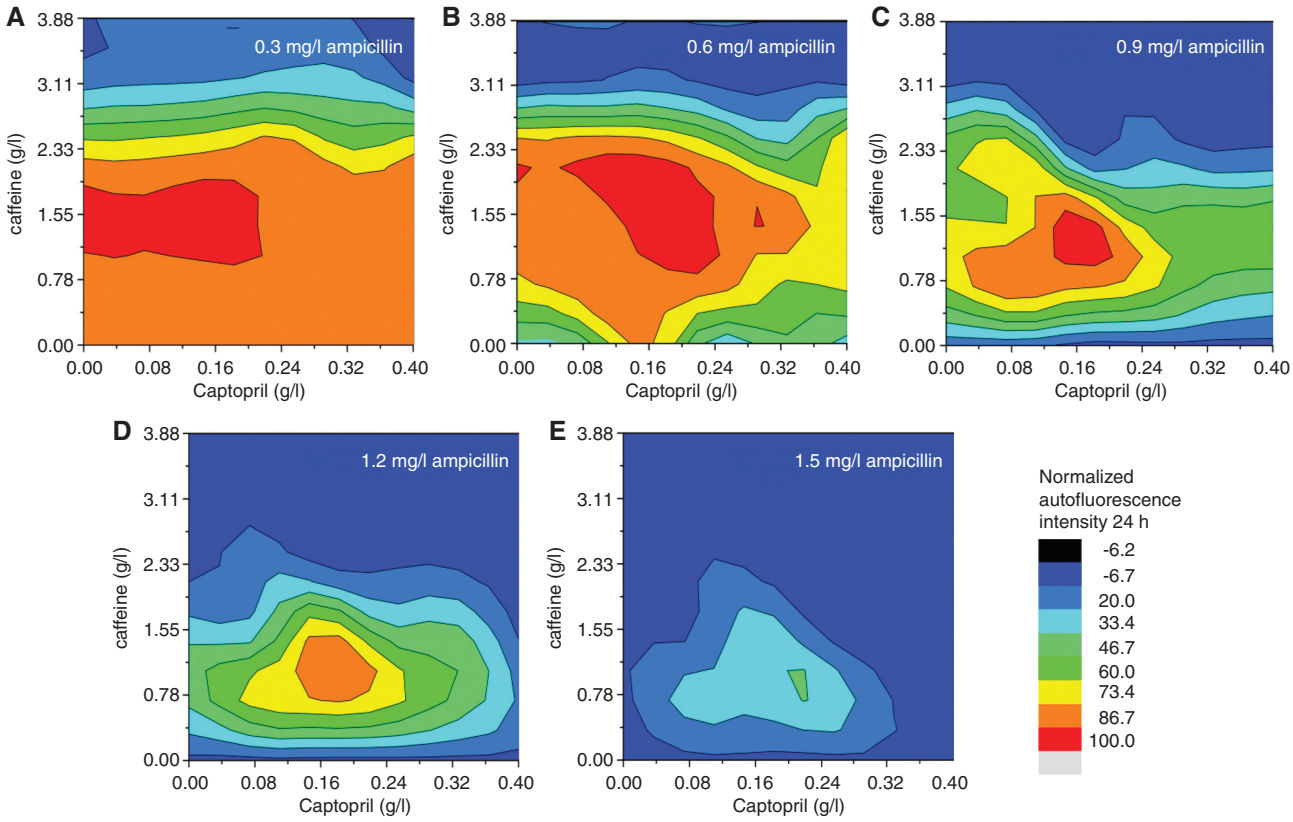

Figure 8 Dose-response relations on autofluorescence intensity after 24 h of *E. coli* cultivation inside microdroplets in three-dimensional concentration spaces of caffeine, captopril, and ampicillin show complex behavior with concentration-dependent stimulation effect and partially antagonistic effect with (A) 0.3, (B) 0.6, (C) 0.9, (D) 1.2, and (E) 1.5 mg/l ampicillin.

concentration space, an antagonistic behavior and formation of activity hills of the response of *E. coli* on caffeine and captopril were found at elevated ampicillin concentrations. The results give an example of strong and unexpected effects for the application of effectors in combination with other substances. These complex responses on the binary and ternary mixtures with captopril, caffeine, and ampicillin may be useful in providing optimal dosing in medical treatments. Overall, this can even lead to improvements on the development of automated techniques for large experimental series in advanced biotechnology.

Acknowledgements: The authors gratefully acknowledge the funding from the German Federal Environmental Foundation under contract no. 20009/009. Furthermore, the authors thank Dr. Jürgen Arning (Bremen University, Center for Environmental Research and Sustainable Technology) and Dr. Alexander Groß (Technical University of Ilmenau, Institute of Chemistry and Biotechnology) for the useful discussions. Many thanks to Steffen Schneider and Frances Möller for their excellent technical assistance.

Received August 28, 2013; accepted October 7, 2013; previously published online November 21, 2013

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