Executive Summary:

There is a growing need for effective monitoring of the micro-organisms and bioprocesses used in the sustainable production of fuels, chemicals and pharmaceuticals. The aim of the NANOBE - consortium was to develop a compact analysis tool for reaction monitoring applications in the industrial biotechnology industry.

NANOBE system was designed flexible in terms of what analytes to measure. However, in the NANOBE project the primary example of the application was all along the production of organic acids in yeast Saccharomyces cerevisiae. Therefore, lactic acid production in genetically modified S. cerevisiae strain was focused on when testing the whole NANOBE system.

The complete NANOBE system was constructed on the basis of the commonly agreed structure of the system and the functions of the individual modules developed in form of several stand-alone units: sampling; sample treatment and delivery; in-situ monitoring of pH, dissolved oxygen (dO2) and carbon dioxide (pCO2); cell counting, sorting and lysis; ELISA (enzyme linked immunosorbent assay) based protein analysis; and finally CE-MS to analyse intra- and extracellular metabolites. The planned full setup occupied an area of 2x2 m2 including the bioreactor and its peripheries. Most of the required space was occupied by auxiliary components such as pumps, valves or electrical power supplies. The virtual system control software (Labview) controlled the individual analysis and core system modules.

In total, only four analysis cycles were carried out with the integrated system at the very end of the project. This was mainly due to the prolonged analysis cycle of the system and repeating plumbing problems. The monitored dissolved oxygen concentrations were in good agreement with the values measured using conventional sensor. The pH and CO2 sensors worked otherwise well but there were some issues with the optical sensor attachment that can be easily solved in future. The sample was successfully transported from the bioreactor to the ELISA and MS modules in two cycles out of four. One out of the two successful system operation cycles resulted in successful data readout in both the ELISA and MS modules. The results from the ELISA analysis suggested that the dilution was not totally reliable. The CE-MS analysis results were qualitatively in good agreement with data from the manually-taken sample using the same Microsaic mass spectrometer.

The main achievements of the project were:

1. Two new analysis tools (micro mass spectrometer already in the market and automated platform for unattended ELISA tests at industrialised prototyping level)

2. New innovative methods and devices developed for dead-volume free µL-scaled sampling and sample handling. The 20 µl sample volume is most probably the smallest sample volume of which so many different analytes can be measured.

3. Developed digital microfluidics for handling 1 µL droplets that push the boundaries of working with really small sample volume so that the initial sample can be separated for several analytical devices.

4. Demonstration of tools for counting and sorting of dead and alive yeast cells with added cell concentration estimation.

5. New tools developed for lysis of the yeast cells.

6. New optical sensor technology developed for autoclavable sensors for pH in the range of 3-6
7. More robust optical sensors for measuring dissolved CO_2
8. One patent application.
9. 19 scientific journal publications and 15 conference presentations.

Overall, the on-line monitoring tools developed in the NANOBE project can help to increase the production rate, yield and concentration of the final product of a fermentation process. These improvements in process monitoring may be crucial for the economic viability of a new bio-based product.

Project Context and Objectives:

Project context

Biotechnological processes are increasingly used for the production of chemicals, fuels and materials. The world market for non-food biotechnology-based products was 45 billion in 2006. Advances in genome analysis, systems biology and synthetic biology enable the engineering of cells as efficient hosts for production of a variety of compounds for various industrial sectors. These include bioactive compounds and drugs for pharma industry, fuels such as bioethanol, platform chemicals and biopolymer precursors for the chemical industry, as well as industrial enzymes for industrial applications. These developments are generating an even greater number of micro-organisms and other cells which can be used as production organisms . The expanding number of tailor made production organisms and processes will necessitate better measurement and control of the processes in order to ensure the highest possible productivity.

In an attempt to reduce our dependency on oil, the EU s Technology Platform for Sustainable Chemistry emphasises fuel production using biotechnology and the development of efficient production processes for chemicals. Sustainability means that these bioprocesses need to be highly efficient and use minimal amount of resources. To achieve these goals one needs to understand the performance of the production organism during the process, and be able to continuously monitor and control the process itself.

The development of efficient bioprocesses requires rapid, high-throughput analysers. For example, the selection of the most optimal production strains is limited by the absence of integrated, on-line tools for monitoring of strain performance during the process. Another bottleneck is the lack of rapid high-throughput systems for optimisation of production conditions. Consequently, closed loop feedback control of the production process is rarely implemented. Multi-parameter analyses, particularly for special compounds and intracellular biomarkers, require tedious manual sampling and various very slow off-line analyses.

The increasing number of production organisms and bioprocesses sets demands for fast and versatile product measurement tools for applications ranging from strain screening to large-scale production. Overall, the requirements are to reduce the efforts needed for process development, running time and the cost from the initial idea to the production scale. Even one month shorter development time can be worth of millions of euros for a pharmaceutical product if it helps to bring the product earlier to the market. Faster process development confers competitiveness. Entering the market place too late is quite often a disaster for the market penetration of a new drug.

In addition, the United States Food and Drug Administration (FDA) process analytical technology (PAT) initiative is about to revolutionize biotechnology-based processes used for the production of pharmaceuticals. The PAT initiative provides for real-time monitoring and control of the state of the production process within a certain 'design space' as opposed to the conventional 'one recipe' -strategy. The PAT initiative is driving demand for reliable real-time monitoring tools in bioprocesses.

1. Organism development creates a great number of strain variants. Real-time analysis of the performance of the strains will aid in screening of strains, and selection of the best production organism in miniaturized or small scale bioreactors.

2. Production conditions are optimised using real-time measurement devices in bench-top bioreactors to accelerate scale-up to full production.

3. Real-time measurement devices can be applied in process control, monitoring, and quality control of the production phase.

4. Overall, the use of the real-time measurement devices has potential to shorten the whole development process by approximately 40%.

Improved bioprocess control, and faster organism development drives a need for a device system which fulfils the following requirements:

a) The ability to take rapid real-time measurements of product and biomarker levels during cultivation, thereby eliminating time-consuming manual analysis. Frequent measurements can be taken without sample storage. In addition, automated data collection allows identification of those time intervals at which selected samples should be stored (e.g. for future quality assurance / quality validation requirements).

b) A versatile analyte range, adjustable to measure various parameters and molecules (including intra-cellular analytes) depending on the process. The analyte range to be measured by the system should be flexible with only changes to the method and without any changes to the system.

c) The ability to collect representative, low-volume samples from large and small scale cultures without endangering the integrity of the cultivation. In addition, all in situ sensing or sampling devices need to be cleanable and sterilizable before and after each sample. Sample volumes will depend on the sensitivity of the tool but should be as small as possible, in particular when sampling from small scale cultures.

d) Reproducible performance throughout the duration of long cultivations, which is achieved to a great extent via automation and using ex-situ devices. In-situ devices in particular are vulnerable to fouling and drift as they often cannot be changed or cleaned during cultivations. In addition, ex situ devices are easier to maintain and calibrate during long cultivations.

e) A compact size and a reasonable price - both for the infrastructure and the consumables. Lower costs and smaller size enable higher throughput via multiplication, which is needed especially in the organism screening and selection stages. Smaller systems are cheaper to install, and can be sited closer to the process reducing the infrastructure required. However, the size is not the primary criterion of the device development; the overall performance of the system is of greater importance.

Scientific and technical objectives of the project

The aim of the NANOBE project was to construct a versatile tool for the real-time analysis of several compounds and biomarkers in bioreactor processes. The tool was targeted to the control and optimization of production processes, and to acceleration of development of production organisms for applications in industrial biotechnology. Micro and nanofabrication techniques were used to exploit the scaling laws associated with microfluidic devices to reduce analysis time and sample volume. The added value of the NANOBE approach is in the ability to measure certain biomarkers, indicative of cell performance and state (e.g. intracellular stress markers, product expression levels, and specific proteases).

The NANOBE approach aimed to a flexible platform for real-time measurement of both intra and extra-cellular analytes. One motivation was to avoid the need to develop a custom probe for measurement of each individual analyte. The versatile measurement tool would require only a change in method (e.g. a change of reagents or analysis conditions) to enable the measurement of another new analyte. The device was to be designed so that it could be coupled to range of bioreactors, from novel micro-bioreactors to conventional industrial production bioreactors.

Automated sample handling for measurements of intra and extra-cellular analytes was of importance because some key biomarkers exist only inside cells.

A general requirement was sensitivity. Sensitivity minimizes the amount of sample required. Small sample volumes have the advantage that many samples can be taken from a culture without disturbing the process.

The sampling and analysis of individual cells was desirable because we need to record variation in single cell performance, rather than observing the performance of the whole population of cells. In addition, accurate cell counts from a culture were paramount in order to relate productivity to cell biomass.

The application

NANOBE system was designed so that it is flexible in terms of what analytes to measure. In practise this means that different modules of the NANOBE system can be selected and combined for various applications. In the NANOBE project the primary example of the application was all along the production of organic acids in yeast Saccharomyces cerevisiae. This related to several publicly funded research projects at VTT (Technical research center of Finland).

It was seen important to develop the NANOBE system so that it can cope with different analytes, different cell types and different cultivation conditions in the future. However, only one application example was to be focused on when testing the whole NANOBE system. The main application example was lactic acid production in genetically modified S. cerevisiae strain. Strain is based on CEN.PK113-16B (URA3. HIS3. Leu2-3,112. TRP1. MAL2-8c. SUC2) from Dr. P. Kötter at Goethe Universität [K-D. Entian and P. Koetter. in Yeast gene analysis (eds. Brown AJP, Tuite MF), Meth Microbiol vol. 26 pp. 431-49 (1998), Academic Press, San Diego]. Strain has a lactate dehydrogenase gene ldhL from Lactobacillus helveticus on a plasmid. Individual selected analytes are chosen so that they are related to lactic acid production.

The analytes were chosen so that during the cultivation of the strain it is possible to follow how the beginning and the end of the lactic acid production pathway operate at the gene expression, enzyme activity and intracellular metabolite level, in addition to consumption of glucose and production of lactic acid. So far it is unknown for instance how the levels of intracellular pathway components change during different phases of the production process. Measurement of the levels may provide additional information into the cell-level regulation of the lactic acid production and open new possibilities for controlling the process in order to improve the production rate. Lactic acid is the precursor for poly-lactic acid, which is used in the production of biodegradable plastics such as cups and plastic bags.

Project Results:

Main S&T results

Sampling

A significant effort in the NANOBE project was put on developing a sampling system, which allows a time-discrete sampling of dead-volume free µl-scaled samples and a subsequent separation of cells and fermentation broth. This compact sampling and filtration system makes an interface between bioreactors and the rest of the NANOBE measurement tool. The sampling probe needs to be sterilizable. It also needs to fit the industrial standard size. The probe should preferably also be applicable to the sampling of different cell types (e.g. yeast, bacterial, filamentous, plant). In addition, integration of filtration and optical in situ sensing (e.g. in situ sensing for dissolved gaseous analytes) were to be included to create an intelligent probe in a conventional package which will fit bioreactors. The sampling and filtration system supplies the downstream analysis modules with the raw sample for cell counting, the retentate for intracellular substance analysis after cell lysis, and the filtrate for extracellular substance analysis.

The sampling probe and the filtration module were realized by means of conventional technologies, whereas the integrated fluid management was partly realized with conventional and with a new on-chip pinching valving/pumping technology. The beneficiaries involved in the sampling probe development were Institut für Bioprozess- und Analysenmesstechnik e.V. (iba), in Germany; Department of Microsystems Engineering (IMTEK), University of Freiburg, in Germany; and PreSens Precision Sensing GmbH (PreSens), also in, Germany.

Sampling probe specifications

At the beginning of the project the sampling probe specifications were discussed with all beneficiaries and defined for Saccharomyces cerevisiae and Streptomyces fermentation.

Dead-volume free sampling

Iba developed a sampling probe that based on a two-capillary concept patented by them. It could be proved for the sampling specifications of Saccharomyces cerevisiae and Streptomyces fermentation by iba. The sampling probe was designed so that the sample could be embedded into two air plugs, which enabled a time-discrete sampling in µL scale (20µL of yeast and 50µL of Streptomyces) without dead-volume. The sampling probe was successfully tested for fermentations with overpressure of 3bar.

In order to withdraw a defined sample volume from a bioreactor operating with a gas flow rate up to 2vvm, it was necessary to develop a special probe head adapter. This adapter excluded the air bubbles of the fermentation broth from the sampling tube but not from the sensor spots which are additionally located on the probe head. Even with the sampling of the filamentous Streptomyces the standard deviation of the sampling process showed only a very small increase from almost zero to 4%. The

sampling probe was successfully purged with buffer and compressed air so that repeated sampling of Streptomyces over a fermentation period of up to ten days was no problem.

Filtration in µL-scale

In order to separate the cells and the supernatant from each other a filtration module based on conventional technology was developed by IBA. It is thus appropriate for usual sterilisation procedures like sterilisation in an autoclave. Using channel spacers having different heights it is possible to apply the filtration module for taking 20 µL samples as well as for 50 µL samples. It is possible to use filters made of ceramics, polymers or paper. As the filtration element an asymmetric polyethersulfone membrane was chosen for yeast and an asymmetric ceramic filter for Streptomyces. A purging procedure was developed to clean the filter between the subsequent samplings. As the module is connected to the bioreactor by means of a sterile barrier downstream the bioreactor, it is also possible to exchange the filter without danger of contaminating the fermentation broth in case the filter is blocked.

With the conventional sampling and filtration system three different kinds of samples could be generated: (i) a raw sample, (ii) a cell-free supernatant and (iii) a sample with cells which are resuspended in buffer. Because most of the streptomytes secrete their products in the supernatant, the last kind of sample was not generated in this case.

Integrated Braille-actuated fluid management

A microfluidic chip was developed as an optional component to replace part of the tubing-based fluidic network of the sampling module. In order to achieve accurate fluid switching and low-volume pumping in nL- and µL-scale with minimal external components, an on-chip valving technique using actuation with a Braille display was developed. In this technique a thin membrane is deformed by the actuation of a Braille pin, resulting in complete blockage of the microchannel.

The microfabrication process of the PDMS fluidic structure with a thin deformable membrane was established by IMTEK. Test experiments for the valving function was successful with water up to flow rates exceeding the range of interest in the NANOBE project, proving the effectiveness of the developed technique for implementation in the sampling module. Additionally, a sequential actuation of multiple valve elements could be successfully used for fluid pumping. The quasi-peristaltic motion of the fluidic structure resulted in a flow rate of up to 6µL/min.

In the final NANOBE demonstrator the role of the microfluidic chip developed by IMTEK was to perform the splitting of the raw sample in two parts, one for cell counting and the other for filtration, and for continuously dilution of the one part of the raw sample to guarantee a fast and accurate mixing. A fluidic structure was designed and tested to intermittently extract small volumes (100 - 500nL) from a continuously flowing stream. Also, a dilution structure was developed utilizing Braille-actuated active mixing of two continuously flowing streams.

The microfluidic chip for sample splitting and dilution was successfully tested with cell sample. The chip fulfilled its function up to volume ratios of 1:20, both for splitting and dilution using buffer.

In situ analysis of pH, pCO2 and pO2

A further challenge for the sampling probe development was the in situ analysis of pH, pCO2 and pO2. As the existing pH sensor was not suitable for yeast culture due to its limited dynamic range of 5.5 to 8.5, PreSens had to develop a new pH sensor to cover the more acidic pH range from 3 to 5.5. For pCO2 a sensor was developed which covers typical dynamic ranges (1 - 25%) for yeast fermentations. The pre-existing DO sensor was suited also for this type of fermentations, dynamic range 0.02 - 100 % oxygen.

Sensors for all three analytes were never applied in the same fermentation before. Therefore a common sterilization method for all three sensor types had to be developed by PreSens. For the first time an autoclavable pH sensors was demonstrated. The autoclaving conditions were determined by the pCO2 sensor which was more critical than the other two sensors. Autoclaving in the absence of oxygen (ensured by fractionated autoclaving) is required.

Many cleaning procedures were investigated but none was compatible with all three sensors. Therefore a disposable concept had to be developed. The spots were integrated onto polycarbonate discs which could be mounted to the probe head and exchanged after the fermentation.

Integrated sensor and sampling probe

A functional conventional sampling probe for a standard port of 12 mm diameter with three integrated optical sensors for gas/pH monitoring were constructed and manufactured by iba. This is reducing the amount of ports needed in the bioreactor, saves time during assembly and reduces the risk of contamination by reducing amount of components. Optical fibres used to read the optical sensor spots have to be removed before autoclaving and can be easily integrated in the sampling probe after autoclaving.

The construction of the sampling probe was optimized for a usage with any bioreactor independently of the position of the agitation motor by separating the sterile and overpressure barrier from the probe. A tube squeezing valve protects the further downstream modules from high pressure inside the bioreactor during cultivation, and from contamination. The sampling and filtration system can be easily coupled sterile with the sampling probe via this valve. The newly developed probe-head adapter guarantees bubble-free sampling from the fermentation broth without any influence on the accuracy of the sensor measurements. As the raw sample is embedded into air plugs, samples can actually be withdrawn without dead-volume. The volume of the sample can be easily adapted from 10 µL to a few hundreds of microliters if necessary.

Testing of the sampling probe in a lab scale process

The sampling probe with its integrated optical sensors was tested in a lab scale process of yeast at iba regarding to its functionality, biofouling effects, and reproducibility in comparison to a conventional tubular probe as sampling system.

The sampling procedure in the NANOBE setup lasts approximately six and a half minutes. The volume of the sample was determined by two methods: i) measuring the sample length in the tube directly after withdrawing and ii) weighting the sample after pumping the sample through the tube

squeezing valve. With both methods a good reproducibility of sample volume was determined. Additionally, there was almost the same standard deviation (sample length 3.1 %, sample weight 2.9 %). The average volume of the sample directly after its withdrawal was 19.4 µL. As the average volume of the sample in the Eppendorf cup was 20.2 µL the sample volume increased during the transport through the tubing and the valves of 3% on average.

The cell counting was performed with repeat determination by means of a Neubauer counting chamber. During four days of fermentation the cell count was determined. During the first 28 hours the cell counts of both sampling probes were almost the same (difference between the two probes only varied 4% on average). In the next days the differences of the cell counts of both probes first increased, then adjusted to each other before the differences increased again (to 15.5% on average). Therefore it was not a cumulative effect. The observed sample volume increase of 3% within the tube cannot result in such an increase or dilution of the sample, which would explain the differences in the cell counting between the two sampling probes. But it indicates an attachment at the inner sides of the tubings. Adhesion and biofouling is always a topic in microfluidic devices, but only some adhesion on the two-capillary probe was observed. Additionally, the different tubing materials, and the tubing diameters together with the flow rates of the samples could influence the sample content. However, the cell counting method itself showed a high standard deviation, so that the differences are only speculative. Only further investigations could finally explain it.

Cell handling tools

In the NANOBE project the cell handling tools were developed to provide information about the concentration and physiological state of the production organism and to perform cell lysis as well as sample handling. Both Ecole Polytechnique Fédérale de Lausanne (EPFL), in Switzerland, and Centre National de la Recherche Scientifique (CNRS), situated in Lille, France, participated in the development of the cell handling tools.

Cell sorting and counting

Cell sorting and counting devices were developed by EPFL. These devices were designated for processing of samples with cells extracted from the bioreactor by means of the sample probe. The main components are cell sorting by dielectrophoresis and cell counting by impedance measurements. The sorting and counting module allows the separation of living and dead yeast cells and the counting of the separated populations. The viability of the sample is obtained by dividing the number of living cells counted by the total number of counted cells. The cell density is obtained by dividing the number of counted cells by the sample volume.

For yeast cells, both sorting and counting channels were 20µm wide and 20µm high. Using the appropriate voltages and frequencies, living and dead yeast cells could be separated and collected in two different channels [G. Mernier al., Sens. and Act. B (2009), doi: 10.1016/j.snb.2009.11.066].

After they are sorted, the cells flow through microelectrodes arranged in coulter counter configuration. The results provided by the EPFL device were compared with traditional viability measurements using a Trypan blue exclusion assay on a glass slide. The device was able to measure the viability of biological samples such as yeast cells.

The connections of the sorting/counting chip were adapted to be able to connect the device to the other NANOBE subsystems with tubing. The tubing of small inner diameter was used and the tubings were arranged so that the sedimentation at the inlet and outlet of the chip and the dead volumes were reduced.

The remaining dead volume of a few microliters still represented a long transit time at the speed of the sorting system (10-20nl/min). Therefore, EPFL developed a pressure regulated as well as a switchable bypass which allowed the sample to pass faster through the tubing, and only a fraction of the sample was analysed with the sorting/counting chip. The switchable bypass was successfully tested with a sample of yeast cells and suitable values of the order of 1 µl/min for the flow entering the sorting/counting system.

During the last year of the project the cell analyser module was reworked to use impedance spectroscopy instead of cell sorting, in order to reduce complexity and improve reliability. Parameters for counting as well as density and viability measurements were adapted for use with NANOBE in order to enable the use of higher flow rates. The result was good, the new system allowed for considerably higher flow rates of around 1 µl/min without use of a bypass.

Cell lysis

Cell lysis is used to break the cell membrane in order to extract intracellular components for analysis. Both chemical and electrical lysis have been investigated in the NANOBE project, although electrical lysis is preferred because components present in the chemical lysing solution could lead to fouling of the downstream analysis. Cell lysis was investigated by both EPFL and CNRS. EPFL focused on electrical lysis whereas CNRS worked both on electrical and chemical lysis.

Chemical lysis

CNRS studied the chemical lysis of Saccharomyces sereviciae yeast cells. First, CNRS determined with Cellytic from Sigma Aldrich if chemical lysis would be compatible with the technology used in the sample transceiver device (i.e. electrowetting on dielectric; described later in this report) and with the NANOBE specifications. The experiments showed that i) the minimal reaction time for the lysis of 80% of the cells is 15 minutes, ii) for a 10-fold dilution, the lysis efficiency after 30 minutes drops below 50%, and iii) the contact angle of Cellytic on Cytop is very low, even after 100-fold dilution, probably because it contains surfactants. Moreover it was not possible to displace more than a 1000-fold dilution. Based on these results, it was seen impossible to integrate chemical lysis in the sample transceiver platform.

Electrical lysis

Electrical lysis makes use of high electrical fields to disrupt the cell membrane. Higher electrical fields yield better lysis efficiency, but can also lead to the formation of bubbles at the microelectrode surface. An alternating voltage at a frequency of 50 kHz was used by EPFL to reduce this effect while still achieving efficient cell lysis.

Specific microchannel designs can be optimised to locally concentrate the electrical fields in the lysis regions. Three possible designs can be used for electrical field concentration: electrical field applied across or along the flow, or using the 'liquid electrodes' design.

These three designs have been evaluated by EPFL using red blood cells, as the lysis of those cells is easily observable optically by the diffusion of haemoglobin out of the cell. The third design with so-called 'liquid electrodes' (planar electrodes placed in dead-end channels perpendicular to the main channel) was chosen for the NANOBE project. In this design the current density and thus the risk of electrochemical reactions is reduced. In addition the electrical field is uniform on the side of the main channel and the length of the lysis region and the transit time of the cells is largest of these three options without reducing the electrical field. This leads to better lysis efficiency and higher throughput.

Two different flow cytometry methods were used to evaluate the lysis efficiency with yeast cells: cell sorting by dielectrophoresis [G. Mernier et al., Lab Chip, vol. 10, pp 2077 (2010)] and multiple-frequency impedance measurements [G. Mernier et al., Sens.Act. B, doi: 10.1016/j.snb.2010.10.050, (2010)]. At a flow rate of 8nl/min, the lysis efficiency achieved was 99%. At a flow rate of 120nl/min the sorting was less efficient but the effect of the lysis voltage was still very clear at 100 Vpp. Overall, the lysis with the EPFL chip was possible with high cell concentrations (up to 2x108 cells/ml) at least up to 120nl/min.

According to the NANOBE project specifications, the cell lysis chip will receive samples with cell concentrations between 3.3x106 and 1.8x108cells/ml at a flow rate of 1.5µl/min. Therefore, the lysis system developed by EPFL was evaluated also at high flow rates. Based on the first results, a chip with a longer lysis region was developed and tested. Living yeast cells, suspended in 30x diluted PBS were exposed to 56Vpp (50 kHz) at low flow rates (several nl/min) and the impedance was measured in coulter counter configuration to verify the efficiency of the lysis. The measurements indicated that the intracellular components were successfully extracted from the cells. However, an effective lysis of the cells at higher flow rates (from 300nl/min on) required the application of higher voltages (100V) which resulted in bubble creation at the electrodes and clogging of the channel. The long term operation of the device was thus impossible and the device was not suitable for the NANOBE project purposes.

To overcome the bubbling and clogging problems, EPFL adopted a new technology based on 3D carbon electrodes. EPFL demonstrated high lysis efficiency (90%) with high flow rate (up to 50ul/min) while the lower flow rates (below 20µl/min) tended to show slow bubble creation and cell sedimentation inside the microchannel. Despite of the good lysis results with optimised operation the EPFL device was not well compatible with the NANOBE system specification and suffered reliability problems.

CNRS had developed parallel electrical lysis technologies in the NANOBE project. The lysis device was made of conductive silicon layer sandwiched between two isolating glass slides. Silicon layer was etched to form microfluidic paths, and electric field could be applied between the silicon channel walls. These three-dimensional electrodes allow constant electrical field along the full channel height. The chip was powered by a high AC voltage to enable disruption of the tough yeast cell envelope without bubble formation.

Two lysis regions are incorporated in the microchannel. The applied voltage across the channel is sinusoidal with peak-to-peak amplitude of 70V at 200kHz frequency. The fluid flow rate can be up to

2µl/min. The liquid buffer used can be 300-times to 30-times diluted PBS. Cell concentrations in the chip should be 2.107cells/ml at maximum (lysis efficiency 92%) since the higher concentrations decrease significantly the lysis efficiency. This matches with the highest cell concentrations (up to 2.108cells/ml before dilution) in the selected NANOBE application.

Based on these results the CNRS lysis chip was selected to be implemented to the integrated NANOBE analysis system. In order to reach the specifications of the NANOBE project, the flow rate in the lysis chip needed to be 10µl/min. Since lysis yield decreases with increasing flow rate, the throughput issue was solved by fabricating a device with five parallel lysis channels. The flow rate in each channel was set to 2µl/min and the required total flow rate of 10µl/min was reached.

Filtration

Three filtration systems were required in the NANOBE workflow:

1. A filter after cell lysis, removing the cell debris from the lysate
2. A filter before EPFL density/viability chip to remove dust but letting cells flow through
3. A micro-debubbler to avoid air entering into the density/viability chip

EPFL tested commercial filters for the removal of cell lysis debris as well as for protection of the cell counter chip. The commercial inline filters from IDEX were found to be suited for the removing of the cell debris from the lysate (filter pore size 1 µm) and for the removal of dust (filter pore size 10 µm).

Moreover, a micro-debubbler [H. van Lintel et al., Micromachines, vol. 3, no 2, p. 218-224 (2012)] was developed and tested to be put just in front of the cell counter. The micro-debubbler was constructed from a filter piece with glued-on tubes. The debubbler channel was made by drilling a 5 mm long, 0.25 mm diameter hole through the filter material (Tracetrap® PTFE). In the NANOBE assembly it was positioned vertically, just above the chip inlet. The micro-debubbler was tested for a variety of pressures and flow rates and it was found that air bubbles of 10µl were reliably removed at the typical NANOBE flow rates of < 10µl/min. The micro-debubbler was found to be suited for the removal of the air between samples, as well as smaller bubbles.

Sample transceiver

A special sample transceiver was developed by CNRS to perform the delivery of the supernatant or lysed cell samples delivered by the sample probe to the individual analysis modules.

The sample transceiver module uses electrowetting on dielectric (EWOD) technology to manipulate and deliver samples to the analysis system. The liquids are provided and delivered to/from the sample transceiver through micro-channels. In the sample transceiver the incoming liquid is digitised to small droplets (volume 1µl) and all the operations in the sample transceiver are made by moving droplets in the central area of the device. Various bio-chemical operations can be done in digital fluidic mode. At the output end the droplets are combined to specified sample reservoir area from where they can be pumped onwards to the analysis tools downstream the NANOBE system via capillary connections.

The functionalities available in the sample transceiver are droplet generation, sample mixing/diluting and sample exportation.

The sample transceiver was tested with cell lysate (with different cell concentration), supernatants corresponding to different cells cultures, the buffer used for CE analysis and PBS with different concentrations. All these fluids were to be used in the NANOBE application. Based on these tests CNRS defined the conditions on these fluids to be manipulated by electrowetting in the sample transceiver. The surface tension and bio contents of the droplets were the key parameters to enable the manipulation of these liquids. It was observed that too low surface tension or too high bio-concentration of the droplets made the droplet displacement difficult or even impossible. The results showed that the movement feasibility of the supernatant in YNB is better than the one in YPD (10 time dilution and 100 time dilution, respectively). For cell in buffer , the two solutions both need to be worked by 100 time dilution. A wide range feasibility of cell lysate and CE buffer were observed. The results confirmed that with a moderate dilution with water it was possible to handle all the fluids needed for NANOBE application.

As a result of the project, the sample transceiver was able to take in the required 20µl sample with a flow rate of 10µl/min and divide this sample into1µl droplets, move them along the predefined path droplet by droplet, dilute the sample aimed for the CE with CE buffer, and pass the samples to the analysing devices (ELISA and CE) downstream the system. All operation could be done within 5 minutes in agreement with the initial specifications. Its major limitation is the bio-pollution of the internal surfaces. This pollution induces a drift in the wettability properties of the surface and is the cause of reliability problems for long term (few hours) use of the device.

Micro-ELISA analysis of proteins

The NANOBE system has two analysis paths for intra- and extracellular compounds. For the first one, DiagnoSwiss S.A. (DiagnoSwiss) has adapted their micro-ELISA technology to automated immunoassays and to on-line process monitoring of extracellular product protein concentrations. Furthermore, DiagnoSwiss has evaluated the feasibility of mRNA level determination using magnetic beads and electrochemical detection in their ImmuSpeed platform.

ImmuSpeed platform

During the first period of the project, DiagnoSwiss designed and fabricated of an automated instrument adapted to the processing of automated ELISA tests in DiagnoSwiss electrochemical microchips. The microfluidic bead-based ELISA platform, dubbed ImmuSpeed, comprises four plate positions (for the samples, reagents, beads and dilution or mixing of the complex) that are processed sequentially with eight pipetting heads. The pipetting heads process one row of eight wells in parallel and are displaceable in the x direction to allow transfer of solution from one plate position to the other and to the chip. The software was further developed in order to better control the fluid handling and the operation of the robotic station. A new detection procedure was implemented to optimise the electrochemical measurements, and data processing software was developed in order to extract the slope of the measured currents vs. time curves and transform them into concentration values. The software comprises curve fitting calculations and enables to process up to 96 detections.

The feasibility of running bead-based assay with electrochemical detection in microchips was demonstrated by adapting biotin-ALP test to the new features of the platform. The obtained results showed that a dynamic range of more than 3 orders of magnitudes can be obtained with the platform, for a sensitivity limit relying in the ng/ml range and with a good reproducibility (below 5% CV).

Magnetic beads functionalised with protein A was also used for the development of an assay for the dosage of immunoglobulin G (IgG). For all the tests, the developed protocol used magnetic beads functionalized with protein A (Biolabs) and diluted to a concentration of 0.3 mg/mL, which was used as solid phase to capture the IgG molecules. The enzyme-labelled conjugate was a goat anti-human IgG (ALP-conjugated affinipure goat anti-human IgG F(ab')2 fragment specific from Jackson Immuno Research) which was diluted 500 times in PBS buffer pH 7.4, and the standards were prepared from a stock solution of 11.2 mg/mL of human IgG (ChromPure Human IgG whole molecule from Jackson Immuno Research).

The results showed that the developed assay protocol was well adapted for the detection of IgG in this dynamic range. The entire assay time (incubation, detection and regeneration) with the optimised protocol for the final NANOBE application takes 40 minutes and delivers a series of eight data points in that time (two serial samples in duplicates for both supernatant and cell lysate).

Here, IgG and ALP were used as tools for the system development. They are not relevant analytes in the Saccharomyces or Streptomyces based bioprocess monitoring since they are not products of yeast. They were yet used for the characterisation of the coupling of the micro Elisa instrumentation in the NANOBE system.

To test the system before integrating it with the full NANOBE system, sequential assay procedure was mimicked at DiagnoSwiss facilities. Repeated experiments were conducted using three yeast culture media (namely YNB, medium taken 3 hours after inoculation and medium taken at the end of the fermentation). The obtained results showed good calibration of the system for the three media, but a repeatability and a reproducibility varying from 10% to 25%. These results were less accurate than those obtained in buffer solution (where the CV remains below 15%), which was likely to be due to matrix effect and non-specific adsorption in the medium, which generated more variations from one experiment to the other. These results were yet in good agreement with what was expected and what can be obtained with conventional ELISA test in microtiterplate and optical detection.

Coupling with the sample transceiver

The specifications for the coupling of the micro-ELISA platform to the sample transceiver were set according to the fluidic layout of the integrated system, the unit operations of the sample transceiver, and of the micro-Elisa platform and the related timings of the analysis cycles. For the immunoassays, the total analysis cycle was set to 40 minutes, with the sample being sent from the transceiver to the ELISA module every 20 minutes, but with 2 samples in duplicates being analyzed simultaneously with a 40-minute cycle.

The coupling system consisted of a peristaltic pump connected to the sample transceiver in order to transfer the samples in wells of a sample collector and placed in the micro-Elisa platform. The robotic head of the micro-Elisa platform will then transfer the samples into the microtiter-plate placed in the instrument for incubation with the capture antibody and then further analysis by electrochemical detection in the microchip. As the platform is designed to process one 96-well plate automatically, the

system will be stopped after 96 detections (i.e. after max. 24 series of samples) in order to wash the microchip and place new plates and reagents in the platform.

Adaptation for the mRNA analysis

Intracellular mRNA is transcribed from the genes of the organisms. mRNA is in turn translated to proteins most of which maturate to active enzymes. Regulation of this information chain can be controlled at different levels, but the first level is to decide whether a gene is transcribed to mRNA or not. In the selected application example for lactic acid production the aim was to follow expression of 3-6 genes to their respective mRNA so that they relate to measured enzyme activities. The genes of interest were ldhL encoding for lactate dehydrogenase activity, PYK1 (CDC19) and PYK2 encoding for pyruvate kinase activity, and HXK1, HXK2 and GLK1 encoding for hexokinase activity.

In the NANOBE system, investigations were made to evaluate whether expressed mRNA levels of the selected genes can be measured using DiagnoSwiss ImmuSpeed device developed primarily for sandwich assay of proteins, and see whether it could be adapted also for measuring the expression of mRNA molecules.

The development of HXK1 mRNA test with electrochemical detection in microchips was pursued by DiagnoSwiss in order to optimize the assay procedure, determine the limit of detection that can be reached and assess the applicability of the method in yeast culture media. The assay procedure was optimized by modifying the hybridization time, the amount of beads functionalized with the capture probe and the concentration of the detection probe. These investigations showed a limit of detection of 0.4fmol/µl in solution (i.e. 2fmol or 1.2 109 HXK1 molecules) with this microchip approach, which is similar to Plexpress sandwich approach [J. Rautio, Microb. Cell Fact., 2003, 2, 10.1186]. Application to yeast culture media showed a sensitivity of 3.1 in YBN and 6.3fmol/µL in medium taken at the end of the fermentation, yet with a relatively low reproducibility since CV varied between 10% and 40%. Spiking experiments also showed that the measured concentrations were lower than the expected theoretical concentration.

These studies demonstrated the feasibility of transposing the TRAC methodology to the electrochemical micro-chip format and that a reliable assay protocol could be developed for HXK1 mRNA detection. However, the obtained results were only semi-quantitative and, in its current state, the assay performances did not show sufficient sensitivity and reproducibility for direct application to on-line monitoring of yeast cultures. Further efforts should be placed in order to investigate whether the assay performances can be further improved to reach the very low detection limit required for real applications.

CE-separation of analytes

The second analysis path of the NANOBE real-time monitoring tool was a coupled capillary electrophoresis - mass spectrometer (CE-MS) system for measurement of small molecules, such as substrates, metabolic products and intermediate biomarker metabolites.

Originally the capillary electrophoresis (CE) was planned to be applied in multiple ways in the NANOBE project to analyse metabolic compounds, nucleic acids, and fluorescent cells. It could also be used for the detection of intracellular enzyme activities. In yeast fermentation the most important

analytes are extracellular sugars (especially glucose and xylose) and organic acids (lactic, succinic and xylonic acid). In Streptomyces SPP fermentation the most important analytes are polyketides rapamycine, nigericine and/or tacrolimus depending on the strain. The design of the on-line analysis system was such that the (above mentioned) analytes in the cultivation broth can be quantified in concentration range 100 mg/L - 100 g/L. The required sample volume from the sample transceiver to the CE is 1 µl.

During the project VTT designed, fabricated and analysed CE chips with and without mass spectrometer (MS) connectivity option. Laser induced fluorescence (LIF) was used as a detection method in the case of CE chips without the MS option while the MS was naturally the choice for detection in the case of other chip design.

The CE chips were made with silicon based technologies. The microfluidic channels were etched in silicon and insulated electrically with a thick thermal oxide. Silicon based microfluidic structures were then closed with a glass lid. The glass lid included platinum electrodes for conductivity detection and initially also for the high voltage (HV) connection. The electrode side of the glass lid was glue bonded on the silicon chip to close the microfluidic structure. Fluidic and electrical connections to the CE chip went through the silicon.

In the microchip CE designs the separation channel dimensions were set to 75 x 75 µm2. Separation channel lengts were either about 3 cm or about 9 cm. The sample injection to the CE was via a small injection loop in the separation channel. The sample loop length varied between 100 to 500 µm (between 0.5 to 3 nl) for the CE-LIF microchips and between 500-3000 mm (between 3 to 17 nl) for the CE-MS microchips. Conductivity detection had two parallel platinum electrodes across the fluidic channel. The electrode spacing was 50 µm.

The silicon microfluidic channel was electrically insulated using thermal oxide. On the plane surface the thermal oxide can tolerate over 1 kV/µm electrical field strength without a breakthrough. Based on this, the thermal oxide thickness was selected to be 2 µm. This would allow high voltage (HV) of 2kV to be applied over the CE separation channel.

The CE microchip was connected to the MS with a capillary connection between the CE separation channel and the MS ionization chip. On-chip valve structures were used to enable more accurate sample injection to the CE. In one chip designs an incubation chamber was also included prior to the CE channel to allow sample preparation steps before the CE analysis.

Two measurement platforms were fabricated in the NANOBE project to connect the CE microchips with external fluidics and to control the CE analysis. The first platform was developed for the CE-LIF chips. It had chip connection block where the CE-LIF could be easily replaced when necessary (fast connections to fluidics and electronics); external fluidic components (pumps, valves, tubings etc.) that were used to filling the chip with the background electrolyte (BGE) solution, injecting the sample into the chip and the sample loop, and washing the chip between the analyses; HV source connected to the CE chip with external electrodes; conductivity detectors to monitor the sample flow in the channels; and laser induced fluorescence measurement system (laser source and photomultiplier tube detector) to measure the separation efficiency. The whole system was controlled with a single LabView program.

The second measurement platform was for the CE-MS chip and targeted to be used in the on-line monitoring as a part of the total integrated NANOBE system. It had a chip connection block; external fluidic components (pumps, valves, tubings etc.); multiport valves before and after the chip to control

time-wise the sample intake from the sample transceiver and sample output to the MS; HV source connected to the CE chip with external electrodes and conductivity detectors to monitor the sample flow in the channels. Here, both pressure controllers and flow controllers were used to balance the fluidic flows inside the chip and the pressure difference across the separation channel. The whole system was controlled with a single LabView program and the system was capable for continuous on-line operation.

The CE-LIF method was aimed for detection of intracellular metabolites (glucose, lactic acid, pyruvic acid, phosphoenolpyruvate, glucose 6-phosphate) and mRNA. Oregon green and Fluorescein fluorescent staining was used in testing the CE-LIF setup and for analysis of metabolites.

During the testing of the CE-LIF fundamental issues were observed. The HV electrodes that were initially located on the chip (platinum electrodes) eroded during a longer period of operating with the chip. Therefore, external electrodes were set up which increased the distance between the electrodes and reduced the voltage per distance ratio in the CE separation channel. The reduction of the voltage per distance ratio had an effect on the separation efficiency since the electric field in the CE separation channel was now weaker.

It appeared also that the silicon chips were not able to tolerate the required high voltages. If the applied voltage is too low, then there will be no separation of analytes which makes this issue quite severe in consequences. Therefore, much effort was put to improve the quality of the silicon oxide layers, but the problem could not be fully solved. The CE chips functioned unreliably at high voltages. The best measured reproducible value was 600 V over a 2 µm oxide layer. This value corresponds to about 160V/cm electric field strength in the 3cm long CE separation channel.

Measurements show good repeatability and the measurement with different HV settings show reducing analysis time with increasing HV. However, the voltage per distance ratio is now too low to separate the two dyes efficiently from each other.

The measurement platform for the CE-MS microchip was installed to the integrated NANOBE on-line monitoring tool. Even though the CE separation was not efficient in the developed microchip it could, however, to be used for controlling and timing the sample injection accurately due to the conductivity detecting capabilities. This was of importance since the 1µl droplet received from the sample transceiver should not be lost. The CE in this case was done outside the chip in a glass capillary before the MS.

MS-detection of analytes

Microsaic Systems Ltd (Microsaic) developed further its micro-fabricated mass spectrometer technology to be integrated with VTT s chip based CE technology. The objective was to develop the first fully micro-fabricated CE-MS system and to provide online detection and definitive identification of analytes separated by the CE module. In the NANOBE system MS detection is used to analyse extracellular substrates and metabolites.

A stand-alone Spraychip® nanospray module and controller was designed, built and tested by Microsaic. The development of negative ionisation mode was completed before delivering the module to VTT for testing.

VTT successfully completed initial trials of the nanospray unit interfaced to one of their commercial mass spectrometers. D-glucose was injected at the concentration of 50ng/nL which yielded 10nmol of analyte for quantification. The injections were performed at 3.03, 17.22 and 33.91 minutes. The peaks had nice shapes even without processing.

The Spraychip® nanospray module was interfaced to a chip scale breadboard atmospheric interface mass spectrometer developed by Microsaic Systems and the functionality of the integrated system was successfully demonstrated. Following the initial breadboard trials, Microsaic completed the design and development necessary to integrate the elements fully into a more compact and stand-alone nanospray ionisation mass spectrometer module. This integrated MS module was tested at Microsaic Systems facility and the performance optimised ready for application testing.

Extensive testing was done by Microsaic for the spectrometer system prior to the delivery of the MS to VTT. Microsaic performed limit of detection (LOD) testing in both positive and negative ionisation mode. Results were obtained for D-glucose 6 phosphate and Lactic acid by loop injection using SIM mode on the MS system. A limit of detection of 1E-4mg/ml and LOQ of 3.5E-4 mg/ml were obtained. Issues were seen with linearity at very low concentrations. Further tests were planned using an HPLC rather than the loop injection. It was also found that the high concentration buffers were reducing the nanospray performance and causing crystal growth in the ESI source. Non-buffered make-up flow of methanol/water (+FA) gave better results.

Sample capture for storage and off-line analysis

A sample capture for off-line surface assisted laser desorption/ionization - desorption ionization on silicon surface (SALDI-DIOS) analysis was developed at CNRS. In NANOBE, the DIOS chip was planned to be used to store and analyze off-line both extra- and intracellular metabolites and products in Saccharomyces cerevisiae and Streptomyces fermentations. In yeast fermentation the most important analytes are extracellular sugars. In Streptomyces fermentation the most important analytes are rapamycine, nigericine and/or tacrolimus depending on the strain. Limit of detection of these compounds is in the range of nmol-pmol/µL.

The DIOS device is based on the same technology as developed for the sample transceiver. In the literature, DIOS is a technique that has been validated for a wide range of low molecular weight biomolecules (< 3000 Da). It is suitable for the analysis of small molecules such as peptides, carbohydrates, drugs etc. Above this mass, molecules such as proteins cannot be physically ionized and desorbed. Furthermore, these results have shown that, according to the experimental conditions (such as material and liquids), detection limits below femtomolar concentrations can be obtained. It is to be noted that these limits correspond to the use of pure liquids containing only few molecules of interest. In order to reach these experimental conditions, a cell-lysate filter has been inserted in the initial set up for the SALDI chip.

CNRS evaluated possible substrate candidates for the DIOS analysis. The diamond nanowires upon chemical functionalization with low surface energy molecules display a superhydrophobic character with low hysteresis (like silicon nanowires). Furthermore, the presence of functional reactive groups on their surface opens avenues for developing new strategies for surface functionalization. Finally, diamond nanowires showed a very high sensitivity (200 zeptomole for verapamil) for then desorption/ionization of small molecules, including sugars, steroids, peptides, etc.

CNRS evaluated also the ion affinity chromatography (IMAC) technique for functionalization of the nanowires. Indeed, for the DIOS analysis, a given analyte has to be transferred from the droplet to the surface and this can be achieved by a right functionnalization of the nanowires. CNRS has shown that peptides modified with a histine tag can be easily separated from an artificial mixture of his-tagged and untagged peptides and their subsequent mass spectrometry analysis with a high signal to noise ratio.

The superomniphobic surfaces developed by CNRS allow handling of droplets having a low surface tension due to either surfactant or some biomolecules. It was shown that depending on their morphology, the silicon nanowires offered liquid-repellent character with very good robustness over a wide range of surface energy.

Towards the end of the project CNRS studied the interaction of graphene oxide and reduced graphene oxide with bovine serum albumin (BSA). The results suggested that graphene oxide has a high capacity for protein adsorption. This property was finally exploited for the displacement of BSA using EWOD with minimum non-specific adsorption.

The SALDI-DIOS was not part of the final system demonstrator.

System integration

Finally, the complete NANOBE system was developed in form of several stand-alone units that were physically integrated to a complete system. All partners were involved in defining the detailed specifications of all modules and their interfaces while IMTEK was responsible for the collection and management of the system specifications.

The NANOBE system was constructed on the basis of the commonly agreed structure of the system and the function of the individual modules.

The planned full setup occupied an area of 2 x 2m including the bioreactor and its peripheries. Most of the required space was occupied by auxiliary components such as pumps, valves or electrical power supplies.

All system modules were transported to VTT for the system construction. All system modules were installed according to the planned physical layout, and it was checked that all modules operate standalone without flaws. The virtual system control software (Labview) controlled the individual analysis and core system modules. Before final system testing the subunit connections were tested to verify the sample transport between different submodules.

The following modifications to the complete NANOBE system had to be made based on the results of the submodule interface testing:

1. Due to software communication problems with the impedance spectrometer and some issues with the sampling probe, no cell viability measurements could be performed simultaneously with the other components of the system.

2. The high-pressure resistance of the silica capillary connections of the cell lysis chip and the limited pressure tolerance of the microvalves used for flow switching prevented the use of the cell lysis chip in the online monitoring system for the final testing. This is a result of a long

capillary connection between the modules that was not planned at the beginning of the project.

3. The supernatant dilution ratio was necessary to be increased to 1:50 from the original 1:10 to avoid the damaging of the sample transceiver electrodes.

Testing of the NANOBE system

The genetically modified yeast cell strain of VTT was cultivated in the bioreactor and used for interface development and integrated system testing purposes. During the testing the pH and oxygen concentration was monitored with conventional sensors for comparison with the PreSens optical sensors. Cell samples were manually sampled with a syringe at the same time as the automated sampling at the IBA sampling probe. Off-line analysis of the manually-taken samples were performed using the CE-MS and ELISA modules and with off-line HPLC for comparison.

Integrated NANOBE system

As explained above cell viability analysis was not performed although diluted raw sample was delivered through the cell counting chip. Also, the pH and CO_2 sensors did not give reliable values due to defects in the optical fibre and suspected displacement of the sensor spots at the tip of the sampling probe. The O_2 sensor continuously monitored the concentration at an interval of 10 seconds.

The software triggering of the sample transceiver and CE modules were performed manually. At the sample transceiver, the arrival of the sample liquid front was monitored with the eye and the start signal for transceiver control was initiated by hand. At the CE module, the controlling software was triggered in the same way by hand when the sample droplet exited the transceiver into the tubing at the CE peristaltic pump.

Despite the original plan of completing the online analysis cycle in 25 minutes with a 20-minute cycle, the finally tested analysis cycle turned out to require 80 minutes. In the course of the timing and flow rate adjustments during the interface development, all process steps at which the sample or washing liquid is delivered into external microfluidic chips needed to be prolonged considerably. The resulting online analysis sequence was such that the sample was delivered to the transceiver approximately 45 minutes after automated sampling. The time dedicated to the sample preparation in the sample transceiver was around 6 minutes. The CE-MS analysis and ELISA measurement of the supernatant would finish at 60 minutes and 100 minutes after sampling, respectively. The diluted raw sample reached the cell counting chip approximately 15 minutes after sampling, although the impedance measurements and viability analysis were not performed online.

In total, only four analysis cycles were carried out with the integrated system. There were two cycles in which the sample was successfully transported from the bioreactor to the ELISA and MS modules. One out of the two successful system operation cycles resulted in successful data readout in both the ELISA and MS modules. The results from the ELISA analysis suggest that the diluted supernatant from IBA sampling/filtration module may have been more concentrated than the theoretical 1:50. If this could be confirmed, it can explain the difficulty to transport the sample in the sample transceiver. The CE-MS analysis results were qualitatively in good agreement with data from the manually-taken sample using the same Microsaic mass spectrometer. The monitored dissolved oxygen concentrations

were in good agreement with the values measured using conventional sensor. The oxygen monitoring continued for two days without major drift.

The failure modes in the three unsuccessful runs were all different. In cycle 1, the sample reached the inlet of the mass spectrometer but did not reach the detector due to clogging of the vacuum interface. The ELISA module also failed to proceed beyond the sample/bead conjugate incubation due to software failure on the master PC side. In cycle 2, several electrodes on the sample transceiver was damaged due to repeated use from interface development to sample testing. In cycle 4, an unexpectedly viscous sample was transported from the IBA sampling/filtration module to the sample transceiver which indicates that the supernatant dilution of 1:50 ratio was not achieved, most probably because of air bubble generation at one fluidic junction. This is also an explanation for the discrepancy between the IgG concentration in the online sample and manually-taken sample in the ELISA measurements.

In summary, the complexicity of the whole NANOBE system caused several irregularities in the sample transport, which could not finally eliminated. The reason for this is that the development of all modules had to be finished before the interfaces could be developed. It was tried to adjust the interfaces with the state of development of the modules, but this was time consuming and expensive, so that it could not always be done.

Testing of an Alternative integrated System Configuration

Online operation of the integrated system was also tested. In this configuration, the CE module was directly connected to the supernatant outlet of the sampling/filtration module. This is one variant of the system for applications that do not require ELISA measurements. Since there is no need of sample distribution to multiple measurement paths, the sample transceiver is also omitted.

The delivered volume and the dilution ratio of the supernatant were kept at 20 µL and 1:50, respectively. A fresh batch of cells were cultivated in the bioreactor, but without IgG spiking since the ELISA module was not included in the system. A total of three sampling events and system operation cycles were performed.

The outcome of the alternative configuration test was similar to that in the full system test - one set of measurement data was obtained in three test cycles. One failure mode was the clogging at the vacuum interface of the mass spectrometer, and the other was leakage at one fluidic connection between the sampling/filtration module and the CE module.

Results on IgG determination

For the two samples delivered by the sample transceiver, only one sample resulted in an electrochemical signal corresponding to an enzymatic reaction. The row detection signals and the calculation of the effective IgG concentration in the various processed samples, the second signal gave a negative slope corresponding to a blank point. The measurable data point corresponds to an IgG concentration of 5.2µg/mL. This result can be explained either by the lack of control of the dilution (supposed to be set at 1:50 but evaluated during the experiments between 1:10 and 1:30) or by a bad distribution of the delivered samples in the two wells (in deed the sample was delivered from the sample transceiver output with two capillaries to the collector wells). Nonetheless, the detected

concentration lies within the 20% confidence interval that should be taken into account for the accuracy of the developed IgG test.

Otherwise, the manually-taken and filtered samples were measured at concentrations of 3.1 ± 0.4µg/mL for the undiluted yeast culture medium and at concentrations of 2.0 ± 0.1µg/mL and 2.2 ± 0.2µg/mL for medium diluted 10-folds and, respectively, 20-folds. The difference between the spiked concentration and the measured concentrations in the undiluted and diluted samples may be explained by a partial decomposition of the IgG molecules during the fermentation, by a loss of IgG during the filtration of the medium, by dilution errors and by artefacts between the signals obtained for calibration points measured in buffer solution and the real samples (matrix effect decreasing the detection signal in the medium). Unfortunately, the lack of experimental data does not allow one to understand the discrepancy between the expected and the measured IgG concentrations.

These limited number of data points prevents to identify clearly the source of errors, to qualify the real performance of the developed assay and to benchmark the results against measurements that could be obtained with the same samples using conventional microtiter-plates with optical detection. The obtained results yet show CV varying from 5 to 12%, which is in good agreement with the 15% error margin determined for the detection of IgG in the ImmuSpeed platform. This result thus confirms the applicability of the micro-Elisa approach to on-line monitoring of cell cultures, although further work is yet required after the end of the project to assess the accuracy, robustness and long-term use of the platform in real conditions.

CE-MS analysis of extracellular metabolites

During the testing of the integrated NANOBE system, altogether 5 samples out of 8 (4 with the integrated system, 3 with the alternative integrated system, and 1 sample manually captured after the sample transceiver) were successfully analyzed on-line with the CE-MS system while being part of the whole Nanobe system. In addition, all of the 8 samples were analysed off-line as well. Absolute quantification was not possible for three reasons 1) the optimization of the CE-method was not complete (peaks were too wide to identify small concentrations), 2) dilution of the sample upstream in the Nanobe system was not accurate eventually, 3) ionization at the MS varied resulting in large uncertainties. Furthermore, the delivery of the sample from the bioreactor was too slow. Quantification problems 2 and 3 could have been and can be solved by using internal standard in the cultivation. Normally internal standard is not used directly in the cultivation as it may affect the performance of the cells or it may be consumed by the cells.

The off-line samples taken at the same time as the Nanobe on-line samples were analyzed with off-line HPLC and the results in terms of which metabolites were detected were quantitatively the same as what was observed with the on-line CE-MS analysis.

On-line ethanol extraction and CE-MS analysis of intracellular metabolites

The analysis of intracellular metabolites was not possible with the Nanobe setup, because the time in the Nanobe setup for the treatment and lysis of the cells would have been too long and the metabolism of the cells would have had time to adapt to the conditions in the sampling system and would not have

reflected the status of the cells in the reactor. A separate setup was built to demonstrate that the analysis of intracellular metabolites was possible with the on-line CE-MS device.

For the release of intracellular metabolites from yeast cells, hot ethanol extraction was used. Sample from the bioreactor came in contact with 81 °C hot ethanol within 30 s of leaving the reactor; precipitating proteins and nucleotides and dissolving lipids; hence stopping the metabolic reactions immediately. The reaction mixture was subsequently cross-flow filtered on ice to cool down the reaction mixture after approximately 30 s residence time and the permeate was used for CE-MS analysis. With this method, also the extracellular compounds can be analyzed as well. Because there was not enough time for CE method development, it was decided that the sample was delivered to MS with loop injection and no CE separation was performed. It would be easier to detect individual compounds from the TIC if all of the analytes arrive to the MS at the same time in one large peak.

Several difficulties occurred during these tests, vac chips in the MS got clogged and there was also some pressure build-up caused by the narrow tubing. It was also noticed that the m/z values varied between analyses in the mass spectrometer which made identification of the peaks difficult.

Results indicate that the extraction was successful and intracellular metabolites, such as sugar phosphates, can be detected with the MS.

In the future research of the CE-MS, the CE method development will be continued. Higher CE voltages and temperature control would increase sensitivity and make the CE peaks sharper. Non-aqueous BGE s would help with the ionization of analytes in electrospray and might also help to prevent the clogging of vac chips. The use of internal standard would be essential for the quantification since there is always variation in the MS signal due to day-to-day variation in the efficiency of ionization.

Overall the project succeeded to connect the MS detection developed by Microsaic to the CE separation by VTT and this CE-MS system to the rest of the Nanobe system. It was possible to perform the analysis using only 20-microliter sample provided via the sample transceiver developed by CNRS. Only 20 nL of this sample was injected to the CE-MS analysis, which is roughly 1 per mil of the 20-microliter sample taken initially from the reactor. These are all very tiny volumes. For instance, when comparing to existing on-line HPLC system for the analysis of extracellular metabolites, the on-line HPLC required 4 mL of sample for filtration and 20 microliters was injected to the HPLC column - the same volume, which was the total sample volume in the Nanobe system [N. Tohmola et al., Biotechnol. and Bioprocess Eng., Vol. 16, pp. 264-27 (2011)]!

Furthermore, VTT was able to develop a method for on-line ethanol extraction in order to enable on-line analysis of intracellular metabolites in connection of the CE-MS system. Measurement of extracellular and intracellular metabolites in connection to bioreactor cultivations on-line was demonstrated for the first time.

However, the methods are not yet quantitative and further work is required. Problems encountered in flowing the electrophoretic solvents used in the CE directly to MS are a major issue that needs to be solved by developing the CE system and the method as well as the ionization.

Microsaic s MS as instrument was very easy to operate; one of the most advanced features of the device was the software. Compared to conventional mass spectrometers changing parts was much easier and faster. Also, the vacuum was reached very rapidly, in just 30 minutes. The electrospray of

this device was very promising, because it was able to produce stabile electrospray despite very low liquid flow rates of CE system.

Cell analyser tests with VTT s own sampling system

As the tests of the cell counter/analyser chip were not successful in the integrated Nanobe system, VTT set up a simpler system for connecting the EPFL chip to a bioreactor. The flow scheme of the setup includes 1) 5-fold dilution of the sample using PBS buffer by different pump flow rates, 2) 15 µL injection loop in a commercial injection valve, 3) PBS to drive the sample from the injection loop into the chip. PBS flow was maintained steady using a pressurized and pressure-controlled vessel.

A yeast cell sample delivered from a bioreactor to the cell counting/viability chip was preformed. The result of the chip-based analysis was 3.9 million cells/mL. In a manually taken sample from the same time point the measured optical density was 4.0 at 600 nm and the cell count 40 million cells/mL with Nucleocounter, which is based on Propidium Iodide staining. There is a disagreement of factor 10 between the off-line and on-line results. VTT has used the Nucleocounter also in other cultivations and is rather confident that the ratio of OD and cell count is roughly 10 million cells/mL at OD1. Thus, there is reason to believe that the cell count with a chip produced too low cell number estimation in these tests. There are several possible reasons that can cause the too low cell count (flow rate, flow profile, dimensional variation between chips, cell sedimentation at low flow rates, blockages in the injection valve).

The viability estimation made with the chip also fluctuated considerably (+/-10%) between measurements. The measurement of viability was lower than expected as in the Nucleocounter assay the viability was around 90%. The reasons for this difference and especially fluctuation are more difficult to understand, but most likely the same factors as described earlier may have affected also the viability measurement.

The developed impedance analysis system can efficiently discriminate living and dead yeast cells and determine the cell density from the average cell speed. The system can function with the sampling probe and gives good results with yeasts. Unfortunately the combination of different problems starting from existing commercial instruments led to the fact that there was no time for producing results to really demonstrate that the cell and viability measurement chip works in the Nanobe system. Results outside the Nanobe system have been presented and published and show that the technology works. When connected to a bioreactor with a simpler system some measurements for cell density and viability directly from the cultivation could be done. However, there is great promise with this technology as cell density and viability measurements are the top priority for measuring in bioprocesses.

Tests of the optical probes

The high fluctuation of the Presens probe was due to high measurement frequency and the fact that the probe was so close to the wall of the reactor that air bubbles could attach to the sensor spot. This problem can be easily solved by different orientation and location of the probe.

Galilaeus tested also the probe including pO_2 sensor and the probe including three different sensors measuring pCO_2, pH and pO_2 simultaneously to collect experience and to test the functionality in a

more demanding process environment. The probe including pO2 sensor was installed in 300L fermentor that locates in an industrial pilot hall with other pilot scale fermentors and downstream equipment. The conditions are rather challenging for instrumentation due the high humidity and temperature changes.

The results and experiences with pO2 sensor were very promising. The measuring values with pO2 sensor of PreSens correlate very well with traditional method. The Presens sensor is durable for 3-4 sterilization times, which is acceptable for the disposable sensor. Compared to the high price of conventional pO2 probe PreSens sensor is rather competitive. The same applies with the probe with three sensors especially as only one port of the fermentor is needed.

Conclusions

The NANOBE integrated system was constructed only a couple of months before the end of the project, occupying one laboratory in VTT. All hardware was connected with each other as planned, with the exception of the cell lysis chip. The resulting system created a long pathway (and thus longer than expected tubing) to some system parts such as the EWOD, which reduced the timing precision and made inclusion of the lysis chip impossible. The fully integrated system was operated for online handling and analysis of genetically modified yeast fermentation.

After a significant amount of software modifications, the fluidic interfacing between the individual modules functioned successfully with acceptable repeatability of the transported sample volume. However, due to the long tubings the timing of fluidic transport to and from the sample transceiver saw non-negligible deviations (due to compressibility of air plugs), which made fully automated operation of the entire system impractical. Therefore, triggering of the module operation was performed manually although automated triggering was implemented in the master program of the system.

Online sample handling and analysis using the integrated system (not including the cell analysis and lysis) functioned flawlessly in one run out of four attempts. Failure modes in the unsuccessful runs were all different, appearing in different sections of the system. This means that the robustness of the modules have to be increased.

Comparisons of the measured mass spectrum from the online and reference offline analyses indicated that the sample has been successfully transported through the entire system without major contamination from the various splitting, mixing and cleaning steps during the automated operation. The ELISA readouts of spiked IgG concentrations showed a significant deviation (50%) from the offline measurement, but the two were on the same order of magnitude. Considering that the dilution ratio of the supernatant sample in the sampling/filtration module is yet to be quantitatively evaluated, the outcome is encouraging for further development of the system.

Overall, the NANOBE concept of an automated online bioanalysis system based on microfluidic core components was proven to be promising in principle. It was shown that a minute amount of sample with a volume of 20 μL can be analysed online in two paths without any manual handling. Two different configurations of the system components were tested, both showing results that are comparable to conventional off-line methods, which was a good demonstration of the modular interchangeability of the system. Improvements in robustness and accuracy of sample handling are keys for achieving constant operation for online monitoring over the full duration of fermentations.

Potential Impact:

The socio-economic impact and the wider societal implications of the project so far

The NANOBE project delivered an integrated real time bioprocess analysis system for measuring of extra- and intracellular compounds. It provided a system for bioprocess monitoring that was not existent at the time when the project was started. The NANOBE system is well beyond the state-of-the art in its concept and individual components. Smart systems integration led to increased functionality, since the sampling is now solved in a way which enables the real on-line analytics during the bioprocess. Different lab-on-a-chip modules enable measurement of various analyte types such as cells, low molecular weight compounds, proteins, and specific mRNAs. Some of the modules are already developed for multiplex analysis. (e.g. optical sensors of PreSens) The system provides improvement in terms of automation, analysis time and sensitivity. Further development is needed to increase the robustness of the integrated NANOBE system.

The analysis platform can significantly improve real-time feedback control of large-scale production processes as well as screening and optimisation of production organisms and conditions. It can be coupled to any size of a bioreactor, from small microreactors designed for strain screening, to laboratory scale bioreactors for process optimisation, and to large full scale industrial production bioreactors. The platform is designed to be flexible so that it can be exploited as the whole platform for multiple analyses or as individual components, i.e devices for analysis of a certain parameter only. Flexibility of the system was demonstrated in the project by using a couple of different system configurations in the testing of the system with real bioprocesses.

The project combined in an innovative manner special expertise in microfluidics, nano- and microfabrication techniques, chip-scale mass spectrometry, photonics, electronics, sensor technologies, and biotechnology.

There is an increasing need for more rigorous bioprocess monitoring due to the increasing number of tailor-made production organisms and bioprocesses that will be required for sustainable production of fuels, chemicals and pharmaceuticals. Development of novel production organisms is benefited from the recent advances in genomics, metabolic engineering, synthetic biology and systems biology, which in turn provide tools and understanding to design analysis methods for those cellular and process parameters and biomarkers that are critical for the most efficient performance of the process. The increasing number of non-traditional bioprocesses with high demands on energy efficiency and yields sets a demand for multiparameter analyses that also enable better feedback control. In addition, using the NANOBE platform, right time points for sample collection for more comprehensive off-line (genome-wide) analyses can be chosen. With the NANOBE system also individual cells can be analysed. This provides means to count non-viable and nonproductive cells in the population. On the other hand cells with intracellular biomarkers (e.g. based on fluorescence) that indicate cell stress etc. can be counted. The biomarkers can also be used for cell sorting and for instance selection of cells with best viability and productivity for further cultivations. It is also noteworthy that the technologies developed allow synchronisation of (yeast) cells. This is of fundamental importance since until now the great majority of biological data has been generated from populations where the cells are in different points of the cell cycle and have different physiology. Thus, it is to be noted that the NANOBE analysis platform is not only useful for monitoring and control of production processes but is an excellent tool for strain screening and process optimisation, as well as for basic studies on cell physiology and cellular phenomena including systems biology analyses. Overall, the on-line monitoring tools developed in the NANOBE project can help to increase the production rate, yield

and concentration of the final product of a fermentation process. These improvements in process monitoring may be crucial for the economic viability of a new bio-based product.

NANOBE platform offers reduced process development times for existing bioprocesses. This will enable the cost reduction e.g. in pharmaceutical industry. Firstly, time is saved for custom manufacturing companies in their process development services. Secondly, the time that it takes for a drug to reach the market will be reduced. This is even more important, since one month earlier in the market for a 1 billion $ blockbuster is worth of 50 million $ for pharmaceutical industry. Thirdly, time savings in product development also confers competitiveness. Entering the market too late is quite often a disaster for market penetration of a new drug. Also e.g. product purification (Down Stream Processing) and process characterization at small scale prior to production of large scale GMP batches (that presently take from weeks to several months), can be done much quicker by use of on-line real time assays, that will be implemented and tested in this NANOBE project. The FDA PAT Initiative is demanding a higher amount of analytical methods during the bioprocess similar to the analytics in the downstream processing. The NANOBE project is directly addressed this challenge by providing a real time and on line measurement platform for multiple analytes.

A further cost effective point is the possibility of miniaturization (one will need less chemicals), which results in a higher parallelization of bioprocess approaches. Furthermore, some modules need less space (in a lab or in a bioreactor) as the known systems; e.g. MS, and the combination of gas/pH sensors with the sampling probe. Last one results in saving ports for other analytics in the bioreactor, saving time during assembly, and reduces the risk of contamination by reducing the amount of components.

Bioreactor is an essential tool for bioprocess research and development. It can be used for wide range of applications varying from general process optimisation to gene mapping tasks. This fact also leads up to a wide range of different type bioreactor designs suitable for different applications. The real time measurement platform developed in the NANOBE project can be realised in a way that it will suit different types of bioreactors and different types of cultivations. The experiences and conclusions obtained during the NANOBE project are of general value for bioprocess monitoring, and very useful for other research areas like cell and tissue engineering, lab-on a chip development, and standardisation.

Although the measurement platform developed in the NANOBE project was tested only with a certain yeast based bioprocess, the perspective still is to apply it to monitor also cultivations based on different organisms in the future. The extracellular analyses developed in NANOBE are very generic and can be used with most organisms. The complexity of the culture medium may however cause adjustment in the device cleaning and analysis methods.

The main dissemination activities and exploitation of results

Dissemination activities

In the NANOBE project the dissemination of knowledge was done in multiple ways. An essential part of the dissemination of the results of the project results was to publish them in major international scientific journals and conferences. Numerous scientific journal papers have been published, accepted for publication or submitted by the end of the project. In addition several conference presentations (both oral and poster) were held in international conferences and workshops.

Industrial platform

An Industrial platform (IPF) was established in the beginning of the project. It comprised of representatives of application and equipment manufacturing companies. The following companies participated in the industrial platform:

1. Galilaeus, Finland, www.galilaeus.fi, represented by Dr. Ari Båtsman (also a beneficiary)
2. Plexpress, Finland, www.plexpress.fi, represented by Dr. Jari Rautio
3. Medicel, Finland, www.medicel.com, represented by Jouni Ahtinen
4. Valio, Finland, www.valio.fi, represented by Dr. Kristiina Kiviharju
5. DSM, The Netherlands, www.dsm.com, represented by Dr. Sybe Hartmans
6. Novozymes, Denmark, www.novozymes.com, represented by Dr. Kim Hansen
7. Süd-Chemie, Germany, www.sud-chemie.com, represented by Dr. Andreas Kusterer
8. Roche, Germany, www.roche.com, represented by Dr. Stefan Buziol

The members of the IPF had an access to monitor the progress of the project. Three workshops were arranged to disseminate the project objectives and results to the IPF members. The material presented to the IPF members have been distributed to all members by e-mail.

Project website

Project website was set up in the beginning of the project. The public extranet can be found in http://www.vtt.fi/NANOBE.

Journal publications and conference attendance

All the dissemination activities during the project are listed below. Altogether, the NANOBE project produced 19 reviewed journal publications, 15 conference presentations and 17 other presentations to disseminate the project results.

List of peer reviewed journal publications:

1. G. Mernier, N. Piacentini, R. Tornay, N. Buffi, P. Renaud, 'Cell Viability Assessment By Flow Cytometry Using Yeast As Cell Model', Sensors and Actuators B, vol. 154, pp. 160-163, doi:10.1016/j.snb.2009.11.066, (2011).
2. G. Mernier, N. Piacentini, and P. Renaud, 'Continuous-flow electrical lysis device with integrated control by dielectrophoretic cell sorting', Lab Chip, vol. 10, pp 2077 (2010).
3. G. Piret, H. Drobecq, Y. Coffinier, O. Melnyk and R. Boukherroub, 'Matrix-free Laser Desorption/Ionization Mass Spectrometry on Silicon Nanowire Arrays Prepared by Chemical

Etching of Crystalline Silicon', Langmuir vol. 26 (2), pp 1354-1361, DOI: 10.1021/la902266x (2010).

4. G. Mernier, W. Hasenkamp, N. Piacentini and P. Renaud, 'Multiple-frequency Impedance Measurements in Continuous Flow for Automated Evaluation of Yeast Cells Lysis', Sensors and Actuators B, doi:10.1016/j.snb.2010.10.050, on line (2010).

5. T. P. N. Nguyen, P. Brunet, Y. Coffinier, and R. Boukherroub, 'Quantitative testing of robustness on super-omniphobic surfaces by drop impact', Langmuir, vol. 26, pp., doi: 10.1021/la103097y (2010).

6. G. Mernier, N. Piacentini and P. Renaud, 'Multiple-frequency Impedance Measurements in Continuous Flow for the Evaluation of Electrical Lysis of Yeast Cells', Procedia Engineering, Vol. 5, pp. 37-40, doi: 10.1016/j.proeng.2010.09.042 (2010).

7. G. Piret, D. Kim, H. Drobecq, Y. Coffinier, O. Melnyk, P. Schmuki, and R. Boukherroub, 'Surface-assisted laser desorption/ionization mass spectrometry on titanium dioxide (TiO2) nanotube layers', The Analyst, vol. 137, pp. 3058-3063, doi: 10.1039/C2AN35207A (2012).

8. F. Lapierre, G. Piret, H. Drobecq, O. Melnyk, Y. Coffinier, V. Thomy, and R. Boukherroub, 'High sensitive matrix-free mass spectrometry analysis of peptides using silicon nanowires-based digital microfluidic device', Lab Chip, vol. 11, 1620-1628, doi: 10.1039/C0LC00716A (2011).

9. C. Wu, F. Bendriaa, F. Brunelle, and V. Senez, ' Fabrication of AD/DA microfluidic converter using deep reactive ion etching of silicon and low temperature wafer bonding', Microelectron. Eng., vol. 88, pp. 1878-1883, doi: 10.1016/j.mee.2010.12.001 (2011).

10. Y. Coffinier, S. Szunerits, H. Drobecq, O. Melnyk, and R. Boukherroub, 'Diamond nanowires for highly sensitive matrix-free mass spectrometry analysis of small molecules', Nanoscale, vol. 4, pp. 231-238, doi: 10.1039/C1NR11274K (2012).

11. Y. Coffinier, N. Nguyen, V. Thomy, and R. Boukherroub, 'Affinity Surface-assisted laser desorption/ionization mass spectrometry for peptide enrichment', submitted to Analytical Chemistry.

12. G. Perry, Y. Coffinier, V. Thomy, and R. Boukherroub, 'Inhibiting protein biofouling using graphene oxide in droplet-based microfluidic microsystems', Lab Chip, vol. 12, pp. 1601-1604, doi: 10.1039/c2lc21279j (2012).

13. G. Mernier, S. Majocchi, N. Mermod, and P. Renaud, 'In situ Evaluation of Single-cell Lysis by Cytosol Extraction Observation Through Fluorescence Decay and Dielectrophoretic Trapping Time', Sens. Act. B, vol. 166-167, pp. 907-912, doi: doi.org/10.1016/j.snb.2012.03.057 (2012).

14. N. Piacentini, G. Mernier, R. Tornay, and P. Renaud, 'Separation of Platelets from other Blood Cells in Continuous-flow by Dielectrophoresis-Field-Flow-Fractionation', Biomicrofluidics, vol. 5, 034122 online (2011).

15. G. Mernier et al., 'Improving the throughput of on-chip cell electrical lysis and extraction of intracellular compounds using 3D carbon electrodes', submitted to Lab Chip.

16. G. Mernier et al.,'Characterization of a novel impedance cytometer design using dielectrophoretic focusing', submitted to Lab Chip.

17. H. van Lintel, G. Mernier, and P. Renaud, 'High-throughput micro-debbublers for bubble removal with sub-microliter dead volume', Micromachines, vol. 3, pp. 218-224, doi: 10.3390/mi3020218 (2012). High-Throughput Micro-Debubblers for Bubble Removal with Sub-Microliter Dead Volume

18. R. Dufour, G. Perry, M. Harnois, Y. Coffinier, V. Thomy, V. Senez, and R. Boukherroub, 'From micro to nano re-entrant structures: Hysteresis on superomniphobic surfaces', accepted for publication in J. of Adhesion Science and Technology, Special Issue, (2012).

19. M. Dupre, Y. Coffinier, R. Boukherroub, S. Cantel, J. Martinez, and C. Enjalbal, 'Laser Desorption Ionization Mass Spectrometry of Protein Tryptic Digests On Nanostructured Silicon Plates',

20. J. Proteomics, vol. 75, pp. 1973-1990, doi: 10.1016/j.jprot.2011.12.039 (2012).

List of conference presentations:

1. J. Rossier, 'Microfluidics system for rapid immunoassay', 2nd Swiss Pharma Science Day, September 2 2009, Bern, Switzerland.

2. J. Rossier, C. Vollet, P. Morier, V. Meylan, and F. Reymond, 'ImmuSpeed: Novel Automated Platform for Fast ELISA in Microchips', Medica 2009, November 18.-21 2009, Düsseldorf, Germany.

3. G. Mernier, W. Hasenkamp, N. Piacentini, P. Renaud, 'Multiple-frequency Impedance Measurements in Continuous Flow for the Evaluation of Electrical Lysis of Yeast Cells', Eurosensors XXIV, September 5-8 2010, Linz, Austria.

4. C. Wu, F. Bendriaa, F. Brunelle, and V. Senez, 'Fabrication of AD/DA Microfluidic Converter using Deep Reactive Ion Etching of Silicon and Low Temperature Wafer Bonding', 36th Int. Conf. on Micro & Nanoeng. MNE2010, September 19-22 2010, Genoa, Italy (2010).

5. Grodrian, K. Lemke, R. Römer, G. Gastrock, J. Metze, 'Dead volume free sampling probe in µl-scale', 15th Heiligen-städter Kolloquium, September 27-29 2010, Heiligenstadt, Germany.

6. P. Heimala, V. Senez, H. van Lintel, J. Miwa, K. Lemke, C. Krause, F. Reymond, A. Finlay, and A. Båtsman, 'Nano- and microtechnology -based analytical devices for online measurements of bioprocesses -NANOBE', Microfluidics in Bioanalytical Research and Diagnostics, Sept. 30-Oct. 1, 2010, Helsinki, Finland (2010).

7. F. Lapierre, G. Piret, H. Drobecq, O. Melnyk, Y. Coffinier, V. Thomy, and R. Boukherroub, 'EWOD lab-on-chip for mass spectrometry and fluorescence analysis', The 14th Int. Conf. on Miniaturized Systems for Chemistry and Life Sciences, µTAS2010, October 3-7 2010, Groningen, The Netherlands (2010).

8. G. Mernier, N. Piacentini and P. Renaud, 'Cell Sorting by Dielectrophoresis for Evaluation of Lysis Efficiency in Continuous Flow', The 14th Int. Conf. on Miniaturized Systems for

Chemistry and Life Sciences, μTAS2010, October 3-7 2010, Groningen, The Netherlands (2010).

9. G. Mernier, N. Piacentini, W. Hasenkamp, and P. Renaud, 'A unified approach to evaluation of yeast cell lysis by continuous flow cytometry', Nanotech-Montreux conference, November 15th - 17th, 2010, Montreaux, Switzerland (2010).

10. T. P. N. Nguyen, Y. Coffinier, V. Thomy, P. Brunet, and R. Boukherroub, 'Fabrication of silicon based omniphobic surfaces', Journées Nationales sur les Technologies Emergentes en micro-nanofabrication (JNTE'10), November 24th -26th , 2010, Ecole Polytechnique, Palaiseau, France (2010).

11. Y. Coffinier and R. Boukherroub, 'Sub-bandgap Absorption of Boron-doped Diamond Nanowires for Matrix-free Laser Desorption/Ionization Mass-spectrometry', Analysis of Small Biomolecules Symposium A: Diamond Electronics and Bioelectronics--Fundamentals to Applications IV, 2010 MRS Fall Meeting, November 29-December 3, 2010, Boston, Massachusetts, USA (2010)

12. C. Wu, F. Bendriaa, F. Brunelle, and V. Senez, 'Sample Preparation Unit for Online Bio-Processes Monitoring ', The 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences (microTAS 2011), October 2-6, 2011, Seattle, USA (2011).

13. P. Heimala, J-P. Pitkänen, V. Senez, R. Boukherroub, H. van Lintel, G. Mernier, J. Miwa, K. Lemke, G. Gastrock, C. Krause, F. Reymond, A. Finlay, P. Edwards, and A. Båtsman, 'NANOBE Nano and microtechnology based analytical devices for online measurements of bioprocesses, Biotechnica', Oct. 11-13 2010, Hannover, Germany (2010).

14. G. Mernier, 'Continuous-flow separation of platelets from blood by Dielectrophoresis-Field-Flow-Fractionation', NanoBioTech Montreux, Nov. 14-16 2011, Montreaux, Switzerland (2011).

Exploitation of results

Both general advancement of knowledge and commercially exploitable research and development results have been generated in the project. General advancement of knowledge relates to the developed technologies, devices and fabrication methods. Significant progress has been made in understanding biopollution and understanding of the interaction between small biomolecules in a liquid phase and nanotextured solid surfaces. Generated knowledge will be exploited in scientific research and development for various biological, chemical, environmental and life science applications by the beneficiaries. Generated new knowledge will be integrated also into the student education.

One patent application was filed in the project. With the new patent with the topic 'Sampling probe for withdrawing samples from aerated liquids' iba shows that the sampling probe consists of a wide application range.

The developed sampling port combining three analytes and sampling is believed to gain interest even without the attached analytical devices of NANOBE. The consortium hopes that positive feedback

from the IPF and from other industries will arise in the near future and lead therefore to a commercialisation of this part of NANOBE.

PreSens, DiagnoSwiss and Microsaic Systems will also exploit commercially those results of the research and development work that are the most mature. PreSens will profit from developments on the individual sensors for dO, dCO2 and pH. Diagnoswiss will use the prototype of automated microchip platform for further demonstration of immunoassay applications, with the aim of obtaining a finalised first-series instrument applicable for beta-testing in view of finding a strategic partnership for commercialisation of the platform or transfer of the technology for R&D applications. Microsaic Systems has incorporated the technology developed during the NANOBE project into a new product, the 3500 MiD. This miniature mass spectrometer was launched at the Lab Automation show in January 2011.

The end user companies have shown interest escpecially on the single component developments in the NANOBE project. These include the sampling probe, mass spectrometer, mRNA analysis and optical sensors (especially pH sensor in the low pH range).

Overall, the on-line monitoring tools developed in the NANOBE project could help to increase the production rate, yield and concentration of the final product of a fermentation process. These improvements in process monitoring may be crucial for the economic viability of a new bio-based product.

List of Websites:

Project public website

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