Lab on a Chip

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PAPER

Development of a microfluidics biosensor for agarose-bead immobilized *Escherichia coli* bioreporter cells for arsenite detection in aqueous samples†

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Contamination with arsenic is a recurring problem in both industrialized and developing countries. Drinking water supplies for large populations can have concentrations much higher than the permissible levels (for most European countries and the United States, 10 μg As per L; elsewhere, 50 μg As per L). Arsenic analysis requires high-end instruments, which are largely unavailable in developing countries. Bioassays based on genetically engineered bacteria have been proposed as suitable alternatives but such tests would profit from better standardization and direct incorporation into sensing devices. The goal of this work was to develop and test microfluidic devices in which bacterial bioreporters could be embedded, exposed and reporter signals detected, as a further step towards a complete miniaturized bacterial biosensor. The signal element in the biosensor is a nonpathogenic laboratory strain of *Escherichia coli*, which produces a variant of the green fluorescent protein after contact to arsenite and arsenate. *E. coli* bioreporter cells were encapsulated in agarose beads and incorporated into a microfluidic device where they were captured in 500 × 500 μm^2 cages and exposed to aqueous samples containing arsenic. Cell-beads frozen at −20 °C in the microfluidic chip retained inducibility for up to a month and arsenic samples with 10 or 50 μg L^{-1} could be reproducibly discriminated from the blank. In the 0–50 μg L^{-1} range and with an exposure time of 200 minutes, the rate of signal increase was linearly proportional to the arsenic concentration. The time needed to reliably and reproducibly detect a concentration of 50 μg L^{-1} was 75–120 minutes, and 120–180 minutes for a concentration of 10 μg L^{-1}.

Introduction

Contamination of natural waters with arsenic is a recurring problem in both industrialized and developing countries,[1] and drinking water supplies for large populations can have concentrations much higher than the permissible levels (for most European countries and the United States, 10 μg As per L; elsewhere, 50 μg As per L).[2] Systemic and chronic exposure to arsenic is known to lead to vascular diseases, to irritations of the skin and of mucous membranes as well as to dermatitis, keratosis and melanosis. Inorganic arsenic is a human carcinogen and ingestion of inorganic arsenic increases the risk of developing cancer of the bladder, liver, kidney and skin.[3]

Arsenic contamination most frequently has a natural origin since As is a common element of minerals in the earth's crust.[4] Weathering of such minerals can be accelerated by microbial activity, specifically in anoxygenic groundwaters with abundant available respirable organic matter, which leads to high concentrations of dissolved inorganic As species.[4] Dominant inorganic anions in solution are arsenite [As(III) in AsO_2^-] or arsenate [As(V) in AsO_4^{3-}].[5] Sources of arsenic can also be anthropogenic and include the application of organoarsenical pesticides, the combustion of fossil fuels, the use of organoarsenical feed additives, mining activities, the disposal of industrial wastes, and the application of arsenic-containing desiccants and preservatives.[2]

Current laboratory techniques, such as atomic absorption and atomic fluorescence spectrometry, inductively coupled plasma techniques and high-pressure liquid chromatography can accurately measure arsenic in an environmental sample to parts per billion (ppb) concentrations, but are expensive to operate and maintain, and they require fully equipped and specialized laboratories.[6,1] In contrast, regions that have the most extensive arsenic contamination, such as Vietnam, Bangladesh, India and

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Cambodia,[7,4] are also the areas with the least access to such advanced techniques, making the need for in field arsenic detection even more urgent. For this reason, there has been increased interest over the last decade in the development of sensors for the field detection of arsenic.[1] Colorimetric tests, electrochemical sensors and anodic stripping voltammetric probes have been developed and tested in the field.[8] However, large-scale field testing of the most common colorimetric tests showed that they are not sufficiently reliable in the concentration range below 50 μg As per L, giving rise to a large number of false-positive and false-negative results.[9] On the basis of this poor performance, new colorimetric tests have been developed and tested in the field,[10] but these remain controversial because of their bulkiness, their use of toxic chemicals (*i.e.* mercury bromide and zinc), and release of arsine gas during the test.[6] For these reasons various alternative protocols and methods have been proposed. One of the alternatives to the abiotic sensors for arsenic detection is a bacteria-based bioassay.[11] The bacteria-based bioassay exploits the extremely sensitive natural defence system of bacteria against arsenite and arsenate, and couples this defence reaction to the production of an easily measurable output.[12–14]

The bioreporter strain *Escherichia coli* DH5α (pPROBE-arsR-ABS) used for the present work was developed for the detection of arsenic previously.[12] It carries a plasmid DNA with a transcriptional fusion between the promoter P_{ars} of *E. coli* and the gene encoding the enhanced green fluorescent protein (EGFP). The strain also encodes the regulatory protein ArsR, which controls the level of EGFP expression in response to arsenite. Basically, in the absence of arsenite, most ArsR repressor binds to its operator site within the P_{ars} promoter and only allows low basal expression of itself and the downstream *egfp* gene (Fig. 1). When arsenite enters the cell, it binds with the ArsR repressor leading to a conformational change and dissociation of the ArsR protein from its operator. As a consequence, the *arsR* and *egfp* genes are more highly expressed. The EGFP levels attained in the cells over time are proportional to the external arsenite concentration in a range of approximately 10 μg–1 mg As per L.[12,15]

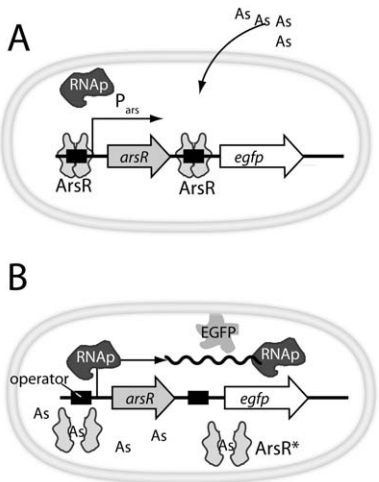

Fig. 1 Schematic outline of the principle of the arsenic bacterial bioreporter. (A) In the absence of arsenite, transcription from *arsR* and the *egfp* reporter gene is repressed by ArsR. (B) When arsenite enters the cell, it interacts with the ArsR repressor that undergoes a conformational change (ArsR*) and dissociates from its operator. In this way the *arsR gene* and the reporter gene are de-repressed, EGFP is produced and fluorescence can be detected.

Whereas several studies showed the proof of principle of the arsenic bioreporter assay, they did not attempt to develop a combined arsenic biosensor instrument in which the cells would be exposed to the sample and their reporter output measured within a potentially small device. The goal of the present work was thus to design a miniaturized arsenic biosensor cartridge, which would be suitable to be inserted in a microfluidics system with an integrated detector. The first immediate research objective was to design, construct and test the efficacy of a small microfluidics cartridge, which could retain the bioreporter cells. Second research objective was to investigate which method for applying and preserving the bioreporter cells would be suitable for such a microfluidics cartridge. We found that the *E. coli* arsenic EGFP bioreporter cells were very easily embedded, applied and stored in small agarose beads of a diameter of ~50 μm, which could be stored within the microfluidic chip at −20 °C. Cell beads demonstrated superb flow characteristics and easy handling on the microfluidic chip, where they were exposed to aqueous samples containing arsenite. The biosensor showed very reproducible EGFP formation at low arsenite concentrations and moreover, allowed time kinetic measurements of reporter signal development.

Materials and methods

Microfluidic cartridge fabrication

The microfluidic cartridge is composed of a polydimethylsiloxane (PDMS) block containing the designed channels and cages (Fig. 2A–C) bonded on a typical microscopy glass slide with a thickness of 1 mm. The PDMS block is fabricated from a silicon mold, which contains the negative imprint of the cages and channels (Fig. S1†). To fabricate the silicon mold, a silicon wafer was structured by dry etching (Bosch process, Alcatel 601E) using a photolithographically structured AZ1512 resist (Clariant) as a mask (Fig. S1A†). Designs were made using Clewin (WieWeb) and transferred to a chrome mask by laser writing. Then, using this mask, they were transferred to the positive photoresist by selective UV exposure.

To fabricate the PDMS block, 10 g of Sylgard 184 silicone elastomer base (Dow Corning) was mixed with 1 g of Sylgard 184 silicone elastomer curing agent (ratio 10 : 1). Then, PDMS was degassed under vacuum for 20 minutes to remove air bubbles, poured on the silicon mold and baked for 2 h at 80 °C to become solid and taking the shape of the mold. Afterwards, the PDMS was peeled off (Fig. S1B†). In order to seal the channels, the PDMS block was irreversibly bonded on a 1 mm thick glass slide by treating the surfaces with oxygen plasma (0.6 mbar, 100 W, 1 min, Diener Electronic—Femto) and placing the imprinted PDMS surface onto the glass. One silicon mold served for several dozen PDMS blocks.

Bioreporter preparation

Starting from a single colony, *E. coli* DH5α strain 1598 (pPROBE-arsR-ABS) was grown in Luria-Bertani (LB) medium in the presence of 50 μg mL^{-1} kanamycin at 30 °C for 18 h and with 180 rpm agitation of the culture flask. The bacterial culture

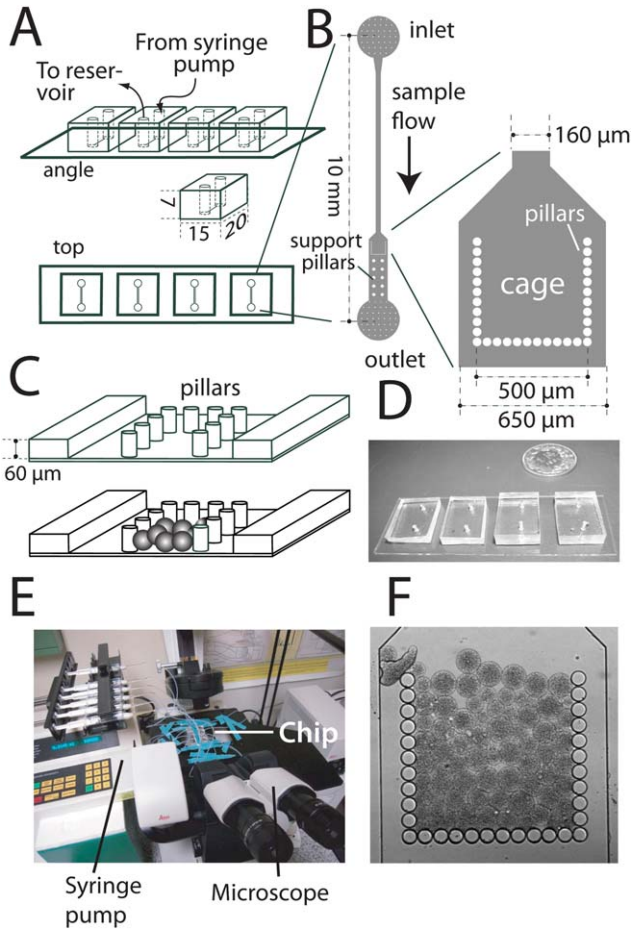

Fig. 2 Working principle of the microfluidic chip. (A) Four PDMS blocks, each containing one or four microfluidic channels, are bonded on a single glass slide. (B) The channel is composed of one inlet, one outlet and a cage. To avoid collapse of the channel, supporting pillars (in white) are designed. (C) The cage traps the cell-beads, while allowing flow through the pillars spaced at 5 µm distance. (D) Picture of the four replica single-lane PDMS chips on glass (size marker is a 5 cent piece). (E) Complete setup of the microfluidics cage with syringe pump mounted on an epifluorescence microscope. (F) Phase-contrast image at 200-fold magnification of the induced cell-beads trapped in the cage.

was then 1000-fold diluted into fresh LB medium plus kanamycin and incubated for a further 24 h at 25 °C and with 150 rpm agitation. At a culture turbidity at 600 nm of between 3 and 4 (representative for early stationary phase cells), cells from 30 mL of culture were harvested by centrifugation at 3000 rpm for 10 min and room temperature. The cell pellet was resuspended into 300 µL of MOPS medium at pH 7.0 (MOPS medium contains 10% [*v*/*v*] of MOPS buffer, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 g glucose per L). MOPS buffer itself was prepared by dissolving, per litre: 5 g NaCl, 10 g NH_4Cl, 98.4 g 3-([*N*- morpholino]propanesulfonic acid, sodium salt), 0.59 g $Na_2HPO_4 \cdot 2H_2O$, and 0.45 g KH_2PO_4. The final concentration of cells in the suspension equalled about 4×10^{11} per mL.

Encapsulation of reporter cells in agarose beads

Material for bacterial cell encapsulation was preheated to 42 °C and a freshly prepared sterile 2.5% (*w*/*v*) agarose solution in deionized water (Agarose D1 low EEO, Conda) was cooled down to 42 °C 20 min before starting. The encapsulation procedure was carried out at 37 °C and the following components were added in sequence with continuous mixing at high speed vortexing (Vortex-Genie 2, Scientific Industries, Inc.). Aliquots of 1 mL of agarose solution, 30 µL of pluronic acid (Pluronic F-68 solution 10%, Sigma) and 200 µL of cell suspension were mixed in a 10 mL sterile glass tube. The cell–agarose–pluronic acid mixture (0.5 mL) was added drop by drop into 15 mL of silicon oil (dimethylpolysiloxane, Sigma) in a 25 mL glass tube. After 2 more minutes of continuous vortexing, the tube containing the cell–agarose–silicon mixture was plunged in a water-ice bath for 10 min. Beads were harvested by centrifugation for 10 min at 2000 rpm and room temperature, after which the oil was carefully removed; first by decanting and draining out (tube standing up side down), then by rinsing with 5 mL of phosphate buffered saline (PBS) solution, followed by bead centrifugation, pipetting oil from the surface and decanting wash solution. This rinsing procedure was repeated once more. Beads were size-fractionated between 40 and 70 µm, first by collecting the flow-through from a 70 µm strainer (Cell strainer 70 µm Nylon, BD Falcon). In a next step the recovered bead solution from 70 µm was poured over a 40 µm strainer (Cell strainer 40µm Nylon, BD Falcon), and beads retained on the filter surface were recovered by rinsing with 6 mL of MOPS medium.

Bead storage

Two procedures of cell-bead storage were tested. In the first procedure, the cell-bead solution was stored outside the microfluidics cartridge at 4 °C in the MOPS solution or frozen at −80 °C in MOPS with 15% glycerol, and introduced into the cartridge when carrying out the arsenic induction assay. For preparation of freezing cell-beads were briefly mixed in the 15% glycerol–MOPS solution, then kept at 4 °C for 1 h to let the glycerol diffuse into the beads, after which they were placed and stored at −80 °C.

In the second procedure, cell-beads were stored within the channels of the microfluidics cartridge. Hereto, ten µL of a cell-bead solution in MOPS (for 4 °C) or in MOPS plus 15% (*v*/*v*) glycerol (for −20 °C) was filled per channel. Storage temperatures were +4 °C and −20 °C. In order to prevent evaporation during storage, the inlets and outlets of the microfluidics cartridge were covered with a PDMS plunger and Scotch tape. Cartridges were stored for periods up to one month and induction of the reporter cells was tested periodically. For arsenic induction assaying a cartridge was removed from storage, equilibrated to room temperature and connected to the syringe pumps with the sample solution as described below. Beads were then flushed at high flow rate (0.5 ml h^{-1}) in the cartridge for 30 seconds, before starting timing of the induction process. We noticed that cell-beads during storage do not necessarily remain within the cage, but distribute across the channel and the cage. They quickly perfused back into the cage, however, as soon as the flow was reconnected.

Viability of cells in agarose beads

To assess bacterial cell viability in agarose beads, a Live/Dead BacLight Bacterial Viability kit (Invitrogen) was used. A volume

of 0.5 μL of both SYTO 9 (green fluorescent, indicative for live cells) and propidium iodide solution (PI, red fluorescent, indicative for dead cells) was mixed with an aliquot of 250 μL of cell-bead suspension and incubated for 15 min in the dark. Afterwards the bead-embedded cells were imaged by confocal laser scanning microscopy (Leica TCSSL) with suitable optical filter sets. The proportion of live and dead cells was calculated from overlays of micrographs taken at SYTO9 or PI fluorescence wavelengths using ImageJ (National Institute of Health).

Arsenic induction assay

Cell-beads were induced in the microfluidics cartridge with arsenite solutions of between 4 and 100 μg As per L, compared to MOPS medium alone as negative control. Appropriate dilutions of a 50 mM stock solution of sodium arsenite ($NaAsO_2$, Merck) were hereto prepared in MOPS medium. The induction solution was taken up with a 5 mL plastic syringe connected to a 1.06 mm diameter PTFE tubing (Fisher Scientific). In the case of cell-beads stored outside the cartridge, 50 μL of cell-bead suspension were aspired at the tubing extremity after filling the syringe with the arsenic test solution. The tube with cell-bead suspension was then connected to the inlet of the microfluidic channel in the PDMS block. A shorter 1.06 mm diameter PTFE tube (Fisher Scientific) glued to an eppendorf was connected to the outlet of the flow lane to collect the outgoing flow. In the case of cell-beads prestored within the microfluidics cartridge the tape and PDMS plunger were removed and the inlet was connected to the tubing and syringe with the arsenic test solution.

The syringe with arsenic test solution was mounted on a syringe pump (Harvard, Pump22 Multiple Syringe Pump), and a constant flux of 50 μL h^{-1} was imposed. EGFP fluorescence from the cells in the beads was followed by digital microscope imaging over a time period of 3 h at 20 °C.

Quantification of EGFP fluorescence in the microfluidics cartridge

EGFP fluorescence from the cell-beads in the cage of the microfluidics cartridge was imaged using a Leica DFC320 cooled black-and-white charge-coupled device camera (Leica Microsystems CMS GmbH, Wetzlar, Germany) mounted on a Leica DMI6000B inverted epifluorescence microscope. Digital images were recorded as 16-bit TIFF every 30 min with an exposition time of 260 ms using the Leica AF6000 program, at 200× magnification and using the GFP BP470/40 filter set (Leica). Images were exported and thresholded at the background signal using ImageJ, after which the average of the signal intensity per unit cage area was calculated. Because the fluorescence in the negative control (MOPS only) also increases slightly with time, we subtracted from all averages the (average) fluorescence value per unit area of cage in the non-induced sample at the corresponding time point. Moreover, in order to compare measurements across different cartridges and on different days, the average (non-induced control corrected) fluorescence for the first point of a time series was subtracted from all subsequent values. This final value is referred to as the *normalized fluorescence intensity* (NFI) in this paper. Figures of merit (Table 1) were calculated on non-corrected raw fluorescence values. All arsenic induction assays on −20 °C stored cell-beads were performed in triplicate simultaneously on a single four-lane PDMS chip. Those on 4 °C and −80 °C stored samples were done in single-lane chips, with multiple arsenic concentrations being tested simultaneously.

Induction of cell-beads in solution and flow cytometry measurements

Flow cytometry on a FACScalibur (Becton Dickinson, Erembodegem, Belgium) was used to independently measure fluorescence intensity of cell-beads (*i.e.*, the collective fluorescence of all cells in a bead) induced or not with arsenite at different concentrations. Cell-bead aliquots of 120 μL that had been stored at −80 °C were thawed and resuspended in MOPS medium, and exposed in a final volume of 1 mL to concentrations of between 4 and 100 μg As(III) per L (diluted from the 50 mM stock in MOPS medium). The mixture was incubated in 10 mL glass tubes for up to 3 hours at 30 °C with 180 rpm rotary shaking. Samples were retrieved after 2 and 3 h for flow cytometry analysis. EGFP expression was analyzed per individual bead and is expressed as the mean green fluorescence intensity (FL1-H, log-scale) as a function of arsenite concentration (μg L^{-1}).

Kinetics

In order to determine the rate of increase in EGFP fluorescence in the biosensor assays as a function of arsenite concentration, we fitted the data using the expression $\ln(y) = \alpha x + b$, with α being the slope, b being a constant, and y the normalized fluorescence intensity as a function of time x.

Results

Microfluidic cartridge

In order to make a miniaturized biosensor device that would contain bioreporter cells and could be exposed to liquid samples, we designed a microcage in a very simple microfluidics cartridge, composed of a PDMS imprint bonded to a transparent glass slide, maintained under constant flow using a syringe pump (Fig. 2). In- and outflow channels to the cage were designed with a height of 60 μm, which we found caused no restriction to the agarose cell-beads even though having a diameter range of 40–70 μm. The cage with a size of 500 × 500 μm^2 was delineated by three rows of pillars on each side interspaced by 5 μm, so that the liquid can flow through. At a distance of 5 μm between pillars individual reporter cells would not be retained since *E. coli* is 2–5 μm long and 1–1.5 μm in diameter. However, by encapsulating thousands of reporter cells in agarose beads, they were effectively trapped in the cage, while the solution passes through (Fig. 2D and E). The whole set-up could contain four PDMS blocks bonded on one glass slide with between one and four lanes each, permitting to measure various samples simultaneously (Fig. 2A and D).

At 50 μL h^{-1}, we found that cell-beads were concentrated in the cage within 20 seconds (Video, ESI†). Flow resistance increased upon cage filling and once the cage was completely filled its flow resistance became much higher than the resistance

Table 1 Figures of merit for the cell-bead bioreporter configuration after different storage conditions and measurement instrumentation

	MDL[a]/μg As per L					RSD[b] (%)					Sensitivity[c] (%)				
Assay[d] time/min	78[e]	108	138	168	198	78	108	138	168	198	78	108	138	168	198
Cage, 4 °C (1 day, out)	0.5	1.1	1.2	1.3	1.8	7.3	10.7	11.1	11.7	13.4	ND	121.7	139.0	165.2	176.6
Cage, −80 °C (4 d, out)	12.7	6.6	1.6	0.6	1.2	70.2	36.7	25.8	17.7	16.4	ND	116.0	116.0	128.6	147.0
Cage, −20 °C (1 day, in)	258	6.4	ND	1.7	1.6	184	17.7	ND	6.2	4.4	104.3	116.3	ND	147.9	162.1
FC, −80 °C (4 day, out)	ND[f]	ND	ND	8.4	ND	ND	ND	ND	8.4	ND	ND	ND	ND	208.5	ND

[a] MDL, method of detection limit. Calculated as the arsenite concentration corresponding to a sample with normalized fluorescence of that in the blank plus three times the standard deviation in the blank. [b] RSD, relative standard deviation. Calculated as the average percent deviation in NFI across all samples compared to the calculated NFI according to the linear regression trend function. [c] Sensitivity. Calculated as the percent increase in NFI at 10 μg As per L compared to the value in the blank at that time point. [d] Cage, cell-beads in microfluidics cage imaged with epifluorescence microscopy. FC, cell-bead signal measured by flow cytometry. 4 °C, −80 °C, −20 °C, storage temperature of cell-beads. 'out': storage outside chip; 'in': storage inside chip. [e] Time of analysis after beginning of exposure of cell beads with arsenite (minutes). [f] ND, not determined.

of the two channels around the cage, and further beads or arsenite sample solution passed beside the cage rather than through it. In this way the number of beads trapped in the cage is the same in each experiment. From this point on arsenite and nutrients in the sample reached the cells by molecular diffusion.

Sensitivity of the bioreporter assay to arsenite

Fig. 3A shows the increase in the mean EGFP fluorescence per unit of cage surface resulting from the induction of the cells within freshly made beads as a function of assay incubation time and at two sodium arsenite concentrations, 10 and 50 μg As per L, compared to a control without arsenite. An induction period of 60 minutes was sufficient to significantly distinguish the EGFP fluorescence signals in both 10 and 50 μg As per L from the negative control (Table 2). Different batches of cell-beads stored for less than one day at 4 °C varied slightly in the absolute signal output at the same induction time and arsenic concentration, which was due to differences in the amount of encapsulated cells (not shown). The mean fluorescence over all cell-beads per time point and arsenic concentration increased linearly proportionally to the arsenite concentration between 0 and 50 μg As per L (r^2 = 0.9576), after which the response saturated (see below). This may actually point to a saturation of the detector (standard exposure setting of the microscope camera) rather than saturation of the inducible response of the cells. The maximum induction ratio for the cell-beads was ninefold for freshly made beads kept for one day at 4 °C (not shown). Flow cytometry analysis of cell-beads indicated a relatively wide range of bead sizes and individual cell-bead mean fluorescence values (Fig. 4E). Inspection of reporter cells within individual beads by laser scanning microscopy showed a regular distribution of cells (Fig. 4A–D), which all were induced to similar levels (not shown), without showing obvious regions within the beads where cells would not respond (*i.e.*, in the innermost part). We can thus conclude that the process of immobilization and the diameter or material of the beads did not inhibit the reporter cells in their response.

Effect of storage conditions

Two procedures for cell-bead storage were tested. In the first the cell-beads were stored outside the microfluidics cartridge (4 °C and −80 °C), whereas in the second procedure the cell-beads were stored within the flow lanes of the cartridge (4 °C and −20 °C). We found that the PDMS–glass cartridge could not withstand freezing and thawing at −80 °C. Cells in beads stored at 4 °C quite rapidly lost inducibility. Although the cells still reacted after 12 days storage, the calculated NFI after 200 min in response to 10 μg As per L decreased by a factor of 15, with the most rapid decline between day 2 and 5 (Fig. S2†). Because of the measurement variability a concentration of 10 μg As per L could no longer be reliably differentiated from a negative control after 9 days. Cell-beads that had been stored at −80 °C retained inducibility much better. An important initial decrease in

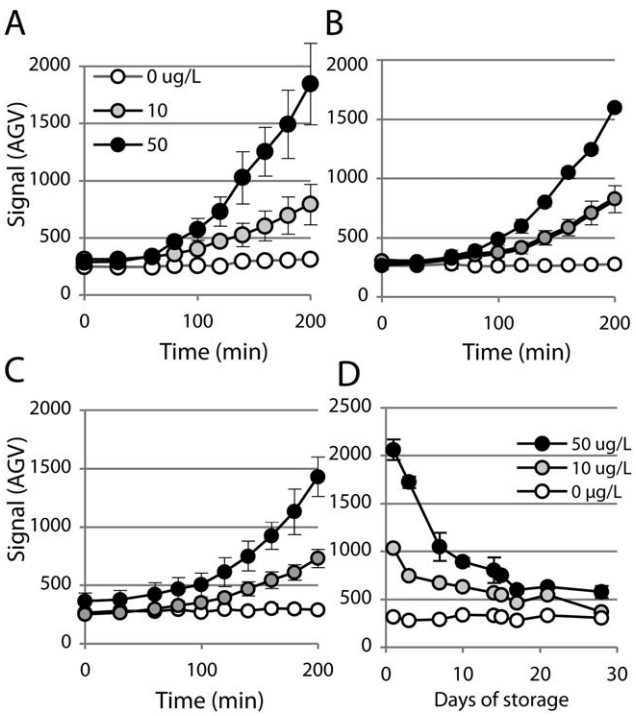

Fig. 3 Raw fluorescence intensities as a function of time and arsenite concentration for differently stored cell-beads in microcage experiments. (A) Fresh cell-beads stored for 1 day at 4 °C. (B) and (C) Cell-beads stored at −20 °C for 3 and 7 days, respectively. (D) Reduction of the signal for 10 and 50 μg As per L after 200 minutes assay incubation for cell-beads stored for different time periods at −20 °C. All data are averages from triplicate assays simultaneously carried out in a four-lane PDMS chip. Error bars denote calculated deviations from the average. Error bars not shown are smaller than the symbol size.

Table 2 Discrimination parameters for bioreporter cell-beads stored at −20 °C

	Minimum time to discriminate signal from background[a]/min		Pair-wise comparison of rate of signal development calculated over time interval[b]								
Storage (day)	10 μg As per L	50 μg As per L	60–120 min			60–200 min			140–200 min		
			0 *vs.* 10[c]	10 *vs.* 50	0 *vs.* 50	0 *vs.* 10	10 *vs.* 50	0 *vs.* 50	0 *vs.* 10	10 *vs.* 50	0 *vs.* 50
1	60	60	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
3	100	80	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
7	100	60									
10	120	120									
14	120	120	$P < 0.05$	$P > 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
17	60	60									
21	160	120									
28	200	140	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

[a] Triplicate simultaneously analysis of flow lanes. [b] Rate parameter α, obtained from linear regression on Ln-transformed signal intensities within the indicated time interval of the induction assay. Pair-wise comparison in two-sided *t*-test with unequal variance on triplicate datasets. [c] 0 *vs.* 10, slopes from the blank compared to 10 μg As per L; 10 *vs.* 50, 10 μg As per L *versus* 50 μg As per L; 0 *vs.* 50, blank *versus* 50 μg As per L.

inducibility occurred directly as a result of freezing, but afterwards the EGFP fluorescence developed at approximately the same kinetics for storage periods of up to 14 days (Fig. 5). In order to determine whether the reason for this lower response would be a decrease in the number of viable bacteria within beads after −80 °C freezing and thawing, we imaged cell-beads stained with the Live/Dead BacLight bacterial viability assay and stored at 4 °C, −20 °C and −80 °C. This demonstrated, however, that more or less independently from the storage temperature, cells in beads contained about 20% of cells staining PI positive (indicative for being dead) (Fig. 4A–D).

Also the increase in EGFP fluorescence signal from −80 °C stored cell-beads after 200 min induction time even at single-lane measurements was linearly proportional to the arsenic concentration ($r^2 = 0.9674$) but with a maximum of five-fold induction (Fig. 6A). The calculated standard deviation in NFI values for cell-bead batches prepared at different times but stored at the same conditions for the same time period, and assayed with 10 μg As per L for 200 min at room temperature in the microcages, was 20% (data not shown).

Storage in the microfluidic chip

We then examined inducibility of cell-beads prepared in the same batch and stored within the microfluidic chip at either +4 °C or −20 °C for periods of up to one month. Similar as for the cell-beads stored outside the cartridge, storage at +4 °C resulted in a rapid decrease in inducibility down to undetectable levels (not shown). In contrast, cartridges stored with cell-beads at −20 °C performed much better and retained inducibility at least until day 28. Also here a decrease in signal development over storage time was observed (Fig. 3B–D), but kinetic data suggest that this was essentially a result of time delay in the onset of induction rather than an overall loss of inducibility (Fig. 7 A and B). As a result, the time needed to significantly discriminate a signal of 10 or 50 μg As per L from the blank increases with prolonged storage time (Table 2). As before, calibration curves for cell-beads stored in the microcartridge at −20 °C were almost linear in the range of 0–50 μg As per L ($r^2 = 0.9997$) after which the signal leveled off until 100 μg L^{-1} (Fig. 6A). The calculated method of detection limit (MDL) based on signal intensity after 200 min equalled 1.6 μg As per L (Table 1).

Response kinetics

Because the signal development over time seemed relatively constant for frozen cell-bead batches (on chip or outside chip), we decided to investigate the kinetic response data as a function of storage time and arsenic concentration. As a proxy for the kinetic response we determined the slope of the signal increase in a semi-log plot (Fig. 7C). Interestingly, also the slope of the signal increase correlated linearly proportional to the arsenic concentration in the range of 0–50 μg L^{-1} (Fig. 7C). We found similar slopes for independently stored cell-bead batches at −80 °C outside chip and at −20 °C on chip, however, slopes decreased by ~30% during one month with the most dramatic decrease in the first 7 days (Fig. 7A and B). When examining kinetic responses in the time window 60–120 min of induction instead of 60–200, we noticed that in most cases 10 and 50 μg As per L could be differentiated from the blank or from each other by a significantly different slope, except at 28 days storage (Table 2). Due to the delayed response, at longer storage times more appropriate kinetic windows (*e.g.*, 140–200 min) resulted in better discrimination (Table 2). Standard deviations on the slopes for the 60–200 time window for triplicate values were slightly better than for end-point at 200 min values (~10%).

Discussion

The goal of the present work was the design of a microfluidics cartridge able to contain bacterial bioreporter cells, to expose the cells to liquid samples with arsenic and to obtain an easy read-out of the signal. The bioreporter cells we employed here had been engineered previously and had been shown to produce fluorescence from EGFP in response to arsenite.[12] To contain the reporter cells in the microfluidics cage we embedded them in 40–70 μm agarose beads. The microcage was filled with cell-beads in less than 1 minute, allowed exposure of the cell-beads to arsenite, and permitted a signal amplification as a result of hundreds of cell-beads accumulating at one position. By using the cell-beads in the cage as sensor element we were able to discriminate arsenite concentrations in the range of 0–100 μg As per L with a calculated method of detection limit (MDL) of 1–1.6 μg As per L. Previously, assays with this particular bioreporter strain resulted in MDLs between 5 and 8 μg As per L after 2 h

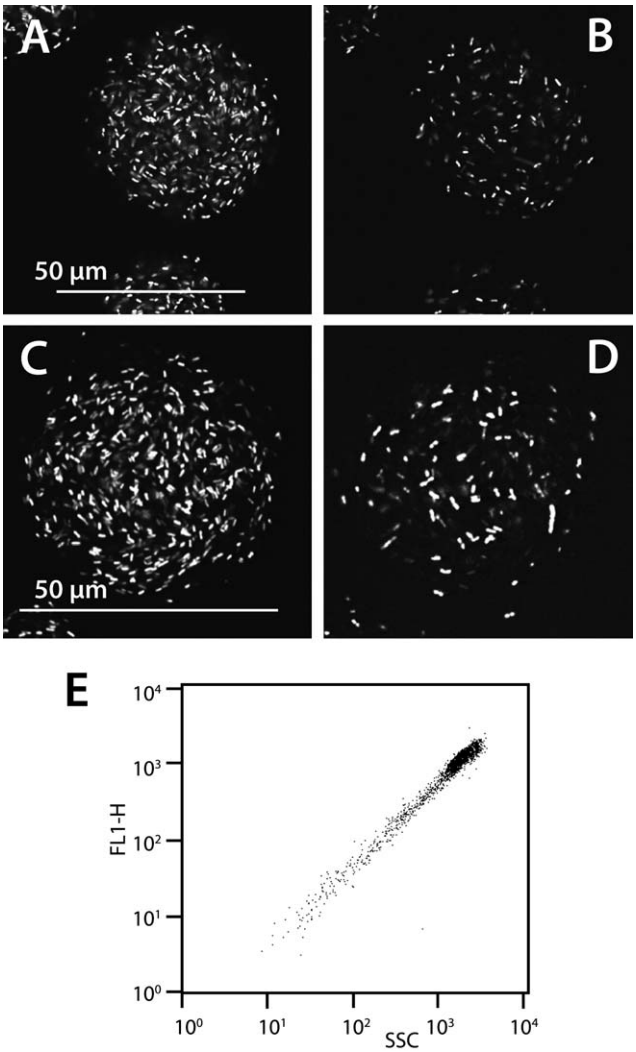

Fig. 4 Viability and fluorescence variability of cells in beads. (A) Reporter cells in beads stored at 4 °C for 4 days stained with SYTO9 (indicative for being alive). (B) Idem, stained with PI (indicative for being dead). (C) Reporter cells in beads stored at −80 °C and stained with SYTO9. (D) Idem stained with PI. (E) Cells in bead variability of fluorescence and side-scatter. All cells in beads were induced with 10 µg As per L for 120 min. Images (A)–(D), confocal laser scanning microscopy. (E) Flow cytometry scatterplot showing fluorescence of beads (FL1) *versus* side-scatter, an indicator of bead size and granularity (SSC).

incubation in case the signal was measured with steady state fluorimetry or epifluorescence microscopy on single cells,[15] which shows that the microfluidics cage method provides an improvement and allows more confident measurements of arsenite samples below 10 µg As per L.

Whereas as such the microfluidics cage method may be seen as a simple particle trap for bioreporter cell-beads with a small improvement in MDL compared to other fluorescence-based detection methods, the real advantage of the cage lays in a simplified liquid handling and prefilling system for bioreporter tests. We tested therefore two potential storage scenarios: outside or inside the chip, with or without freezing. Bioreporter cells could be quite stably maintained within beads with loss of around 20% viability upon freezing and thawing. Reactivation by arsenite remained highly reproducible for beads stored at

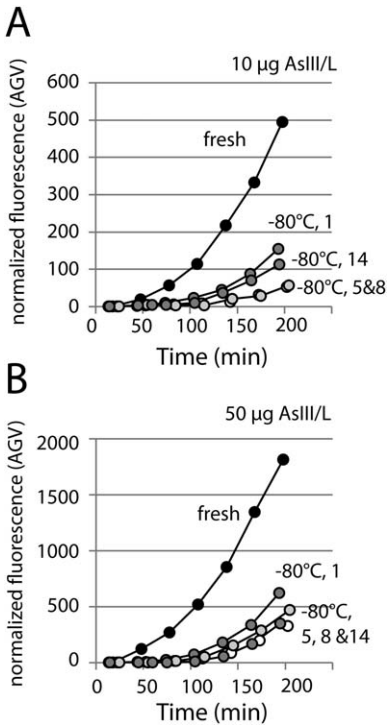

Fig. 5 Effect of storage of cell-beads at −80 °C on signal development in response to arsenic. (A) Normalized fluorescence intensity of cell-beads in microcages exposed to 10 µg As per L as a function of incubation time, for batches stored for different time periods (1, 5, 8 or 14 days, as indicated). (B) Idem, for cell-beads exposed to 50 µg As per L. Data points from microcage experiments derive from a single flow-line analysis (estimated error ∼15%).

−80 °C, whereas the time needed for reactivation increased for prolonged storage at −20 °C. Storage at 4 °C in a few days resulted in loss of inducibility at 10 µg As per L. The fact that cells are not dead after storage but display longer lag times before inducing the reporter system suggests that they lack metabolic energy for immediate reactivation. Thus, there is room for further improvement of reactivation time if the exact reason for the energy imbalance would be understood. For practical importance, cell-beads could be prefilled and stored at −20 °C in the microfluidics cartridge with satisfactory performance even after one month storage, in terms of differentiating concentrations of 10 and 50 µg As per L from each other and from a blank without arsenic.

In terms of the measurement errors, fluorescent signals produced by arsenite-exposed cell-beads from a single batch that had been stored at −80 °C showed an average deviation of 15%. Batch to batch variations in cell-bead preparations resulted in variations of 20% on the NFI obtained with the same arsenite concentration and the same induction time. The overall average relative standard deviation (RSD) over all measurement points of the arsenite response curve is slightly better for the −20 °C stored cell-beads on chip (4–6% after 180 min) than for the 4 °C cell-beads (a little over 10%, more or less independent of the incubation time), and −80 °C stored cell-beads (a little over 15% after 168 min, Table 1). For the −20 °C stored cell-beads on chip (triplicates) this is in the order of RSDs previously calculated for steady state fluorimetry measurements (on six replicates).[15] This

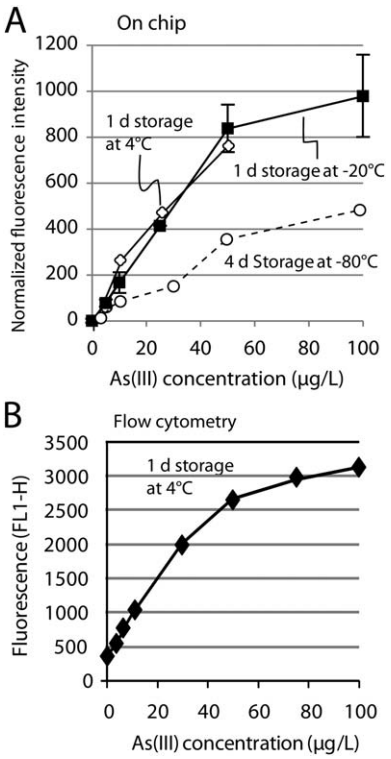

Fig. 6 Signal calibration as a function of arsenic concentration based on the 200 min end-point measurement. (A) Calibration curves obtained for cell-beads in microcages after storage for 1 day at 4 °C (open diamonds, single flow lane analysis), after storage for 1 day at −20 °C (closed squares, triplicate lane analysis), or after storage for 4 days at −80 °C (open circles, single flow lane analysis). (B) Calibration curve obtained with cell-beads stored for 1 day at 4 °C using triplicate suspension assays and flow cytometry analysis of bead fluorescence. Error bars indicate calculated deviation from the average. In case error bars are not visible they are smaller than the symbol size (except for the open diamonds and circles, which are single lane assays).

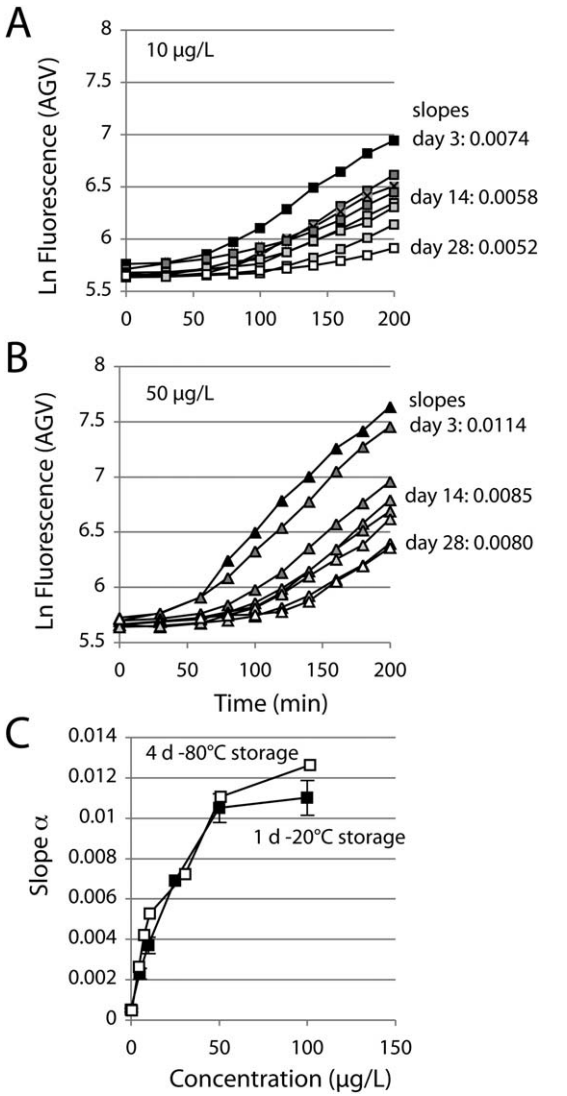

Fig. 7 Kinetics of signal development in assays with stored cell-bead chips. (A) Ln-transformed signal intensities after exposure of cell-beads stored at −20 °C on chip over prolonged storage times to 10 µg As per L. Relevant slope values for the 'linear' increasing parts of the curves are shown. (B) As for (A) but at 50 µg As per L. (C) Calibration curve of calculated slopes as a function of As concentration, for cell-beads stored at −20 °C on chip (triplicate assays, closed squares) or for cell-beads stored at −80 °C outside chip (single lane assays, open squares).

RSD shows that fewer calibrations would be sufficient to accompany each sampling series. One could thus envision using a four-lane microfluidics cartridge with one lane containing a calibration standard and the other three analyzing triplicate samples.

The microcage biosensor can be used in endpoint measurement mode (*e.g.*, data in Fig. 6A), which after 100–200 min assay time is sufficient to obtain an MDL of 1.6 µg As per L in the case of cell-beads stored at −20 °C for 1 day and for cell-beads stored at −80 °C (Table 1). Longer incubation definitively improved the sensitivity of the signal at 10 µg As per L up to around 170% for all storage conditions of the cell-beads (Table 1). In contrast to end-point measurements, arsenite concentrations in samples can also be inferred from continuous monitoring of the fluorescence signal produced by the cell-beads over time. In this case the relationship between the fitted slope of Ln-transformed signal intensities (α) and arsenite concentration is considered (Fig. 7). So, for example, at any incubation time point subsequent to time = 0 the slope constant (α) can be estimated from the preceding points, and we found that for most measurements with the cell-beads the induction interval 60–120 min is sufficient to significantly discriminate the slopes at 10 and 50 µg As per L from each other and from the blank (Table 2). Likely, when more measurements can be performed at shorter time intervals than in this case (60 min), the rate parameter can be estimated correctly after a shorter total incubation period. In this case analysis of samples can be stopped as soon as the rate parameter is reliably estimated, which could save time.

In our experiments we chose to embed cells in agarose mini-beads according to a procedure that has been mentioned previously but improperly described by Zengler *et al.*[16] Agarose beads satisfied all the conditions needed to perform microcage measurements: (1) no bead crashing was ever observed during its handling in the chip; (2) agarose is a very porous polymer, so arsenite and nutrients needed for the cells can easily diffuse into the beads; (3) agarose is transparent thus there is no problem for

optical observation. Moreover, the amount of cells per bead is easily controllable by changing the concentration of cells in the original suspension. Different methods for bacterial cell encapsulation have been described, employing various kinds of hydrogel matrices, like alginate,[17] sol–gel matrices[18] and agar.[19] Automated methods for cell encapsulation exist as well, which make use of DNA-arraying instruments that deposit and immobilize nanodrops of cells suspensions.[20,21] The procedure for agarose embedding has the advantage that it is cheap, does not require complicated equipment and produces very small beads suitable for incorporating in microfluidics devices.

Bacterial reporter cells have also been incorporated in microfluidic platforms before, using embedded structures in *e.g.*, silicon[22] or glass.[23] We find here that a PDMS device can be reproducibly constructed, starting from one silicon mold, and hundreds of identical chips can be fabricated inexpensively. Moreover, PDMS can be easily connected to external tubings, because of its material flexibility. Disadvantage of PDMS for bioreporter assays is that in particular organic target compounds may directly interact with the material and are 'lost' for the analysis. However, arsenite and heavy metals would rather adsorb to glass than to plastics and we did not find any indications for loss of arsenite through adsorption to PDMS.

In order to trap cells using a microfluidic chip, different structures have been proposed: U-shaped traps,[24] dam structures which retain cells along the channel by defining a net cross-flow over the dam,[25] and microwells on the bottom of a channel that can capture cells by sedimentation[25] or by lateral capillary force.[26] The advantage of our pillar-made microcage is that it is simple to fabricate (only one etching step) and does not require extra steps in terms of flow control: once the syringe pump is switched on, the cage fills with a constant number of cell-beads and an arsenic flux is imposed. In contrast, the pillars are relatively wide apart and single reporter cells would not be contained in the device. The pillar cage system allowed us to concentrate cell-beads in a small space where the fluorescence intensity is easily quantified. The advantage in having a filter cage instead of a filter wall is that the number of beads accumulated against the filter cage is constant, and therefore during each experiment the beads are undergoing the same conditions in terms of nutrients and arsenite distribution.

In conclusion, we have demonstrated that with a microfluidics cage device and reporter cell-beads one can quantitatively measure arsenite in aqueous solutions, well below the current drinking water limit of 10 μg As per L. Importantly, we show that cell-beads can be stored within the microfluidic chip at −20 °C for periods of at least one month while retaining inducibility. As for this kind of storage a common freezer is sufficient, this is a crucial step forward to make the use of such chip devices possible in a field situation. Moreover, as handling of the cell-beads is not required anymore, the device is potentially utilizable by a user who is not familiar with the laboratory techniques. We further showed that if necessary, long-term maintenance and inducibility of reporter cell-beads can be achieved by freezing in 15% glycerol at −80 °C, from where they can be easily reintroduced into the microcages. This work only focused on the development of the microfluidics cage and the details of measuring the reporter signal in cell-beads, but still used a high-end epifluorescence microscope for reporter signal detection. In order to achieve the longer term goal of a simple complete biosensor instrument, we are now focusing on the development of an optical detection system and an instrument housing to integrate the microfluidics cage.

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References

1 E. Diesel, M. Schreiber and J. R. van der Meer, *Anal. Bioanal. Chem.*, 2009, **394**, 687–693.
2 D. K. Nordstrom, *Science*, 2002, **296**, 2143–2144.
3 C. Abernathy, R. L. Calderon and W. R. Chappell, *Arsenic Exposure and Health Effects*, Elseviert Science Ltd., London, 1999.
4 S. Fendorf, H. A. Michael and A. van Geen, *Science*, 2010, **328**, 1123–1127.
5 C. K. Jain and L. Ali, *Water Res.*, 2000, **34**, 4304–4312.
6 D. Melamed, *Anal. Chim. Acta*, 2005, **532**, 1–13.
7 M. Berg, H. C. Tran, T. C. Nguyen, H. V. Pham, R. Schertenleib and W. Giger, *Environ. Sci. Technol.*, 2001, **35**, 2621–2626.
8 S. Laschi, G. Bagni, I. Palchetti and M. Mascini, *Anal. Lett.*, 2008, **40**, 3002–3013.
9 M. Rahman, D. Mukherjee, M. K. Sengupta, U. K. Chowdhury, D. Lodh, C. R. Chanda, S. Roy, M. Selim, Q. Quamrussaman, A. H. Milton, S. M. Shahidullah, M. T. Rahman and D. Chakraborti, *Environ. Sci. Technol.*, 2002, **36**, 5385–5394.
10 C. M. Steinmaus, C. M. George, D. A. Kalman and A. H. Smith, *Environ. Sci. Technol.*, 2006, **40**, 3362–3366.
11 P. T. Trang, M. Berg, P. H. Viet, N. Van Mui and J. R. van der Meer, *Environ. Sci. Technol.*, 2005, **39**, 7625–7630.
12 J. Stocker, D. Balluch, M. Gsell, H. Harms, J. S. Feliciano, S. Daunert, K. A. Malik and J. R. van der Meer, *Environ. Sci. Technol.*, 2003, **37**, 4743–4750.
13 D. L. Scott, S. Ramanathan, W. Shi, B. P. Rosen and S. Daunert, *Anal. Chem.*, 1997, **69**, 16–20.
14 S. Tauriainen, M. Karp, W. Chang and M. Virta, *Appl. Environ. Microbiol.*, 1997, **63**, 4456–4461.
15 M. Wells, M. Gösch, R. Rigler, H. Harms, T. Lasser and J. R. van der Meer, *Anal. Chem.*, 2005, **77**, 2683–2689.
16 K. Zengler, G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short and M. Keller, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15681–15686.
17 D. O. Fesenko, T. V. Nasedkina, A. V. Chudinov, D. V. Prokopenko, R. A. Yurasov and A. S. Zasedatelev, *Mol. Biol.*, 2005, **39**, 84–89.
18 J. R. Premkumar, R. Rosen, S. Belkin and O. Lev, *Anal. Chim. Acta*, 2002, **462**, 11–23.
19 J. H. Lee, R. J. Mitchell, B. C. Kim, D. C. Cullen and M. B. Gu, *Biosens. Bioelectron.*, 2005, **21**, 500–507.
20 H. Tani, K. Maehana and T. Kamidate, *Anal. Chem.*, 2004, **76**, 6693–6697.
21 T. Elad, J. H. Lee, S. Belkin and M. B. Gu, *Microb. Biotechnol.*, 2008, **1**, 137–148.
22 E. K. Bolton, G. S. Sayler, D. E. Nivens, J. M. Rochelle, S. Ripp and M. L. Simpson, *Sens. Actuators, B*, 2002, **85**, 179–185.
23 I. Inoue, Y. Wakamoto, H. Moriguchi, K. Okano and K. Yasuda, *Lab Chip*, 2001, **1**, 50–55.
24 L. Y. Wu, D. Di Carlo and L. P. Lee, *Biomed. Microdevices*, 2008, **10**, 197–202.
25 M. Yang, C.-W. Li and J. Yang, *Anal. Chem.*, 2002, **74**, 3991–4001.
26 A. Hademhosseini, J. Yeh, S. Jon, G. Eng, K. Y. Suh, J. A. Burdick and R. Langer, *Lab Chip*, 2004, **4**, 425–430.