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Microfluidic biosensing systems
Part II.† Monitoring the dynamic production of glucose and ethanol from microchip-immobilised yeast cells using enzymatic chemiluminescent μ-biosensors

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A microfluidic flow injection (μFIA) system was employed for handling and monitoring of cell-released products from living cells immobilised on silicon microchips. The dynamic release of glucose and ethanol produced from sucrose by immobilised *Saccharomyces cerevisiae* cells was determined using microchip biosensors (μ-biosensors) with either co-immobilised glucose oxidase–horseradish peroxidase (GOX-HRP), or alcohol oxidase–horseradish peroxidase (AOX-HRP), catalysing a series of reactions ending up with chemiluminescence (CL) generated from HRP-catalysed oxidation of luminol in presence of *p*-iodophenol (PIP). The yeast cells were attached by first treating them with polyethylenimine (PEI) followed by adsorption to the microchip surface. The cell loss during assaying was evaluated qualitatively using scanning electron microscopy (SEM), showing that no cells were lost after 35 min liquid handling of the cell chip at 10 μl min^{-1}. The enzymes were immobilised on microchips *via* PEI-treatment followed by glutaraldehyde (GA) activation. The GOX-HRP μ-biosensors could be used during five days without any noticeable decrease in response, while the AOX-HRP μ-biosensors showed continuously decreasing activity, but could still be used employing calibration correction. The glucose and ethanol released from the immobilised yeast chips were quantitatively monitored, by varying the incubation time with sucrose, showing the possibilities and advantages of using a microfluidic system set-up for cell-based assays.

1 Introduction

The cell constitutes the smallest living building block in higher organisms, containing all functions and information for life. The cell, as such, is thus a very useful entity in various biochemical assays to get deeper insight of the molecular machinery, where drug discovery is one of the main driving forces.[1,2] A clear trend is seen going from the use of receptor–ligand binding assays to receptors reconstituted in living cells. The latter approach makes it possible to distinguish between agonists, inverse agonists and neutral antagonists.[1,3] Furthermore, whole signalling-pathways can be studied if the ligand has multiple interaction points downstream from the signalling events.[1] Cells from the target species are, however, not always used; instead biochemical systems are reconstituted in a different organism, where yeast (*Saccharomyces cerevisiae*) is one of the most common models. One important reason for its popularity is that it is a unicellular eukaryote organism with highly tractable molecular biology.[4] A practical aspect is that it can be cultivated and manipulated without having extensive sterile facilities, which is needed for mammalian cell cultures.

Another trend in medicine, biological and biochemical research disciplines is the emerging use of microchip platforms for chemical analysis,[5–10] as pointed out in Part I.[11] Entailing these advantages and facing cell-based assays, micro systems and miniaturised assays can offer lower consumption of cell culture, real-time continuous monitoring of intracellular or secreted biomarkers, automated liquid flow handling of one or several cells. The environment can be controlled very precisely in terms of addition and removal of medium and reagents, thus minimising cellular stress. The continuous flowing format also leads to no waste products from the cells being accumulated and since cells can be confined in very narrow microstructures, small amounts of secreted compounds may reach high concentrations, and thus be more easily detected. Several of these aspects have recently been reviewed elsewhere.[12]

To explore the possibilities and features of microfluidic biosensing systems we here report the second part of two on our work towards an *in vitro* microchip-based system for monitoring of cell released products and cell dynamics. As a simple and robust model system, *S. cerevisiae* cells were immobilised on silicon flow-through microchips and inserted in a microfluidic system. The presented microfluidic system registers the production of glucose and ethanol released from the yeast chip, after stimulation by sucrose, using two different chemiluminescent (CL) enzyme-based flow-through microchip

biosensors (μ-biosensors), developed and described in Part I.[11] These were composed of either glucose oxidase (GOX) or alcohol oxidase (AOX) co-immobilised with horseradish peroxidase (HRP), and placed downstream from the yeast cell microchip. The yeast were first stimulated with sucrose and the formed glucose or ethanol was then transported to the μ-biosensor and oxidised by GOX or AOX under the formation of hydrogen peroxide (H_2O_2). H_2O_2 then participated in the HRP-catalysed CL reaction, together with luminol and *p*-iodophenol (PIP) and the produced light detected *via* a photomultiplier tube (PMT), corresponding to the amount of glucose or ethanol released by the yeast cells.

2 Experimental

2.1 Chemicals

Immobilisation reagents glutaraldehyde (GA) 25% grade I, polyethylenimine (PEI, molecular weight 750,000) 50% (w/v), and sodium cyanoborohydride were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP, 1000 units mg^{-1}, type VI-A), glucose oxidase (GOX, from *Aspergillus niger*, 100–250 units mg^{-1}) and alcohol oxidase (AOX, from *Hansenula sp.* 20–40 units mg^{-1} and *Candida boidinii*, 0.54 units mg^{-1}) were from Sigma Chemicals Co. Luminol 97% and *p*-iodophenol (PIP) 99% for the enzyme μ-biosensor CL reaction were from Aldrich Chemical Co. (Milwaukee, MI, USA). Hydrochloric acid (HCl) 37%, ammonia (NH_3) 25%, dimethylsulfoxide (DMSO) and hydrogen peroxide (H_2O_2) 30% were from Merck (Darmstadt, Germany).

2.2 Buffers and solutions

100 mM Tris/HCl buffer pH 7.0 was prepared from a 100 mM Tris stock solution adjusted to pH 7.0 with 6 M HCl. The Tris stock solution was also used for preparation of 10 mM Tris/HCl pH 7.0 and 20 mM Tris/HCl buffer pH 8.0. Succinate buffer was prepared from 10 mM succinic acid adjusted to pH 6.0 with 2 M NaOH. 2 mg ml^{-1} PEI solution for enzyme and cell immobilisation was prepared in water and pH was adjusted to 7.0 with HCl. The carrier flow in the μFIA system contained 9 g l^{-1} sodium chloride (NaCl, Merck) in water. Stock solutions of 50 mM luminol and 150 mM PIP were prepared in DMSO. The μ-biosensor reagent buffer was prepared in 20 mM Tris/HCl pH 8.0 containing 250 μM luminol and 150 μM PIP. For assaying the invertase activity of bakers' yeast, 1 mM sucrose (ICN Biomedicals Inc., Aurora, OH, USA) in water was used. Furthermore, to assay the released ethanol from cultivated and immobilised yeast, 58 mM sucrose (equals 20 g l^{-1} sucrose, which is commonly used as standard concentration in cell culture mediums) in water was used. Calibration solutions of glucose (0.1, 0.3, 0.6, 1.0, and 1.3 mM) and ethanol (5, 20, 40, 60, 80, and 100 mM) were prepared from α-D-(+)-glucose (Sigma Chemicals Co.) and ethanol 99.7% (Solveco Chemicals AB, Täby, Sweden) dissolved in water. Ultra pure water was obtained from a Milli-Q water purification system (Millipore, Bedford, WY, USA).

2.3 Microchip fabrication

The flow-through silicon microchips for use in enzyme μ-biosensors (described in Part I[11]) and for yeast immobilisation were fabricated by chemical wet etching of silicon, as described by Laurell *et al.*[13] and Drott *et al.*[14] and were made porous, as described in Part I.[11] The microchips for yeast immobilisation (Fig. 1a) were 13 mm long and 3.1 mm wide, consisting of 28 parallel V-grooves, 10 mm long, 100 μm wide at the top and 71 μm deep, with each end falling into inlet and outlet basins. The pitch between the grooves was 10 μm. The total volume of one yeast cell microreactor was approximately
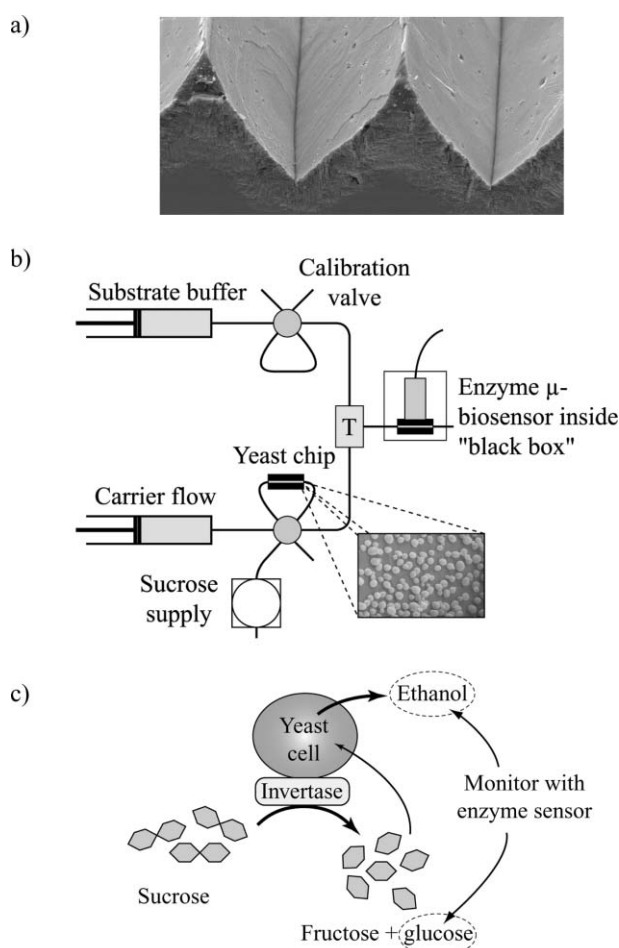
Fig. 1 (a) A flow-through silicon microchip with V-grooves used for immobilisation of yeast cells: channels 100 μm wide at the top and 71 μm deep. (b) Picture of the μFIA system set-up. The reagent buffer was 20 mM Tris/HCl pH 8 (containing 250 μM luminol and 150 μM PIP), and carrier flow was 9 g l^{-1} NaCl. A dual syringe pump at 10 μl min^{-1} delivered both carrier and reagent buffer. The sucrose was supplied *via* a peristaltic pump set to 10 μl min^{-1}. The yeast microchip was connected to a six-port injection valve instead of the injection loop. The sensor calibration solutions were introduced at the second six-port valve, located at the reagent buffer flow stream, having a 2 μl injection loop. Both carrier flow and reagent buffer were mixed at the mixing T, and further downstream was the enzyme microchip sensor-PMT detection unit placed. The sensor was placed in a 'black-box' to shutout light from the surroundings; (c) Yeast cell model: the cells are stimulated with sucrose, which is broken down by the periplasmic enzyme invertase into glucose and fructose. The two latter sugars are transported into the cell and consumed, resulting in formation of ethanol. Both invertase activity (*via* glucose) and ethanol production is monitored continuously using the μ-biosensors.

1.9 μl. This microchip construction was also applied for long-term studies of a reporter gene modified human cell line.[15]

2.4 μ-Biosensor preparation

Prior to immobilisation of enzymes, all silicon microchips were cleaned according to the following: (1) Boiling for 1 min in a mixture of 3 ml 25% NH_3 and 3 ml 30% H_2O_2 in 10 ml water, (2) rinsing with water, and (3) 1 min boiling in 3 ml 37% HCl, 3 ml 30% H_2O_2 and 10 ml water. The microchips were then rinsed and stored in water until use.

The μ-biosensor immobilisation procedure was the same as in Part I,[11] however, here only PEI immobilisation was used for both GOX-HRP and AOX-HRP sensors, however, AOX from two different origins was tested. Briefly, PEI-based μ-biosensors were prepared as follows: A layer of PEI was adsorbed on the microchip surface by immersing the cleaned

microchips in 2 mg ml^{-1} PEI solution pH 7.0 over night at room temperature (RT). The microchips were rinsed with succinate buffer to remove non-adsorbed PEI and then placed in a 2.5% (v/v) GA solution prepared in succinate buffer for 1 h at RT. After thorough rinsing with succinate buffer to remove traces of GA, the microchips were ready to be immersed into enzyme solutions for 18–24 h at +4 °C. For glucose μ-biosensors the enzyme solution was composed of 4 mg ml^{-1} GOX and 1 mg ml^{-1} HRP in succinate buffer. Similarly, the ethanol μ-biosensors were immobilised in a solution of AOX, containing either 4 mg ml^{-1} *Hansenula sp* or 7 mg ml^{-1} *Candida boidinii*, and 1 mg ml^{-1} HRP. After enzyme attachment the μ-biosensors were rinsed and placed in 100 mM Tris/HCl pH 7.0 for 1 h at RT to block residual aldehyde groups. The Schiff bases were then reduced with 2 mg ml^{-1} sodium cyanoborohydride solution in 10 mM Tris/HCl and the reaction mixture was allowed to proceed for 1 h at RT. The μ-biosensors were then carefully rinsed and stored in 10 mM Tris/HCl buffer at +4 °C until use.

2.5 Preparation and immobilisation of yeast cells on silicon microchips

Yeast cells from three sources were used: For optimisation of the microfluidic system two different baker's yeast (yeast for "sweet doughs" with low invertase activity (LIA) and yeast for "normal doughs" with normal invertase activity (NIA), Jästbolaget, Sollentuna, Sweden) was used, while the yeast strain *S. cerevisiae* CEN.PK113-7D (*MATα, MAL2-8^c, SUC2, leu2, trp1, his3, ura3*) was used for further measurements. The latter was grown as follows: 100 ml synthetic deficient medium (composed of 6.7 g l^{-1} yeast nitrogen base without amino acids, 20 g l^{-1} sucrose, 50 mg l^{-1} of each lysine, methionine and tryptophan, and 250 mg l^{-1} leucine, all chemicals from Sigma Co.) were inoculated with a loop full of yeast cells from solid medium. The culture was grown over night at 37 °C and stirring. At harvest the optical density (630 nm) was approximately 0.5. The yeast was rinsed three times with cold 9 g l^{-1} NaCl solution and then immersed in 2 mg ml^{-1} PEI solution so that the final yeast concentration was 5 mg ml^{-1}. The PEI treatment was performed for 2 h at +4 °C in a test tube with gentle shaking. Finally the yeast was washed three times and suspended in cold NaCl solution to reach 5 mg ml^{-1}. In the last step, the yeast suspension was poured over the microchips, which had been cleaned by the same procedure as described above for the enzyme immobilisation. The yeast was allowed to adsorb to the microchip surface for 40 min at +4 °C. After thorough rinsing with cold 9 g l^{-1} NaCl solution, the cell microchip was inserted in the μFIA system for assaying.

In a similar way, the cell microchips with bakers' yeast were prepared by suspending approximately 100 mg yeast to final concentration of 10 mg ml^{-1} in cold NaCl solution. After three washings the yeast was suspended in PEI solution and treated in the same way as described above for the cultivated yeast.

2.6 Microfluidic set-up and assay procedure

Two microfluidic set-ups were explored, *i.e.*, the microfluidic sequential injection analysis (μSIA) system already presented in part I,[11] and the microfluidic flow injection analysis (μFIA) system depicted in Fig. 1b. The carrier flow of the μFIA system was 9 g l^{-1} NaCl solution, delivered at 10 μl min^{-1} by a sp260p dual syringe pump (World Precision Instruments Inc., Sarasota, FL, USA). The syringe pump was also equipped with a second syringe containing μ-biosensor reagent buffer, which was 20 mM Tris/HCl pH 8.0 with 250 μM luminol and 150 μM PIP, as optimised in Part I.[11] This buffer was added continuously to the flow stream at 10 μl min^{-1} at a mixing-T before the enzyme μ-biosensor. The yeast cell microchip was inserted in place of an injection loop on a six-port valve (Rheodyne, Rohnert Park, CA, USA) in such a way that sucrose could be pumped on the microchip with a peristaltic pump (Gilson Minipuls 2, Villiers le Bel, France) at 10 μl min^{-1} for 1.5 min. The flow was then stopped for a certain incubation time, which allowed the cells to consume the sugar and respond by producing glucose and ethanol. Next, the valve was switched and the carrier flow entered the microchip and transported glucose and ethanol formed by the yeast to the enzyme μ-biosensor. The valve was switched back to the original position when the sample had passed over the μ-biosensor (1.5 min) and another portion of sucrose was added over the yeast, followed by incubation *etc*. Depending on which μ-biosensor was used (GOX-HRP or AOX-HRP) one of the two analytes was oxidised, producing a corresponding amount of H$_2$O$_2$ that was detected in the CL reaction catalysed by HRP. Directly after insertion of a new yeast microchip in the flow system, sucrose was pumped continuously at 10 μl min^{-1} over the chip for 5 min (to the waste). This was always done for each newly prepared cell chip, to remove any loosely bound cells and discard them to the waste. Both the yeast microchip and μ-biosensors were incorporated into the system *via* specially designed holders of transparent poly(methyl methacrylate), described elsewhere.[16] A photo-multiplier tube (PMT, model no. HC135-01, Hamamatsu Photonics K. K., Japan), mounted on top the microchip sensor, registered the CL signal, and the whole sensor unit was placed in a 'black box' to shut out light from the surroundings (see Fig. 1b). The PMT signal was acquired using an in-house developed computer-software. To calibrate the μ-biosensors, while also performing incubations on the yeast microchip, a second six-port valve equipped with a 2 μl loop was placed on the reagent buffer flow line just before the mixing-T. The volume of the injection loop was the same as the volume of the cell microchip (1.9 μl). Quantitative data was extracted from the CL signal curves by determining the peak heights. The concentration of formed glucose or ethanol during incubation was determined by using the corresponding calibration curve.

3 Results and discussion

During the last five to six years several research groups have explored the task to handle, use, and assay living cells in the flow format. The group of Ruzicka has worked with flow injection analysis (FIA) systems for monitoring cellular activity based on micro-bead-immobilised adherent cells for monitoring oxygen consumption[17] and agonist–receptor interactions.[18,19] Furthermore, SIA systems were developed for real-time monitoring glucose consumption of murine hepatocytes immobilised in a micro-bioreactor using an enzymatic two-step reaction for determining glucose and spectrophotometric detection.[20] This was further developed to, besides glucose, also include lactate extrusion in another sequential injection system for monitoring mouse hepatocytes.[21]

Microfluidic systems have been developed for the purpose of both assaying and handling of cells.[12] However, most of the microfluidic cell assay systems are based on suspended cells[22–27] and only a few are using immobilised cells.[28–30] In fact, most mammalian cell lines used in molecular biology are adherent, which means that they need to be cultivated on a solid surface in order to perform well and to multiply. Moreover, continuous assaying of the same cell population is a prerequisite to obtain information of the dynamic changes in cellular response to a stimulus. Cell-based assays are often in the time range of several hours, which means that the cells must be entrapped to allow monitoring of the same cell population in a microfluidic system. Although yeast is not an adherent microorgansim, this problem was solved by PEI-assisted adsorption to a silicon

microchip. Attachment was accomplished by treating negatively charged yeast cells with the positively charged polybasic aliphatic amine PEI followed by adsorption to a clean silicon microchip surface, which carries negative charges from hydrolysis of acidic silanol groups. PEI has been used to immobilise different biocatalysts[31] and was used to develop microchip affinity protein sensors,[16,32] similar to the enzyme μ-biosensors used in this study (see also Part I[11]). This approach allowed continuous sampling of metabolites from the yeast cell microchips. In this mode, the fluid surrounding the cells could be manipulated for transport of reagents or removal of cell-released products, while keeping the same cell population.

In the microfluidic system reported in this paper yeast was used, in which the extra-cellular invertase activity and ethanol production during respiration were monitored. When yeast is exposed to sucrose as the sole carbon source, the cells respond by producing the enzyme invertase (β-fructofuranosidase, EC 3.2.1.26), which is located in the periplasma (see Fig. 1c). Invertase converts sucrose into glucose and fructose, which are transported into the cell. The sugars might be metabolised by respiration or alcoholic fermentation, if oxygen supply is limited or sugar concentration is high (the Crabtree effect[33]).

The μSIA system developed in Part I[11] of this study was intended to be used as the liquid handling technique, due to the possibility of measuring several reactions simultaneously (*i.e.*, glucose and ethanol) with the same system. The system was thus tested to assay the yeast microchips, however, this configuration led to problems, *e.g.*, the μSIA sometimes entered air bubbles in the system which were trapped on the cell chip and could only be removed by flushing with high flow rate with the result that cells were dislocated from the chip. Second, the multiposition valve could only move position stepwise, which made the liquid handling much too slow to allow incubation times less than approximately 10 min. The latter proved to be very important especially for monitoring glucose release from yeast. Thus, a μFIA system shown in Fig. 1b was set up, resulting in a functioning and faster, but less versatile system. This μFIA yeast monitoring system was evaluated in two ways: A qualitative approach using scanning electron microscopy (SEM) looking at the adherence of cells by visually comparing the yeast cell density on the surface, before and after measurements, and a quantitative approach where the continuous production of glucose and ethanol from the microchip-immobilised yeast cells was monitored, using the enzyme μ-biosensors placed downstream from the yeast microchip.

3.1 Adherence of yeast cells to silicon microchips

SEM images were used to evaluate loss of cells due to the continuous flowing environment. Fig. 2a shows a cell microchip directly after immobilisation not yet inserted in the flow system. Fig. 2b and c show two microchip that have been exposed to an initial 5 min rinsing at a flow rate of 10 μl min^{-1}. Fig. 2d and e show two microchips subjected to first 5 min initial rinsing, then 1, 2, 3, 4, and 5 min incubation with sucrose following glucose assaying, which includes 20 min liquid handling, and means the presence of the chips for a total of 35 min in the flow system.

Considering the SEM images of yeast chips that were inserted and assayed in the μFIA system (Fig. 2b–e), there is a loss of cells at the top between the V-grooves, when compared to the unused yeast chip in Fig. 2a. This loss is mainly an effect due to the lid and the flexible membrane of the flow cell, which are compressed on top of the microchip and touches the upper part of the microchip edges between the channels, thus pushing the cells away from this region. Due to this, a 5 min rinsing after insertion of each yeast chip was inserted to discard any dislocated or loosely bound cells before assaying. These SEM
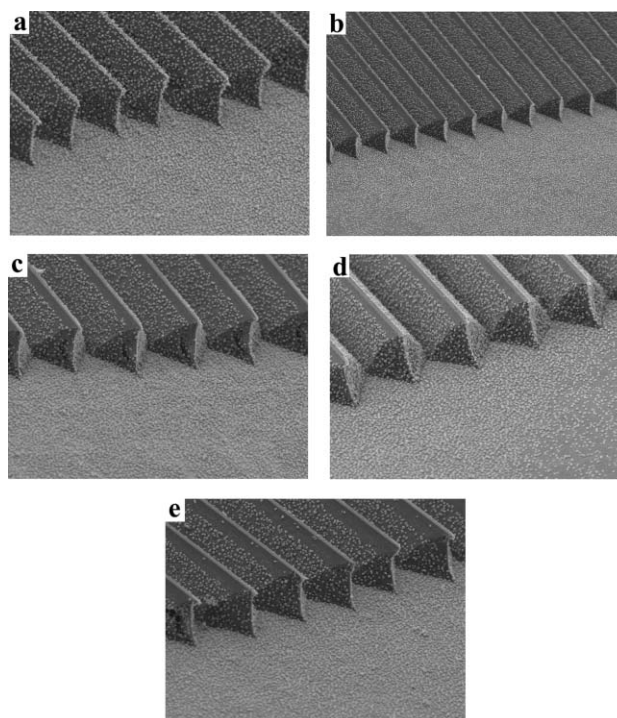
Fig. 2 SEM images of cell microchips subjected to different treatments. (a) Image of a cell chip directly after immobilisation, while (b) and (c) shows two different chips that both have been inserted in the flow system and exposed to initial 5 min rinsing. Finally (d) and (e) shows two microchips that been in the flow system and have had 5 min initial rinsing and a sequence of 1, 2, 3, 4 and 5 min incubation assays.

images will be considered further when discussing the repeated incubation assay cycles below.

3.2 Monitoring invertase activity – glucose production from immobilised yeast

Depending on the composition of carbon sources available, the periplasmic invertase activity is up or down regulated. If both glucose and sucrose are available, the former is preferred and results in down regulation and thus low invertase activity.[34] The *in vivo* invertase activity can either be assayed by enzymatic determination of formed glucose[35] or by reducing sugars (both glucose and fructose are determined).[36]

In our experiments, three types of yeasts were investigated; one laboratory yeast strain (see Section 2.5 for details), and two kinds of bakers' yeast; one used for baking ordinary bread (here denoted as normal invertase activity (NIA) yeast) while the other is used for doughs with high sucrose content. According to the manufacturer, the latter bakers' yeast has a lower invertase activity to avoid inhibition by the formed glucose and fructose due to the high sucrose content in the dough (thereby denoted as low invertase activity (LIA) yeast).
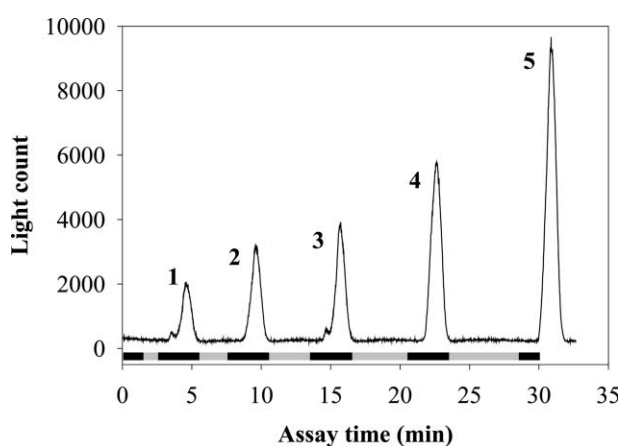
Due to the better sensitivity of the PEI-based GOX-HRP μ-biosensors found in Part I,[11] this was the sensor used in the following work. The PEI-GOX-HRP sensors were characterised by generating calibration plots (see Fig. 3) from injections of glucose standards of 100 to 1300 μM into the μFIA system in Fig. 1b. Linear calibration plots for the PEI-GOX-HRP sensor were obtained by log-log transformation of data. During a five days usage period the PEI-GOX-HRP μ-biosensor showed the following regression data: 5.12, 5.36 and 5.01 in line slope and corresponding intercepts of 2.56, 2.44 and 2.30, obtained at day 1, 3 and 5, respectively. All coefficients of correlation were 0.999. The operational time for each calibration and the following assay of glucose
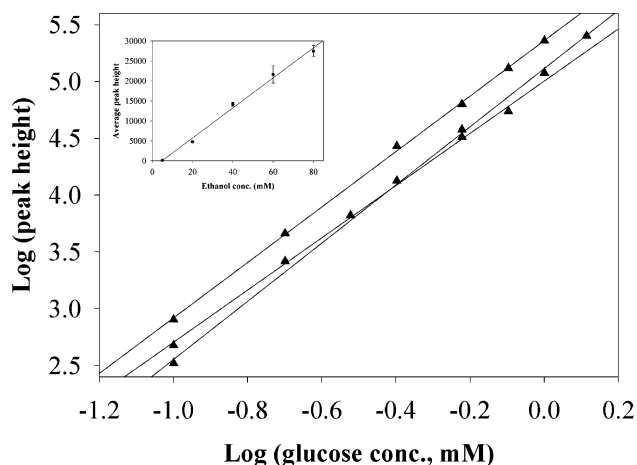
Fig. 3 Calibration characteristics for the PEI-based microchip sensors: the main graph is for three GOX-HRP calibrations performed on three different days and the inset shows an AOX-HRP calibration.

liberated from yeast cells lasted between 12–14 h, during which no loss in sensor activity was found. The μ-biosensor (excluding the yeast chip) was also tested if 1 mM and 58 mM sucrose solutions interfered with the detection but did not result in any μ-biosensor signal.

The continuous signal read-out from the PEI-GOX-HRP μ-biosensor during 1–5 min incubation of a yeast (cultivated) microchip with sucrose is shown in Fig. 4. Right above the time axis is a time-graphic representation of the system events (the very first 5 min pumping to remove loosely attached cells is not included, since it was only done once for each chip). The first event in the assay cycle was 1.5 min filling with sucrose followed by stopped flow and 1 min incubation. Then the valve was switched and the carrier removed the formed metabolites from the yeast during 1.5 min. The assay cycle was thereby finished and the valve was switched back for another loading of sucrose. Thus, in-between two incubations was 2 × 1.5 min of liquid handling. The computer-connected PMT-μ-biosensor unit monitored what arrived with the carrier, recorded a peak, and at the same time sucrose was loaded on the yeast microchip for a consecutive incubation. As expected, the peaks increased

with increasing incubation time because more sucrose was broken down by invertase. Incubation (5 min) with 9 g l^{-1} NaCl solution was also tested but did not result in any signal. The two baker's yeasts, *i.e.*, normal- (NIA) respective low invertase activity (LIA) yeast microchips, were assayed in the same way and the produced glucose concentration plotted *versus* the incubation time is shown in Fig. 5. The curves represent the average plots of three (NIA) respective two (LIA) repeated incubation assays cycles with the same chip. As expected, the glucose production was lower from the chip with the cells with LIA, compared to the NIA cell, and all performed incubation assay cycles showed the same behaviour of increasing glucose production with increasing incubation time. The SEM images (Fig. 2d and e) show two cell microchips that each has been subjected to a series of 1, 2, 3, 4 and 5 min incubation assay cycles (*i.e.*, 35 min in total including all liquid handling steps). From this, no visible cell loss is observed after a full incubation assay cycle, or any decreased invertase activity in terms of decreased signal intensity during glucose determinations (Fig. 5).

When the incubation time was extended up to 30 min for the two kinds of bakers' yeast chips both curves approached 1 mM glucose (data not shown), which was the theoretical maximum that could be produced from 1 mM sucrose. The difference in sucrose hydrolysis of the two yeasts was not significant, which could be due to the low sucrose concentration (1 mM) used, as compared to realistic conditions, *e.g.*, in cell media for yeast where 58 mM sucrose (*i.e.*, 20 g l^{-1}) is the common standard concentration.

The stability of the system was determined by 12 consecutive 5 min incubations. The resulting average glucose production was 0.15 mM having relative standard deviation 16% (data not shown). One contributing factor to the signal variation can be that the timing of the incubations was performed manually, however, in light of the fact that we perform measurements on living cells, the signal variation is not bad.

3.3 Monitoring the dynamic ethanol production from immobilised yeast

When inserting the PEI-AOX-HRP sensor (AOX from *Candida boidinii*) in the μFIA system (Fig. 1b), the sensor stability was very poor and lost all activity within 12 h. The

Fig. 4 Continuous signal read-out from the PMT during a set of 1, 2, 3, 4, 5 min incubations for cultivated yeast (58 mM sucrose). The peak heights are increasing due to the increasing production of glucose. In-between the signal curve and the time axis is a graphic time stock showing the system events during the whole assay. Black areas indicate the time used for liquid handling and the grey incubation times. The time stock starts with supply of sucrose during 1.5 min (black). Next follows 1 min incubation (grey) and a 3 min liquid handling event (black), which in more detail consists of 1.5 min removal of formed glucose for transport to the sensor and 1.5 min addition of fresh sucrose. Then follows 2 min incubation (grey) and so forth.
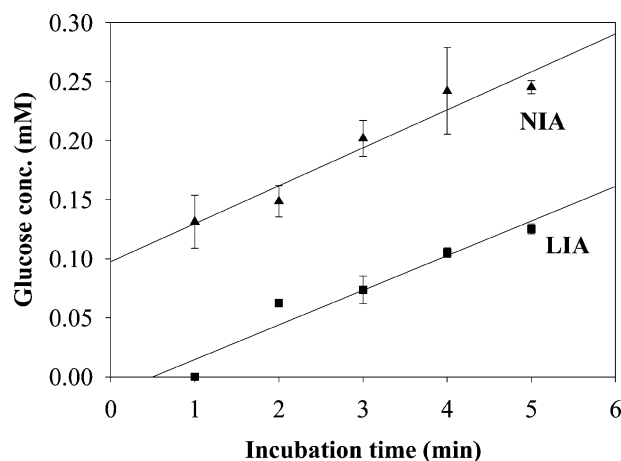

Fig. 5 The plot shows invertase assay for the two sorts of baker's yeast, one having normal invertase activity (NIA) and a second having low invertase activity (LIA), using 1 mM sucrose. The data from the two cell microchips were extracted as peak heights and recalculated into concentration using a glucose calibration curve, constructed immediately after the incubation assays. Three subsequent 1–5 min incubations were performed for the NIA chip and two for the LIA. An average concentration was then calculated for each incubation time for the two cell chips and plotted *versus* incubation time. The error bars show the standard deviation for each incubation time chip (not all are visible behind the data point dots).

explanation why this sensor was less stable in the present μFIA than in the previous μSIA system (operational stability of 8 h and a half-life of 2–3 days) is very likely due to that the enzyme in this configuration was continuously flushed with reagents (luminol and PIP) at an elevated pH of 8, needed for the CL reaction. In the μSIA system, on the other hand, it was exposed to the same reagents and pH in a discontinuous format and only for comparatively short time periods. The instability of AOX is a phenomenon that has been reported several times before and attempts have been made to improve both operational and storing stability.[37,38] Moreover, the species from which the enzyme origin seems to be of importance.[37] As an alternative, AOX from *Hansenula* sp. was thus immobilised in the same way as *Candida boidinii* and the resulting μ-biosensor inserted in the μFIA system. This resulted in a sensor with much better signal intensity, which could be used for measurement during one day (*i.e.* 12 h continuous operation) in the μFIA system. In between sucrose incubations 20 mM ethanol standard was continuously injected, following calculation of μ-biosensor correction factors, to account for any signal decrease with time. The *Hansenula sp.* PEI-AOX-HRP sensor was also tested with 58 mM sucrose but did not show any response.

Ethanol liberated from yeast was determined for cultivated yeast microchips only. The sucrose solution was 58 mM and to obtain the maximum ethanol production it was de-aerated continuously with helium gas. Before insertion in the μFIA system the immobilised yeast chips were subjected to two different pre-treatments; either by immersing in 9 g l^{-1} NaCl solution or in synthetic deficient cell medium (see Section 2.5 for detailed composition), both performed at RT and for 20 min. It is obvious from Fig. 6 that pre-incubation in cell medium had a positive effect since the production is more than three times higher compared to the yeast chip pre-treated with NaCl solution. The reason for this might be that the immobilisation procedure can have forced the cells into a resting state; the immobilisation took approximately 3 h, during which the yeast was kept in PEI solution and 9 g l^{-1} NaCl at +4 °C. The initial ethanol concentration obtained after 10 min sucrose incubation stays more or less the same over the whole tested time range. In fact the upper curve (chip treated in cell medium before measurements) decreases slightly after 40 min incubation. The lower curve (9 g l^{-1} NaCl treated chip) tends to rise slowly.
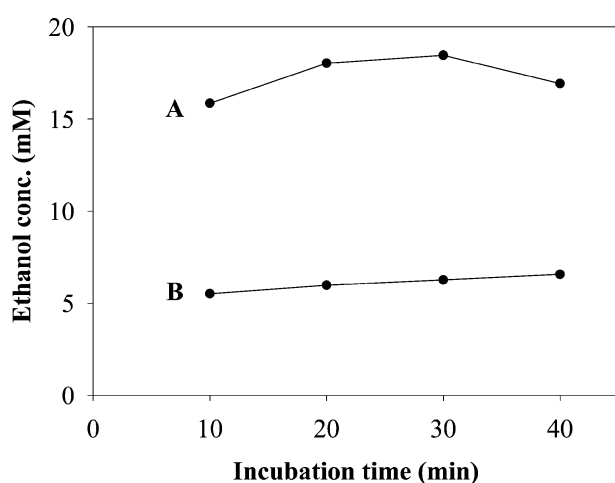
Fig. 6 Ethanol production assay for two microchips with cultivated yeast. Curve A shows the production when one of the two chips was immersed in SD medium for 20 min before insertion in the flow system and assaying. Curve B shows production from the second microchip, which was treated in the same way as chip A but instead using 9 g l^{-1} NaCl solution. The sucrose concentration was 58 mM.

The maximum ethanol production that can be obtained from a single yeast microchip can be estimated in rough terms by counting the cells in Fig. 2d and e and considering that the biomass of one cell is 1.5×10^{-11} g biomass per cell.[39] The total number of cells on a chip can be estimated to approximately 1.8 millions, which results in a total biomass of 2.7×10^{-5} g biomass per chip. In general, the maximum ethanol production for yeast is 2 g ethanol g biomass^{-1} h^{-1}, which can be recalculated into 0.7 mmol ethanol g biomass^{-1} min^{-1}. Thus under these terms, the maximum ethanol production per chip should be 1.95×10^{-5} mM chip^{-1} min^{-1}. The volume of one chip is 1.9 μl while the maximum concentration change is 10 mM chip^{-1} min^{-1}. From Fig. 6, the ethanol concentration is 5 and 15 mM, respectively, obtained during 10 min and recalculated into conversion per minute we obtain 0.5 and 1.5 mM chip^{-1} min^{-1} for the curves respectively, which are below the maximum conversion. This seems quite realistic, since a maximum rate of 2 g ethanol g biomass^{-1} h^{-1} only occurs under optimal conditions, which was not the case in our system, using 9 g l^{-1} NaCl as carrier flow without any nutrients except sucrose during the incubation.

4 Conclusions

The performance of a microfluidic system is highly dependent on the liquid handling technique employed. Initially a μSIA system was set-up (part I[11]), but proved to be insufficient for cell monitoring purpose, mainly due to slow performance. As an alternative the μFIA system presented in this paper was developed, however, functioning well but less versatile. The studied microfluidic system was based on stimulation of immobilised yeast chips with sucrose and subsequently monitor the periplasmic invertase activity *via* glucose formation and ethanol production that followed from respiration. The two cell products (glucose and ethanol) could be continuously and quantitatively monitored by flow-through μ-biosensors with co-immobilised GOX and HRP or AOX and HRP, which catalysed a system of reactions ending with the HRP catalysed CL oxidation of luminol, enhanced by PIP. The GOX-HRP μ-biosensor could be used during five days without any significant loss of activity, whereas the AOX-HRP sensor needed to be continuously corrected for loss of activity with time. The latter was probably an effect from the rather harsh conditions by continuously supplying the μ-biosensor with CL reagent buffer pH 8.0, which was a consequence of the μFIA format that had to be used.

The yeast cells were immobilised *via* PEI and the robustness of this procedure was evaluated qualitatively by SEM pictures and quantitatively by monitoring the glucose activity with time, and indicated no significant loss of cells or activity after 35 min in the system at 10 μl min^{-1} (including incubation and liquid handling steps).

Acknowledgements

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