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IMPLEMENTATION OF MICROREACTOR TECHNOLOGY IN BIOTECHNOLOGY

BOOK OF EXTENDED ABSTRACTS

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Dear Colleagues,

Welcome to the 4th international conference Implementation of microreactor technology in biotechnology – IMTB 2017 in one of the most beautiful parts of Slovenia!

The IMTB conference aims to provide a platform for people from industry and academia, working in the cross-section of microfluidics, life sciences, analytics and bioprocess engineering. The interdisciplinary feature of the IMTB conferences enables participants to present and discuss their latest results, developments and strategies within these complementary fields and to integrate them to gain new accomplishments for industrial implementation.

This event is a sequel of the IMTB 2010 Conference held in Ljubljana, Slovenia, in September 2010, the IMTB 2013 Conference, which took place in Cavtat, Croatia, in May 2013, and the IMTB 2015 Conference, which was held in Opatija, Croatia, in May 2015. Organizers of this conference series are collaborative research groups from the Faculty of Chemistry and Chemical Technology of the University of Ljubljana, Slovenia, and the Faculty of Chemical Engineering and Technology of the University of Zagreb, Croatia.

Invited prominent speakers from leading research institutions and industry together with participants of various backgrounds including chemical, mechanical and electrical engineering, medicine, pharmacy, chemistry, biochemistry, microbiology and biotechnology, will present their recent achievements in the field of enzymatic microreactors, cells within microdevices, analytical microdevices and bioprocess intensification and integration within microdevices.

The Scientific Programme of the IMTB 2017 Conference encompasses more than 60 contributions which will be presented in sessions Enzymatic Microreactors, Cells within Microdevices, Analytical Microdevices and Bioprocess Intensification and Integration. The IMTB 2017 Conference gathered almost 90 participants from leading research institutions and industry from all over the world in a beautiful Slovenian town Bled, sheltered by picturesque mountains with the island in the middle of an Alpine lake.

We would like to thank all participants who contributed to the high quality of the programme. Furthermore, we gratefully acknowledge members of the Organizing and the Scientific Committee for their valuable contribution and especially Gabriela Kalčikova, Ana Jurinjak Tušek and Filip Strniša for their efforts and commitment. Special thanks go to the COST Action CM 1303 Systems Biocatalysis, sponsors, EU FP7 project EUROMBR, as well as the Section on Applied Biocatalysis of the European Federation of Biotechnology for their support.

We wish you a fruitful and pleasant stay at the IMTB 2017 Conference in Bled and we are looking forward to meeting you at the IMTB2019 Conference!

Polona Žnidaršič Plazl and Bruno Zelić
Co-Chairs of the IMTB 2017 Conference

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Engineering platforms of micro- and extended nano-fluidics
Takehiko Kitamori
The University of Tokyo, Japan

Chair
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Marko Mihovilovic
Technical University Vienna, Austria

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László Poppe, Budapest University of Technology and Economics, Hungary

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Stefano Servi, Politecnico di Milano, Italy

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Helmholtz-Centre for Environmental Research, Germany

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Ulrich Krühne, Technical University of Denmark, Denmark

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University of Southampton, United Kingdom; University of Oviedo, Spain

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Graz University of Technology, Austria

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University of Ljubljana, Slovenia; University of Oulu, Finland

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Politecnico di Milano, Italy; Istituto di Chimica del Riconoscimento Molecolare C.N.R, Italy; Università degli Studi dell'Insubria, Italy

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Technion-Israel Institute of Technology, Israel; Budapest University of Technology and Economics, Hungary

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University of Ljubljana, Slovenia; LentiKat's a.s., Czech Republic

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University College London, United Kingdom

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Politecnico di Milano, Italy; University of Urbino, Italy

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Universidade Lisboa, Portugal; Instituto Politécnico Setúbal, Portugal

AP10 *Multivalency effects on the immobilization of sucrose phosphorylase in flow microchannels for the development of a high-performance biocatalytic microreactor*
Donya Valikhani, Juan M. Bolivar, Martin Pfeiffer, Bernd Nidetzky
Graz University of Technology, Austria

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Politecnico di Milano, Italy; Università degli Studi di Milano, Italy

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Graz University of Technology, Austria; Technical University of Denmark, Denmark

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University of Granada, Spain; NanoMyP®, Spain

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University of Granada, Spain; NanoMyP®, Spain

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University of Nova Gorica, Slovenia; Jožef Stefan Institute, Slovenia

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University of Granada, Spain; University of Oulu, Finland; NanoMyP®, Spain

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University of Ljubljana, Slovenia; Corning S.A.S. Corning European Technology Center, France

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University of Ljubljana, Slovenia

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Anže Belovič, Peter Panjan, Radek Stloukal, Adama Marie Sesay, Polona Žnidaršič PlazlUniversity of Ljubljana, Slovenia; University of Oulu, Finland; LentiKat's a.s., Czech Republic

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University of Southampton, United Kingdom; University of Oviedo, Spain

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University of Zagreb, Croatia

DP8 *Experimental and theoretical evaluation of residence time distribution in miniaturized packed bed reactors with Novozym® 435*
Filip Strniša, Marijan Bajić, Peter Panjan, Tomaž Urbič, Polona Žnidaršič Plazl, Adama Marie Sesay, Igor Plazl
University of Ljubljana, Slovenia; University of Oulu, Finland

DP9 *Implementation of aqueous micellar two-phase systems within a microfluidic device for protein purification*

Filipa A. Vicente, **Živa Brečko**, Mojca Seručnik, João A. P. Coutinho, Sónia P. M. Ventura, Polona Žnidaršič Plazl
Universidade de Aveiro, Portugal; University of Ljubljana, Slovenia

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Goran Jovanovic, Conor Zoebelein, Tanner Bushnell, Davis Weymann, Marina Cameron, Martin Lubej, Matthew Coblyn, Karl Schilke, Mark Dolan, Lew Semprini Oregon State University, USA; University of Ljubljana, Slovenia

Plenary talks

P1

Engineering platforms of micro- and extended nano-fluidics

Takehiko Kitamori*

Department of Applied Chemistry, School of Engineering, The University of Tokyo
**email:kitamori@icl.t.u-tokyo.ac.jp*

Introduction

It is just a matter of time that microfluidics and nanofluidics produce novel values in the society and science, and their contributions will open new markets. Our contribution to both micro- and nano-fluidics is establishing the engineering platforms for general use. Microfluidics is now mainly going into applications. Immunoassay, cell culture, and extraction are the typical cases which cannot be achieved by electroosmotic fluidics nor electrophoresis. This talk gives an overview of our platforms for both micro/nano fluidics, and introduces the recent progress of some applications with the future in mind.

Microfluidics platform

The microfluidics began with electroosmotic fluid control and electrophoretic separation analysis in the early 1990's, in which capillary electrophoresis like a DNA sequencing was tried to be miniaturized on a glass substrate, though capillaries hadn't been replaced by microchannels. Around the same days but a bit later, pressure driven microfluidics, in other words, non-electrophoretic microfluidics was developed, and we were the earliest group that made its base and opened it to the variety of analytical, biological and even synthesis devices in the 1990's, and summarized as micro unit operation MUO in the early 2000's. The methods are as follows:

1) Pressure and surface driven fluid control
2) MUO and their combination for complex multistep chemical processing
3) Thermal lens micro and ultrasensitive detection for almost all species (non-fluorescent)
4) Chemical functionalization of channel surface for above MUO and fluid control

First of all, we developed our original detection method Thermal Lens Microscopy (TLM) in 1993, which is a kind of laser spectroscopy enabling detection of almost all molecules having optical absorption at single to countable number level in liquid. It is suitable for readout of target molecules in micro- and nanochannel for general use. By the way, our first microchannel was fabricated on a slide glass to experiment TLM detection in liquid, because the space under the microscope was very narrow. This microchannel was Y-shaped channel and became the origin of MUO. Therefore, our strategic methods of MUO and TLM were combined from 1995. This combination worked well to develop many kinds of chemical and biological processing, mixing reaction in 1996, cell culture in 1997, extraction in 1998, immunoassay in 1999, and summarized as MUO in 2002. Chemical modification on the surface of microchannel was also the key technology for pressure driven fluid control utilizing affinity between fluid and surface as well for chemical functionalization of the channel surrounding the fluid.

These platforms harmonized to establish our methodology of microfluidics and developed many kinds of applications because of its flexibility: Immunoassay, wet analysis, chemical synthesis, polymer synthesis, cell bioassay, and others. They contain multistep parallel and serial MOUs realizing complicated chemical processing. Some of them have been put into practical use. For example, our microfluidic immunoassay system was commercialized and could save some patients' lives in our University Hospital by utilizing its flexibility, rapidity and high sensitivity. Those applications were demonstrated in the 1990's when the pressure driven microfluidics was still in the embryonic period, and they have been the main activities of microfluidics in the 2000's regardless of droplet or continuous flow.

For the real implementation of microfluidics in industry and science, there are still some technologies to be developed. System technology, design method, and online flow control mechanism are the typical issues. However, we are feeling anticipation that microfluidics will break into a big market and contribute to science, industry, and society.

Nanofluidics platform and its future

Another direction of progress in microfluidics is nanofluidics which is 1000 folds smaller in size than microfluidics. However, the principle of our methodology is basically the same as microfluidics listed above from 1) to 4), though some modifications are needed to adjust to nanoscale scale fluidics. We published a paper concerning the nanofluidic device in 2002 in Analytical Chemistry, and it was the earliest reports of nanofluidics using nanochannels. After that, we have devoted ourselves to the extended-nano fluidic device technology and intensively reported fabrication and detection technologies as well as the unique characteristics of liquids and fluids in the nano scale channels.

In addition to these platforms, we applied the ultimate smallness of extended-nano fluidics for pico, femto, and even atto liter analytical chemistry. For example, atto-liter chromatography, femto-liter and single-to-countable molecules immunoassay, and chromatographic separation at atto-liter sample were successfully demonstrated. At the size interface between micro and nano channels, a sampling channel from single living cell to a nanochannel was realized. And a systemized micro/nano hybrid fluidics was developed. We recently reported a micro/nano hybrid fluidic device in which photocatalyst based fuel supplier system and fuel cell were totally miniaturized and integrated on a glass chip.

By using nanofluidics devices, unique properties of liquid and fluid were found. For example, viscosity of water is five times larger than normal water, dielectric constant becomes 1/7, proton conductivity is two hundred times larger, fluid at the channel wall is slipping and its velocity is not zero, and so on. Water molecules in a glass nanochannel strongly interact with the channel wall, and the nature of molecule as a particle becomes remarkable in nano scale condensed phase.

Future application of nano/micro hybrid fluidics may realize ultimately small research tools to many scientific fields. For example, a "single cell single molecule analysis" is the most attractive device for bio and medical researcher. The analytical chemistry using colorimetry with calibration line may become to molecular counting, that is the "absolute analysis". Nanofluidics may bring evolutional progress in science.

P2

Flow chemistry @ the chemistry & biology interface

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Bioorganic synthetic chemistry represents a border crossing discipline at the cross-roads of chemistry and biology. The combination of biotechnological methods with modern catalysis enables strong synergies towards sustainable technologies. In addition, the molecular understanding of biological processes opens up novel approaches to affect biology *per se*.

The re-introduction of continuous processing conditions on lab-scale has facilitated particular synergies in this transdisciplinary field, providing a number of unique case-studies with major impact by flow-chemistry. Several aspects of these border crossing fields will be addressed in selected examples within this lecture:

i) Process intensification by implementation of continuous flow regimes enables highly selective exploitation of renewable resources towards platform chemicals implementing high safety standards[1].
ii) The combination of continuous flow-chemistry and biocatalysis (especially focusing on redox-biotransformations) in a cascade set-up offers a powerful tool towards the production of high-value products[2].
iii) Discovery of novel pharmacological tools by diversity oriented utilization of microwave- and flow-chemistry in compound library design opens the door towards a fundamental understanding of biological processes; progress within prospective future therapies for diseases based on concepts of regenerative medicine will be presented[3].

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P3

Microfluidic droplets as tools for high-throughput biology: enzyme evolution, recruitment and discovery based on catalytic promiscuity

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'Promiscuous' enzymes possess additional activities in addition to their native ones, challenging the textbook adage "one enzyme – one activity". The observation of strong promiscuous activities in the alkaline phosphatase (AP) superfamily - where one active site can catalyse up to six chemically distinct hydrolytic reactions with promiscuous second order rate accelerations between 10e9 and 10e17 - suggests that even broadly promiscuous catalysis can be rather efficient. To efficiently explore the interconversion of promiscuous enzyme, we use picoliter water-in–oil emulsion droplets produced in microfluidic devices as high-throughput screening reactors. We present new workflows that allow screening of >10e6 clones and permit successful selections from single protein and metagenomic libraries, where lower throughput approaches have failed.

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P4
Slug-flow microfluidic bioreactors for production of special chemicals

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Microfluidic slug-flow systems are characterized by moving discrete volumes of a dispersed phase (slugs, droplets) in a continuous phase. The resulting flow regime then approximates the plug flow with uniform residence time of all droplets (slugs). The interfacial area between two immiscible liquids (or gas/liquid) in slug-flow systems is large and the diffusion distance between the interfacial surface and slug core is short. Moreover, an internal circulation within slugs occurs, which further increases intensity of the interfacial mass and heat transports and accelerates transport-limited chemical reactions.
In this talk, I will report on several experimental slug-flow microbioreactors developed in the Laboratory of Microchemical Engineering, University of Chemistry & Technology, Prague. We constructed a modular microfluidic system consisting of different slug flow generators, reaction capillaries and phase separators. The system was tested in the enzyme synthesis of particular chemicals. The obtained results confirmed benefits of microfluidic platforms at least in several aspects if compared to classical bioreactors.
Enzyme hydrolysis of soybean oil by the enzyme lipase was selected as one model reaction system. Lipases represent commercially important enzymes that catalyze various hydrolytic, esterification and transesterification reactions. Lipases are widely used in food, dairy, detergent, and pharmaceutical industries and they are tested for the production of biopolymers, biodiesel, enantiopure chemicals, agro-chemicals or flavor compounds. Because lipases typically operate at oil-water interfaces, slug-flow arrangements provide large interfacial/reaction area with advantage. It will be shown that the integrated slug-flow microreactor allows for the fast production of free fatty acids and monoglycerides with a relatively high substrate conversion[1]. Moreover, it was found that energy consumption is significantly smaller than in classical agitated tanks. Decrease of interfacial tension in the course of the enzyme reaction leading to slug-flow destabilization represents a possible problem for the microreactor performance. As we have shown, this problem can be resolved by the introduction of a third fluid phase into the originally two-phase water-oil slug flow, which generates regular two-phase liquid slugs separated by a gas phase[2].

Diastereoselective synthesis of L-phenylserine catalyzed by the enzyme L-threonine aldolase represents another model system that we studied in collaboration with Prof. Hessel at the Technische Universiteit Eindhoven. In this case, slug-flow arrangement allows for the continuous refilling of sparingly soluble substrate (benzaldehyde) into an aqueous reaction mixture. We identified suitable composition of an organic phase to provide stable slug-flow in a wide range of operational parameters. The effects of substrate concentrations, enzyme concentration, and other parameters on the L-phenylserine concentration in the product stream were examined and proper reaction conditions were identified. Experimental results on the L-phenylserine diastereoselectivity demonstrated the importance of properly chosen organic phase. High syn-anti forms ratio can be achieved in 2-methyltetrahydrofuran (MeTHF) in a system with long residence time and low syn-anti forms ratio is provided by toluene environment and short residence time[3].

Finally, I will summarize our recent efforts in droplet addressing and sensing in microfluidic devices by means of imposed electric field. We invented technology for the integration of durable gold microelectrodes in polymeric substrates[4]. Many microfluidic devices with incorporated microelectrodes were designed in our lab for different purposes. Addressing and controlled oscillatory motion of water in oil droplets mediated by faradaic charging was carried out[5]. Addressing of oil in water droplets based on the combination of electrophoresis and the Marangoni effect was successfully tested[6]. The possibility of addressing of water in water droplets in aqueous two phases systems was also proved. In near future, integration of controlled droplet transport and slug-flow or droplet-flow microreactors will be tested. It is expected that electric field will enhance the transport of ionic species between phases and accelerate phase separation.

Keywords: enzyme, microchip, slug flow, lipase, L-threonine aldolase, electric field

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Session A: ENZYMATIC MICROREACTORS

AK
Thermophilic proteins and their applications in microreactors for industrial biocatalysis

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Enzymes used for commercial biotransformation reactions are required to be stable under the industrial conditions employed. The use of naturally thermostable enzymes isolated from 'Hot' environments can be a source of enzymes that are more stable to high temperatures, extremes of pH and exposure to organic solvents (1).

Thermostable enzymes are ideally suited to micro-reactor applications since they are more robust towards immobilisation conditions and once in place within the micro-reactor channels can be used for long periods of time without denaturation. This provides ideal conditions for single micro-reactors to evaluate the substrate specificity and activity of the biocatalyst towards both natural and unnatural substrates in a rapid fashion. The packed micro-reactor could be used for the marketing the biocatalyst to academia and industry.

Rapid optimisation of biocatalytic reactions can be carried out using micro-reactors in a time saving manner. The optimised reactions can be scale-up using in multiple stacked micro-reactors which can be used over long periods of time due to the robust nature of the biocatalyst.

The micro-reactors offer ideal possibilities for the use of reaction cascades where both chemical and biocatalyst steps are arranged in tandem. The thermophilic enzymes are most robust to organic solvents and other components used in the chemical steps.

Many enzymes require different co-factors for their reaction mechanism. Some co-factors are regenerated 'in situ' on the enzyme but others leave the enzyme and have to be regenerated by another enzyme. In this latter case for the coenzymes NADH or NADPH it would be advantageous to have regeneration of the cofactor within the micro-reactor channel.

This presentation will give two examples of thermophilic enzymes that have been successfully either immobilised or packed as cross-linked aggregates within micro-reactors and their substrate specificity and stability evaluated and compared to the free enzyme. The first example used a monolith-immobilised thermophilic L-aminoacylase, from the thermophilic archaeon *Thermococcus litoralis* which has been developed for use in biocatalytic reactions and its stereo-specificity and stability has been evaluated. This enzyme is used for the production of chiral amino acids and their analogues and is used at scale by Dr Reddy's in its free state. We have demonstrated that the enzyme when immobilised in the micro-reactors can be a tool for rapid screening of enzyme specificity and that it shows similar substrate specificity to that reported of the free enzyme (2). This approach has been used to evaluate potential new substrates with N-benzoyl- (L-threonine, L-leucine and L-arginine) and N-acetyl- (D,L-serine, D,L-leucine, L-tyrosine and L-lysine) protecting groups. The order of preferred substrates was found to be Phe > Thr > Leu > Arg for N-benzoyl substrates and Phe_Ser > Leu > Met > Tyr > Trp for N-acetyl substrates. By using this micro-reactor screening approach a significantly smaller amount of enzyme and substrates was required. It was demonstrated that the micro-reactors were still operational in the presence of selected organic solvents, such as ethanol, methanol, acetone, dimethylformamide (DMF) and dimethylsulfoxide (DMSO). The results indicated that a combination of a small amount of an appropriate solvent (5% DMSO) and a higher reaction temperature could be employed in biotransformations where substrate solubility was an issue (3).

The second example used a thermophilic (+)-γ-lactamase from the archaeon *Sulfolobus solfataricus* (4) which was used as a crosslinked precipitate packed in a micro-reactor channel which was plugged at the ends to prevent loss of the enzyme material. This micro-reactor was subsequently used to study enzyme stability, activity, kinetics and substrate specificity. The thermophilic (+)-γ-lactamase retained 100% of its initial activity at the assay temperature, 80°C, for 6 h and retained 52% activity after 10 h. The high stability of the enzyme in this form meant that the micro-reactor it could be used to screen many compounds in a short

period of time. This advantage overcame the fact that the immobilisation process affected enzyme kinetics and activity, which was reduced (by 70%) compared to the free enzyme. In general, the enzyme displayed similar substrate specificity to that found in a previous study for the free enzyme (4) however, enhanced activity was seen towards one substrate, acrylamide (5). Both of the above enzymes do not require a co-factor for activity. We have developed an approach for the co-factor regeneration of NAD(H) for dehydrogenase reactions. This uses a novel controlled pore glass-poly(pyrrole) material which is not only suitable for the co-immobilisation of enzymes and co-factors, but also enables the facile electrochemical regeneration of the co-factor during the reaction. By employing the selective reduction of (*rac*)-2-phenylpropionaldehyde to (*S*)-phenyl-1-propanol as a model, we have demonstrated the successful co-immobilisation of a dehydrogenase enzyme and its co-factor NAD(H). This has been incorporated into a continuous flow reactor facilitating the *in situ* electrochemical regeneration of NAD(H) for a period in excess of 100 h. Using this approach we have developed a reagent-less, atom efficient system applicable to the cost-effective, continuous biosynthesis of chiral compounds (6).

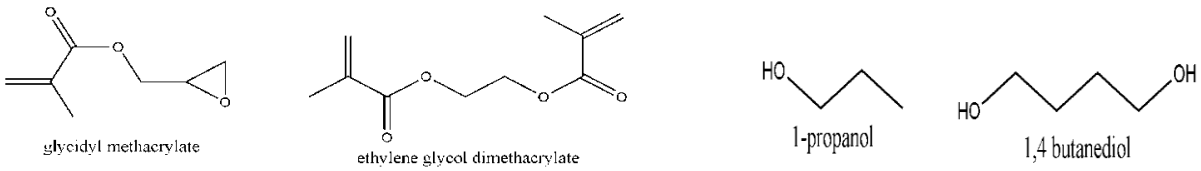

Figure 1: *Thermococcus* L-aminoacylase immobilized onto monoliths. Monomer: GMA:EDMA 3:1, Porogen:Propanol:Butanol:water, 7:4:1.Monomer:Porogen 1:3, with 1% 2,2 dimethyl-2-phenylacetophenone (DMPA) (initiator:monomer), activated using UV light (365nm) for 10 minutes. Enzyme attached by amide coupling.

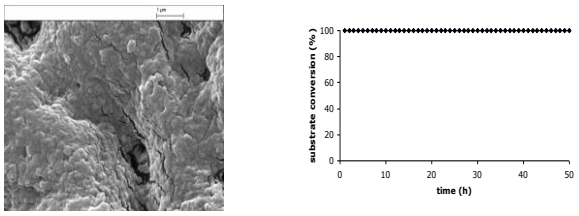

Figure 2: Scanning electron micrograph image of cross-linked enzyme (+)-γ-lactamase from the archaeon *Sulfolobus solfataricus.* The enzyme stability at 50ºC over 50 hours with 10 mM N-Benzoyl-L-Phe as the substrate and flow rate 1 uL min^{-1}

Keywords: Thermophilic proteins, immobilisation, enzyme aggregates, micro-reactor, co-factor recycling.

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AO1

Reaction design for the compartmented combination of heterogeneous and enzyme catalysis

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The combination of a heterogeneously catalyzed reaction with an enzymatic biotransformation in one-pot cascade processes is a concept followed to reduce equipment costs and purification efforts. By saving time and energy due to the reduced number of workup operations needed it can be an important element of the so called "Green chemistry". However, often incompatibilities between the two types of catalysts hamper this approach. Transferring the process into continuous plug flow reactor system could solve these problems. Microreaction technology could provide one solution here. We were interested in the direct conversion of glucose and a range of other sugars into their 2-keto-3-deoxy sugar acid derivatives by combining heterogeneous inorganic catalysis with enzyme catalysis. The reaction involves the gold-catalyzed direct oxidation of hexoses and pentoses by molecular oxygen and the subsequent conversion of the sugar acids through an enzymatic dehydration step, such representing an efficient synthesis route toward bio-based building blocks that contain a vicinal methylene and keto group. By transferring the process from a one-pot batch reactor into a combined stirred tank/ plug flow system high yields were achieved for the 2-keto-3-deoxy forms of gluconate, arabinoate, xylonate as well as galactonate[1].

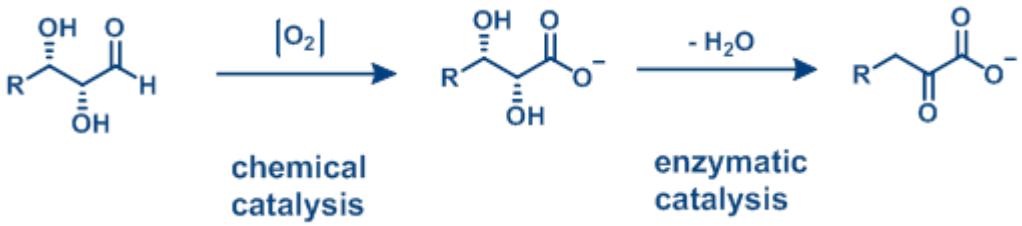

Figure 1: Reaction cascade for keto-deoxy-sugar synthesis

Keywords: chemoenzymatic synthesis, 2-keto-3-deoxy sugar acids, continuous flow

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AO2

Continuous-flow dynamic kinetic resolution of amines and amino acids in microreactor setups

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A continuous-flow cascade reactor system was applied to perform a protease (Alcalase)-catalyzed dynamic kinetic resolution (DKR) of *N*-Boc-phenylalanine ethyl thioester with benzylamine.[1] The thioester was transformed in a serial cascade system of six biocatalyst-filled columns at 50 °C (KR) and five silica gel-filled racemization columns at 150 °C yielding the product in 79% conversion and 98% ee (Figure 1). This was the first example of a DKR for an amino acid derivative in continuous-flow mode using an alternating cascade of packed-bed enzyme reactors and racemization reactors kept at different temperatures.

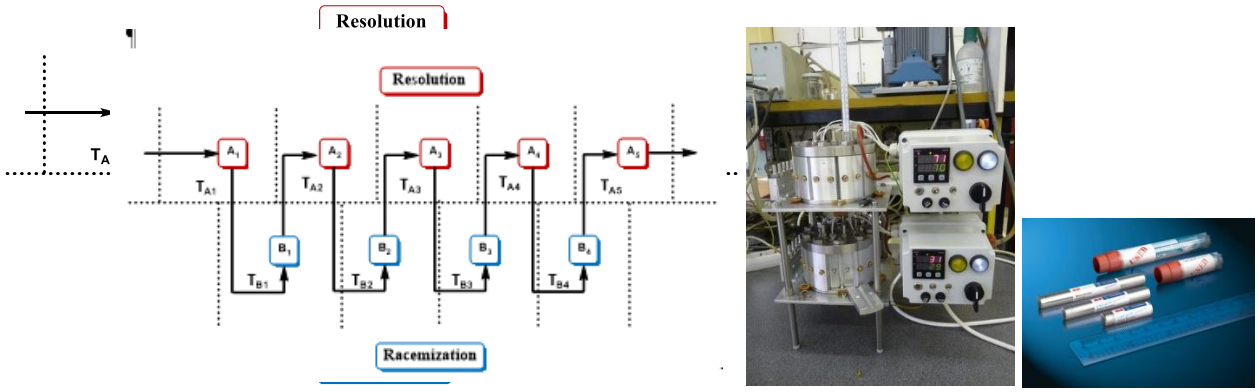

Figure 1: Alternating cascade of reactors for continuous-flow dynamic kinetic resolutions.[2]

The first full dynamic kinetic resolution (DKR) of 1-phenylethylamine under continuous-flow conditions was performed in a two reactor system consisting a lipase-filled and a lipase+racemization catalyst-filled packed-bed microreactor.[3] Six heterogeneous palladium catalysts were investigated in racemization of (*S*)-1-phenylethylamine using ammonium formate as hydrogen source in proper solvents. The most effective Pd-catalyst could perform an almost full racemization at 60 °C which allowed its combination with the immobilized lipase B from *Candida antarctica* (CaLB). The lipase mediated dynamic kinetic resolution (DKR) of 1-phenylethylamine was performed in fully continuous-flow mode using a mixed bed of the covalently immobilized CaLB and the best Pd-catalyst using isopropyl 2-ethoxyacetate as acylating agent resulting in the product in over 99% conversion with 91% isolated yield and 99.6% ee.

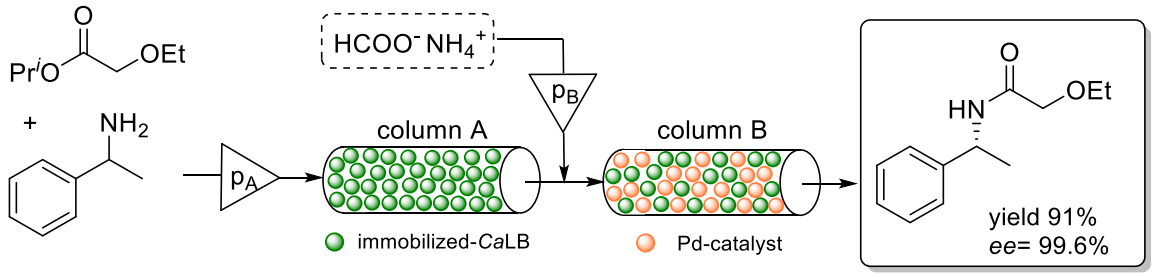

Figure 2: Continuous-flow chemoenzymatic dynamic kinetic resolution of 1-phenylethylamine.[3]

Keywords: Enzyme immobilization, Continuous-flow microreactor, Amine, Amino acid, Dynamic kinetic resolution

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AO3

Overcoming the challenges of cascading enzymatic microreactors

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Continuous processes have gathered a great interest in the last decade in the pharmaceutical industry due to the possibility to have reproducible conversions, and increased space-time yields. This is accomplished by a constant quality of the product output, the suppression of side reactions and by a wider operational window, among others. In processes that involve highly toxic, explosive or hazardous compounds, miniaturization can be a way forward to enhance operation safety and point-of-use generation of reagents. Furthermore, the reduction in scale can help to find novel routes for process intensification and ultimate improve process economics. Microreactors, with their rapid mass and heat transfer, small reaction volumes and short diffusion paths, are promising tools for the development of such processes. These tools have been widely applied in organic synthesis and are now attracting a broad interest at all stages of biocatalytic process design by providing promising synthetic methodologies. Nonetheless, the implementation of microreactors in an industrial set up has its challenges namely the control of reaction conditions.

In this contribution we will present results on how the challenges of implementing microreactor in an industrial environment are overcome, by compartmentalization of reaction conditions, recovery of product and monitoring of reaction progress. We will show how the production of 2-amino-1,3,4-butanetriol (ABT), an important building block of several antibiotics, antivirals and sphingolipids (1), is reduced from ~24 hours to solely 2 hours in a two-step biocatalytic conversion using transketolase and transaminase. Results on continuous production and recovery of chiral pharmaceutical intermediates will be shown, a showcase for the continuous production of high vale compounds.

We will demonstrate, for the first time, real-time pH monitoring of the progress of enzymatic reaction in multiple locations in a microfluidic system, as a first step towards achieving pH control (2). For this purpose, pH sensors with two different pH sensitive dyes (3) were integrated at several positions in the microreactor channel which allowed pH monitoring between pH 3.5 and pH 8.5. Time-course profiles of the reaction pH were recorded for a transketolase and a penicillin G acylase catalysed reactions.

The microfluidic side-entry reactor (μSER) employed (2) allows adjusting the pH of the reaction medium. With pH adjustment, the pH drop of the penicillin G acylase catalyzed reaction was significantly attenuated, the reaction condition kept at a pH suitable for the reaction, and concomitant product yield increased (Figure 1). This represents a further step towards fully instrumented and controlled microfluidic reactors for biocatalytic processes.

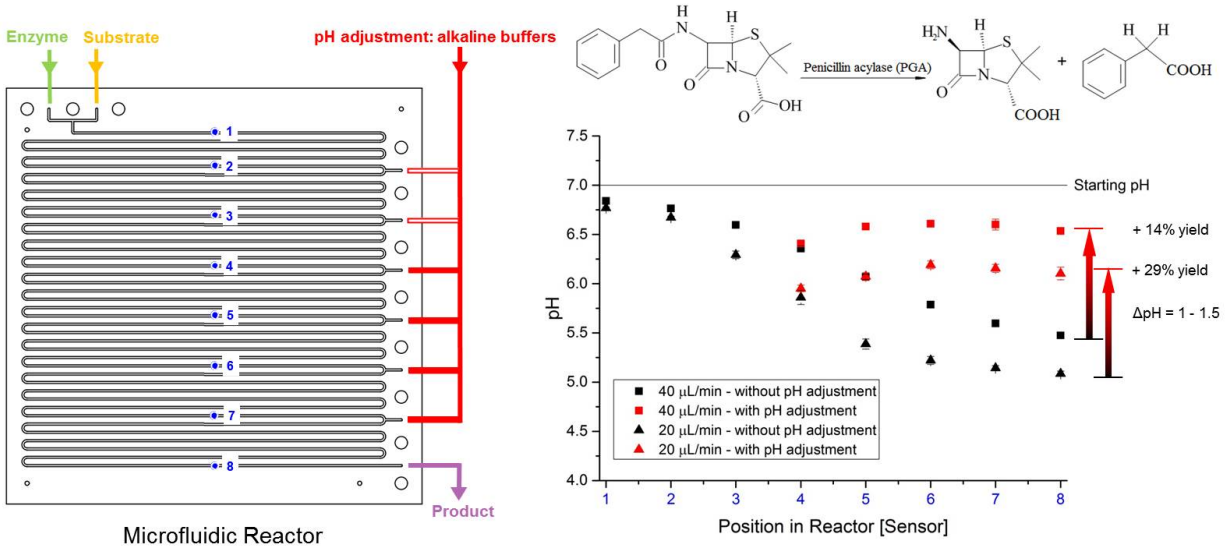

Figure 1: Microfluidic side-entry reactor schematic showing the readout positions of eight sensor spots, one after each side entry (left). pH adjustment of a penicillin G acylase reaction at two different flow rates showing that the stabilization of the pH in the ideal operating range of the enzyme leads to an increase in product yield (right).

Keywords: Optical sensors, microfluidic side-entry reactor, transketolase, penicillin G acylase, online monitoring, compartmentalization, transketolase, transaminase, chiral intermediates

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AO4

Characterization of single-microbeads as isolated microreactors for heterogeneous biocatalysis.

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Heterogeneous biocatalysis is an attractive alternative to perform chemical processes because enable the separation and recycling of the catalyst, facilitating in-flow reactions and thus increasing both efficiency and sustainability of the process (1). In this context, enzyme technologists are encouraged to develop operationally efficient and stable heterogeneous biocatalysts (2), however characterization of the supported enzymes at single-particle level is rather limited. A porous single particle immobilizing enzymes can be observed as microreactor where substrates and products diffuse in and out. Today, we are witnessing how single-cell analyses are aiding the advance of the synthetic biology in chemical manufacturing (3). However, the concept of single-particle is still unexploited in immobilized enzymes in spite of the similarities existing between the cells and particles forming a heterogeneous biocatalyst. In this work, we showcase different enzymatic systems that have been characterized at the level of single-beads to better understand the stability and the kinetic properties of the immobilized enzymes. In this task, fluorescence confocal microscopy and atomic force spectroscopy have allowed us understanding the local effects of the immobilization on the enzyme properties. By using single-bead studies, we have proved that the spatial localization of multi-enzyme systems across the microstructure of single beads determines the performance of certain reaction cascades (4). Moreover, using fluorescence anisotropy and force spectroscopy of single-beads we have discovered the effect of different immobilization chemistries on the stability of the immobilized enzymes (5,6).

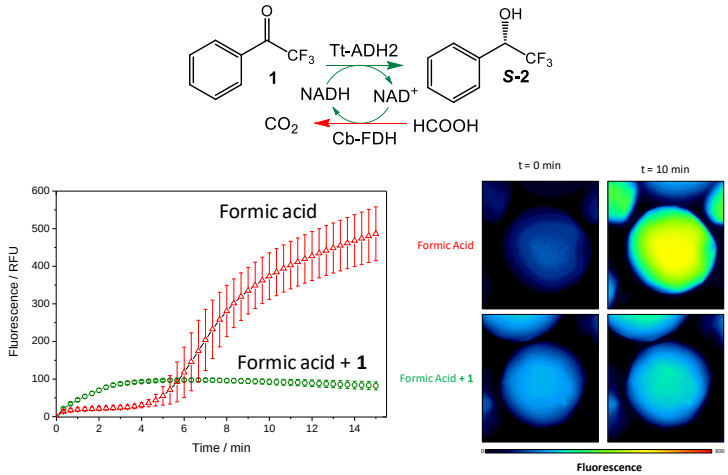

Figure 1. Single-bead kinetics of a bi-enzyme cascade co-immobilized with NAD+ on the porous agarose microbeads.

Most recently, we have used confocal laser scanning microscopy to run single-bead reactions in presence of labeled substrates or cofactors for *in operando* studies that unveil mass transfer or inactivation issues provoked by the immobilization protocol (7) (Figure 1). All these data have been highly valuable for us in order to guide the fabrication of highly efficient heterogeneous biocatalysts. Therefore, the use of analytical techniques at the level of single-beads enormously contributes to the understanding of the heterogeneous biocatalysis and pursues shinning light into the "black-box" of the protein immobilization. On top of that,

running single-beads of porous materials as individual microreactors can open a new path for process intensification and for lad-on-a-chip design with interesting application in chemical manufacturing, biosensing or biomedicine.

Keywords: Enzyme immobilization. *In operando* studies. Multi-enzyme systems. Alcohol dehydrogenase. Lipase.

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AO5
Wall-coated biocatalytic microreactors for bioprocess intensification: opportunities and challenges

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Like in chemical catalysis, there is the clear trend to shift bio-chemical reactions from batchwise to continuous and heterogeneously catalyzed transformations. Recycling of catalysts is simplified and continuous intensified reactor development thus promoted. Microstructured flow reactors constitute an emerging class of microprocess engineering tools for this development.[1] Exploiting microscale effects, microreactors promise intensification of reactions otherwise limited by mass transfer. However, full realization of the intensification requires careful integrated design of heterogeneous catalyst and reactor. First, that implies the achievement of high surface-bound biocatalytic activity (*Cat*) that determines maximum space-time yield (*STY_max*). Second, the degree to which the *STY_max* is utilized depends on how well the fluidics of the microreactor is matched to the requirements of the reaction. This presentation will show advances in the design and characterization of surface-immobilized enzymatic microreactors.

Discussion is built around the comprehensive development of effective O_2-dependent heterogeneous biocatalyst and the transfer of the operation from batchwise to continuous flow. The achievement of high *Cat* is restricted by insufficient knowledge of physical and biochemical factors governing performance of heterogeneous enzyme catalysts.[2] We show how conjoined design of surface and protein is used to facilitate enzyme loading in high quantity and quality on solid phase. [3,4] *In-operando* opto-chemical internal sensing is developed to characterize the biocatalyst's local environment. [3,4] In situ direct determination of the internally available O_2 concentration enables to assess the relative importance of physical and biochemical effects, and distinguish scenarios of pronounced loss of intrinsic activity from diffusional control.[3] Target-oriented selection of porous materials for immobilization is enabled and geometrically mesostructured silica materials enhanced (≥ 10-fold) the catalytic effectiveness at high *Cat* via mass transport intensification.[4] The intensification of *Cat* via enzyme confinement into solids stimulates the idea of miniaturizing the reactor such that microscale effects can be captured and further exploited in a suitable microstructured reactor design. Wall-coated microreactors are used for that purpose.[5] To evaluate the realization of *STY_max* at high conversion efficiency in flow operation, we will show the usefulness of time-scale analysis in which three characteristic times, that is, the mean residence time, the reaction time, and the diffusion time, are considered and their interplay in defining the reactor output is analyzed. Interplay between catalytic surface coating, reactor miniaturization and mas transfer requirements will be discussed and further opportunities for process intensification will be identified.

Keywords: hererogeneous biocatalyts, enzyme immobilized microreactors, oxygen dependent transformations, process intensification, G-L-S transformations.

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AO6

Theoretical and experimental study of surface enzyme kinetics in the microreactor system with immobilized ω-transaminase

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Biocatalytic production of optically pure amines offers high enantio- and regioselectivity and presents a sustainable alternative as opposed to traditional chemical production, which requires the use of toxic and environmentally unfriendly transition metal catalysts.[1] Biocatalysts known so far to produce chiral amines are hydrolases, oxidoreductases, and transferases.[1] Among them, the ω-transaminases (EC 2.6.1.X) have lately been gaining increased attention, due to being the only naturally occurring enzymes capable of stereoselective amination of ketones.[1]

Microreactor technology offers a promising alternative to established production systems and has been successfully introduced in various chemical industries.[2] Continuous process operation, mostly laminar fluid flow, enhanced mass and heat transfer, high surface to volume ratio and a new concept of increasing the capacity by numbering up approach are among major benefits making microscale reactors very promising tools also for biocatalytic processes.[2] For development of continuous bioprocess, biocatalyst immobilization is preferred, since it enables facilitated product separation, as well as the possibility of biocatalyst recovery and reuse.[3]

Microreactor system with surface-immobilized ω-transaminase was developed and used for conducting continuous biotransformation. Enzyme immobilization employs Z_{basic2}-tag appended to the enzyme and is based on ionic interactions between the tag and silicon/glass microchannel surface.[3] To describe the enzyme-catalyzed transamination of (*S*)-(-)-α-methylbenzylamine and pyruvate to acetophenone and L-alanine, the ping-pong bi-bi mechanism was followed.[4] For estimation of enzyme kinetic parameters, batch experiments with free biocatalyst were performed. The time-scale analysis based on a detailed mathematical model combining transport phenomena and surface enzymatic reaction in the microreactor system (Fig. 1) was applied to characterize studied continuous bioprocess with a heterogeneous surface enzyme kinetics.[5] The proposed model was then simplified and at the same time used to define the surface concentration of the immobilized enzyme.[5] Very good agreement between experimental data and predictions of developed microreactor model is observed for various inlet substrates concentrations and different flow rates. Furthermore, the verified model was validated based on additional experiments within two consecutively-connected microreactors with surface-immobilized ω-transaminase.

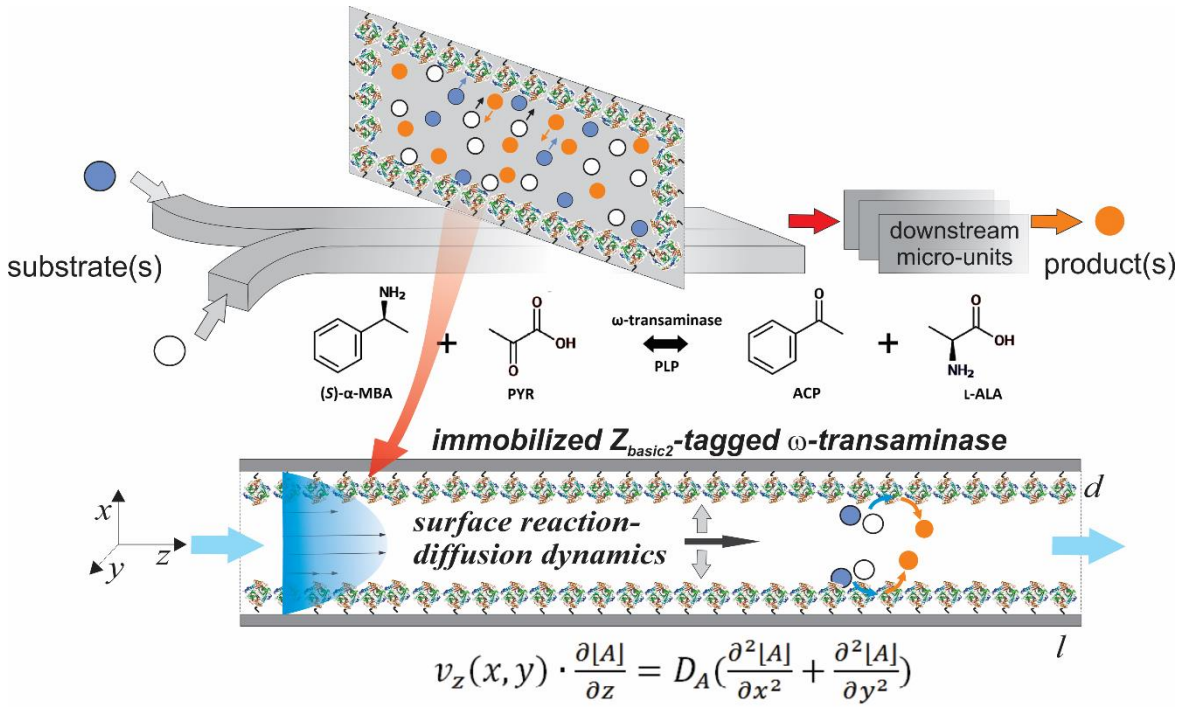

Figure 1: Schematic presentation of the microreactor system with surface-immobilized ω-transaminase.[5]

Keywords: microreactor, mathematical modelling, surface-enzyme kinetics, enzyme immobilization, transamination

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AP1

Multienzymatic and stereoselective cascade process for the synthesis of 2,3-disubstituted tetrahydrofuran precursors: preparation of the most odorous and pleasant roasted meat aroma stereoisomer

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Enantiopure 2-methyl-3-substituted tetrahydrofurans are key synthons for the production of several biologically active molecules such as drugs, flavors, and agrochemicals. Thus, a stereocontrolled and efficient methodology for the obtainment of these products is highly desirable . In this work ene-reductases and alcohol dehydrogenases are used in a two-step multienzymatic cascade reaction (1) for the stereoselective reduction of different α-bromo-α,β-unsaturated ketones to the corresponding bromohydrins. The final products are further manipulated following two diastereodivergent routes, one based on a lipase catalyzed cleavage of the protecting group and the second characterized by a camphor sulfonic acid mediated isomerization of a β-hydroxyepoxide to give the tetrahydrofuran-2-ol and allowed the preparation of different tetrahydrofuran synthons. One of these is finally used for synthesis of the most odorous and pleasant stereoisomer of the roasted meat aroma, i.e., (2S,3R)-2-methyl-3-thioacetate tetrahydrofuran (2).

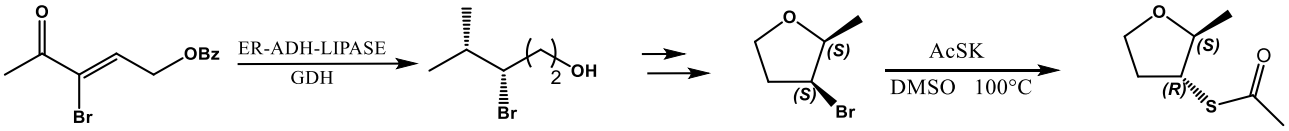

Figure 1: synthetic route for the preparation of th most pleasant stereoisomer of roasted meat aroma.

Keywords: Ene-reductase, alcohol dehydrogenase, cascade reaction, 2-methyl-3-substituted tetrahydrofurans,

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AP2

Use of silica nanosprings and electrospun nanofibers for the development of wall-coated enzyme microreactors

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Wall-coated enzyme microflow reactors have received significant attention towards the realization of continuous flow biocatalytic conversion.[1,2] However, the immobilization of sufficient amounts of enzymes into microreactors is a considerable challenge for development. To overcome this challenge, the enhancement of the binding capacity of the reactor's internal surface is necessary. The integration of high surface area nanomaterials into microchannels appears to be a solution.

In this work we suggest two approaches for increasing the internal surface area within microchannels: the integration of silica nanosprings (NS)[3] and electrospun-metal nanofibers.[4] NS-coated glass slide was integrated in a 2-plate microreactor and functionalized with sulfonic acid groups (NS-SO_3H). At the applied pH, the sulfonate groups act as a negatively charged linker for binding sucrose phosphorylase from *Bifidobacterium longum* (*Bl*SPase) fused to a strongly positively charged mini-protein called Z_{basic2}. This was used as the model enzyme. NS-SO_3H-microflow reactor showed excellent performance metrics with regard to the productivity and the operational stability compared with the untreated glass microreactor. Different mass of NS loadings were used for the enzyme immobilization. The effect of mass transfer in the internal structures on the performance of the microreactor was studied. Mathematical modeling was used to theoretically describe the system.

In the second approach, electrospun-metal nanofibers were integrated into a 2-plate microreactor. In this case, *Bl*SPase fused to His-tag was immobilized on the microchannel surface. The immobilization is based on the coordination bonds between Cu^{2+} ion embedded in the nanofibers and a His-tagged protein. The binding capacity for the enzyme was evaluated and tested for selected biotransformation.

Acknowledgement

Financial support of the EU FP7 project EUROMBR (Grant Agreement No. 608104), COST Action CM1303 Systems Biocatalysis, and of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia through Grants P2-0191 and BI-US/15-16-049 is acknowledged.

Keywords: microreactors, silica nanosprings, electrospun nanofibers, enzyme immobilization, sucrose phosphorylase

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AP3

Synthesis of biodiesel from waste chicken oil using an immobilized methanol-stable lipase

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Biodiesel offers a promising solution for the energy crisis by being a sustainable and renewable alternative for fossil fuels using a wide range of feedstocks. We have previously engineered a lipase from *Geobacillus stearothermophilus* T6 for stability in methanol, obtaining variant H86Y/A269T/R374W (LipT6$_M$) with an 87-fold improved half-life in 70% methanol.[1] In the present study our goal was to convert the methanol-stable lipase variant LipT6$_M$ into a durable, reusable, and economical catalyst for biodiesel synthesis by investigating several immobilization strategies including entrapment in sol-gel, covalent attachment and adsorption to synthetic resins.

Two ternary sol-gel matrices, an octyltriethoxysilane (OTEOS) based aliphatic matrix, and a phenyltriethoxysilane (PTEOS) based aromatic matrix, were evaluated. In addition, the influence of skim milk (SM) and soluble *E. coli* lysate proteins as bulking and stabilizing agents in conjunction with sol-gel entrapment were investigated for the first time (Figure 1). Covalent attachment was tested with epoxy methacrylate, epoxy-butyl methacrylate, and amino methacrylate resins, while adsorption was investigated with octadecyl methacrylate and divinylbenzene methacrylate porous particles. All systems were tested in a hydrolysis assay, esterification activity in hexane, recycling, and biodiesel synthesis from waste chicken oil.

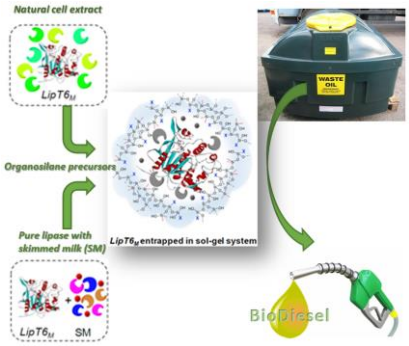

Figure 1: Sol-gel immobilization of LipT6$_M$ for biodiesel synthesis from waste chicken oil.

The immobilization in a sol-gel matrix with phenyl side chains was found to be highly beneficial in terms of recyclability and productivity. The highest activity was obtained by entrapment of the crude *E. coli* cell lysate containing the expressed enzyme, therefore avoiding costly purification efforts and downstream processing. Evaluating two sol-gel matrices having a different hydrophobic nature, revealed diverse silica networks preferences of the same enzyme in its pure form compared to its crude extract state. The presence of protective agents such as SM powder and *E. coli* soluble proteins was found to promote the esterification activity significantly, albeit to a different extent, dependent on the sol-gel matrix properties.[2] The enzyme entrapped in the PTEOS matrix was successfully used for the synthesis of biodiesel from waste chicken oil on a 1-L scale reaching 82% conversion (Figure 2).

Among the systems evaluated for immobilization of the purified enzyme, adsorption to octadecyl methacrylate enabled the highest esterification activity. Nevertheless, the immobilized enzyme lost 75% of its activity in the third cycle. The enzyme immobilized covalently to epoxy-butyl methacrylate was both highly active and

maintained activity in several cycles. Current attempts to improve this covalently immobilized lipase focus on bioimprinting with detergents and substrate-analogs known to have a positive effect on lipases activity.

Figure 2: One-liter bioreactor used for the synthesis of biodiesel from waste chicken oil with sol-gel entrapped $LipT6_M$.

This work was supported by COST Action Systems Biocatalysis, CM 1303.

Keywords: lipase, biodiesel, waste oil, bioreactor, sol gel, COST

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AP4

Functionalized electrospun mats integration in a microreactor for ω-transaminase immobilization

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Microscale technology presents great opportunity for establishment of continuous biocatalytic processes[1]. Very high surface to volume ratio and small dimensions enabling efficient mass transfer in microreactors present a huge potential for surface enzyme immobilization, which is often prerequisite for its competent use. In order to achieve high enzyme loads and stable, but also readily reversible surface immobilization to allow re-use of the microstructured element, functionalization of biocatalysts and inner surfaces is employed.[2]

In this work, an aminotransferase ATA-wt (c-LEcta, Germany) was genetically modified with poly(His)-tag to obtain His-ATA-wt. In order to assemble a microflow reactor, commercially available resealable flow cell platform (Micronit, The Netherlands) was integrated with electrospinning-based nanofiber mat Tiss®-NH_2 (NanoMyP®, Spain) functionalized with tris(carboxymethyl)ethylene diamine and loaded with Cu^{2+} (Figure 1a). After integration of a membrane in the microreactor, the evaluation of reactor volume and efficiency of His-ATA-wt retention after the exposure to enzyme aqueous solution was performed using high pressure syringe pumps for entering the fluids. Furthermore, microreactors with immobilized enzyme were further tested for selected transamination reaction, where an in-line HPLC analysis was used to evaluate reactor performance at various flow rates and substrate concentrations. The stability of a microreactor with immobilized enzyme was tested by performing the continuous biotransformation 24 and 48h after the immobilization.

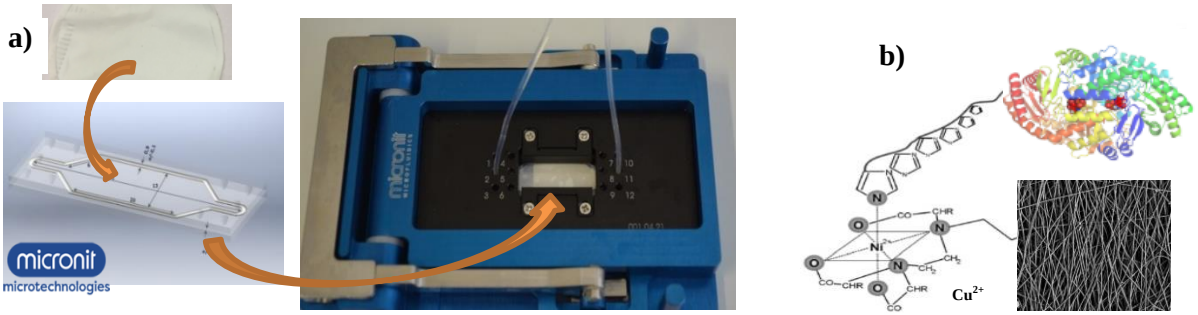

Figure 1: a) Assembly of a resealable flow cell microreactor with nanofiber mat, and b) immobilization principle.

Based on a rather simple and short immobilization procedure, where His-tagged protein was linked to nanofibers with Cu^{2+} based on coordinative bonds (Figure 1b), we were able to immobilize up to 6.6 mg/mL of protein in the microreactor of 68.4 μL internal volume. Up to 90 % conversions of 40 mM (*S*)-(-)-α-methylbenzylamine and 40 mM sodium pyruvate to L-alanine and acetophenone were achieved at 30 °C in the presence of pyridoxal 5'-phosphate within the residence time of 7 min, which shows very high volumetric productivity of the developed enzymatic microreactor. Furthermore, above 90 % of initial productivity could be retained within the tested period.

Acknowledgement

Financial support of the EU FP7 projects BIOINTENSE (Grant Agreement No. 312148) and EUROMBR (Grant Agreement No. 608104), COST Action CM1303 Systems Biocatalysis, and of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia through Grant P2-0191 is acknowledged. S.M. is grateful for the support through the Erasmus+ Programme. The authors are thankful to c-LEcta, Germany, for providing ATA-wt enzyme.

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AP5

Study on scale-up of miniaturized packed bed reactor for ω-transaminase-catalyzed chiral amine synthesis

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Production of bioactive, enantiomerically pure compounds either by direct asymmetric synthesis from prochiral substrates or by kinetic resolution of racemic compounds using biocatalysis as highly selective, diverse and environment-friendly approach, is gaining increased attention. ω-transaminases, which are pyridoxal-5′-phosphate-dependent enzymes that catalyze transfer of amino functionalities from amine donors to ketone groups, were shown to be very effective catalysts in the production of chiral amines, widely used as highly valuable products or key intermediates in the production of optically pure compounds.[1,2]

In order to develop economically relevant biocatalytic process, continuous operation with immobilized biocatalyst is typically beneficial. Packed bed columns with enzymes or cells entrapped within porous particles are often a matter of choice, although problems with transfer limitations within particles and fluid channelling in real reactors might prevent efficient exploitation of biocatalysts. Polyvinyl alcohol lens-shaped particles prepared by LentiKats' methodology, which enable efficient immobilization of biocatalytic material in a porous matrix possessing excellent physico-mechanical characteristics and at the same time providing very good biocatalyst activity and stability, are suitable for application in packed bed reactors.[3,4]

In this study, LentiKats® with immobilized ω-transaminase were used within a reusable miniaturized packed bed reactor (MPBR) consisted of a laminated system of polymeric materials creating microchannels. A systematic scale-up of the MPBR was achieved by increasing individual dimensions of the rectangular- and hexagonal-shaped channels (Figure 1), and reactors were evaluated regarding their efficiency using model transamination reaction. Developed MPBR was also used to establish temperature dependence of the reaction catalyzed with immobilized ω-transaminase yielding 55 °C as an optimal temperature. Furthermore, operational stability of the MPBR with ω-transaminase immobilized in LentiKats® was tested under the continuous flow during the period of 21 days revealing over 80% retained initial productivity. The benefit of using miniaturized reactors in process development study which could be performed in a very short time and at low chemicals consumption was confirmed.

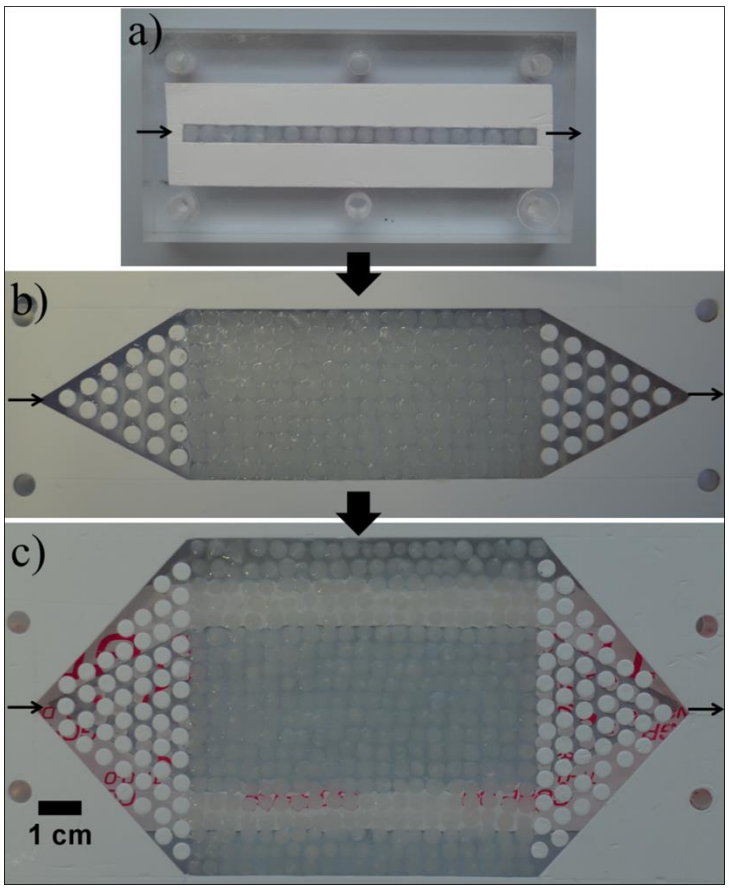

Figure 1: MPBRs presenting the scale-up in channel width, where length and depth were the same: a) an MPBR with cca. 4 mm wide rectangular channel, b) an MPBR with hexagonal channel (rectangular part is cca. 40 mm wide) with triangular inlet and outlet parts containing pillars, and c) an MPBR with hexagonal channel (rectangular part is cca. 80 mm wide) with triangular inlet and outlet parts containing pillars.[4]

Keywords: Miniaturization, Biocatalysis, Immobilization, Packed bed, Scale-up

Acknowledgement

Financial support from the EU FP7 Project BIOINTENSE – Mastering Bioprocess Integration and Intensification across Scales (Grant No. 312148) is acknowledged, while M. Bajić was supported through Ad futura scholarship program provided by the Slovene Human Resources Development and Scholarship Fund (Grant No. 11011-73/2013). ω-transaminase was kindly provided by c-LEcta (Leipzig, Germany). Authors acknowledge Dr. U. Novak and A. Belovič for their generous help in experimental work.

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AP6

Bioreactor for the continuous purification of simvastatin by lovastatin esterase

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The natural product lovastatin and its semisynthetic, more effective derivative simvastatin, are clinically useful drugs for the treatment of hypercholestemia. Generally, chemical synthesis of simvastatin leads to the mixture of those compounds in a 90:10 ratio. This mixture cannot be used as a drug due to the rules of European Pharmacopoeia and it requires separation. This mixture of statins obtained in a chemical step can be readily transformed into ammonium salts and used for biotransformation step (Figure 1). In order to achieve good yield and productivity an enzyme lovastatin esterase should be immobilized on solid support. An efficient separation method based on immobilized lovastatin esterase was employed in a packed bed bioreactor. Moreover, chromogenic substrate has been designed, synthesized and examined as a probe for determination the lovastatin esterase specific activity.[1]

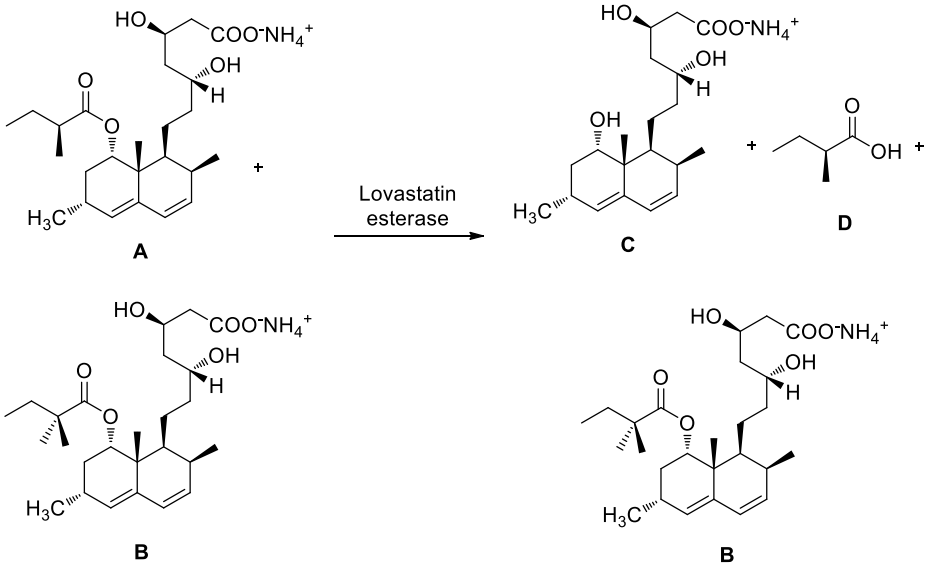

Figure 1: Generation of monacolin J ammonium salt (**C**) and 2-methylbutanoic acid (**D**) during selective hydrolysis of lovastatin ammonium salt (**A**) in the presence of simvastatin ammonium salt (**B)**

The results of synthesis of a series of a new fluorescent probes for assaying lovastatin esterase will be shown together with its evaluation. A details of complete process leading to pure simvastatin using continuous flow bioreactor from lovastatin will be discussed.

We gratefully acknowledge the financial support of the NCN projects 2013/14/M/ST5/02199, 2014/14/M/ST5/00030 and the COST action CM1303.

Keywords: lovastatin, biotransformations, esterase, bioreactor, hydrolysis

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AP7

Simplified immobilisation of histidine-tagged enzymes in poly(methyl methacrylate) microreactors

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Microfluidic reactors, particularly Immobilised Enzymatic Microreactors (IEMR) have drawn considerable attention in recent years as an alternative to complex and time-consuming traditional reactor technologies. IEMRs can offer improved stability and durability, ability of enzyme reuse and removal of product from the reaction system.[1] Despite the many advantages of IEMRs, considerations of microreactor fabrication cost and efficiency of enzyme immobilisation in such systems still exist. Effective immobilisation methods are required to expand the range of enzyme classes that can be used in microfluidic reactors and to maximize stability of the enzyme and amount that can be loaded. In particular, immobilisation methods are required that are fast and easy to implement in rapidly prototyped IEMRs, and which provide enzyme immobilisation that does not constrain the fluidics in the chip.

As polymer-based microreactors are largely used in fast-prototype fabrication, we developed a simplified immobilisation method for histidine-tagged enzymes in a microreactor made out of poly(methyl methacrylate) (PMMA).[2] This 1-step-immobilisation is based on the affinity method of His-tag/Ni-NTA interaction and is achieved by treating a PMMA surface with nitrilotriacetic acid (NTA) without the need for prior amination (Figure 1). This method was compared to the 3-step method involving amination of PMMA, and linking NTA *via* a glutaraldehyde cross-linker shown in Figure 1A.

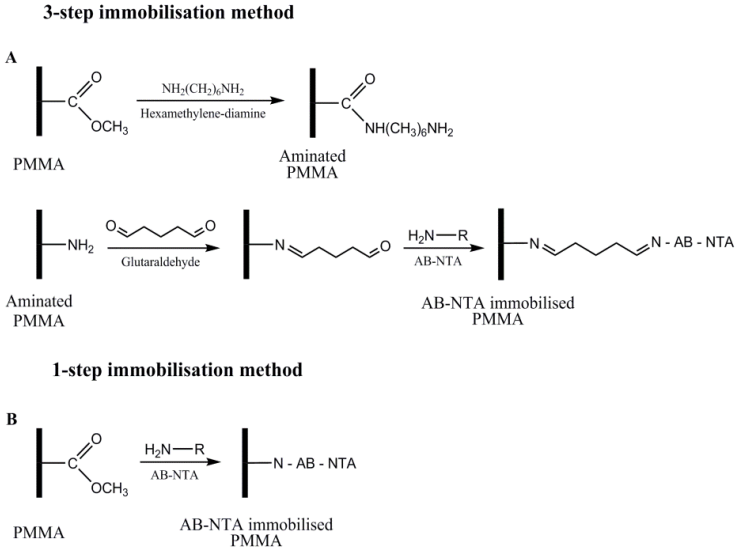

Figure 1: 1-step and 3-step immobilisation chemistries on poly(methyl methacrylate) (PMMA) surface. (A) 3-step immobilisation chemistry. The first line corresponds to amination chemistry of the surface. The available methyl esters of PMMA, under basic pH conditions, are reacted with an electron donor (N) present on the hexamethylene-diamine (HMDA), producing primary amines on the surface. Second line of the scheme represents the linking step of primary amine bonds formed on PMMA surface with amine bonds of AB-NTA molecule using the cross-linker glutaraldehyde. (B) 1-step immobilisation chemistry. AB-NTA molecule substitutes the HMDA step corresponding to 3-step immobilisation procedure, and amine bonds of AB-NTA molecule react directly with the available methyl esters on the PMMA surface, formed under basic pH conditions. These 1-step and 3-step immobilisation procedures produce a functionalized PMMA surface that subsequently is used for immobilising histidine-tagged enzymes.

Additionally, enzyme binding efficiencies of the two immobilisation techniques in microreactors were studied using a His-tagged transketolase (TK) and results showed approximately 7% of the TK supplied was bound using the 1-step method and approximately 11% of the enzyme bound with the 3-step method. To evaluate the catalytic activity, a TK-catalysed conversion of hydroxypyruvate and glycolaldehyde to erythrulose was performed in the microreactors prepared using the two immobilisation methods (Figure 2). Specific activities of TK in microreactors utilizing 1-step and 3-step immobilisation methods gave similar values of 109±14 and 114±19 nmol·mg^{-1}·min^{-1}, respectively. Additionally, the reusability of the microreactor produced *via* the 1-step immobilisation method was tested and the immobilisation surface was shown to be reusable with at least 85% of initial productivity being maintained over three cycles.

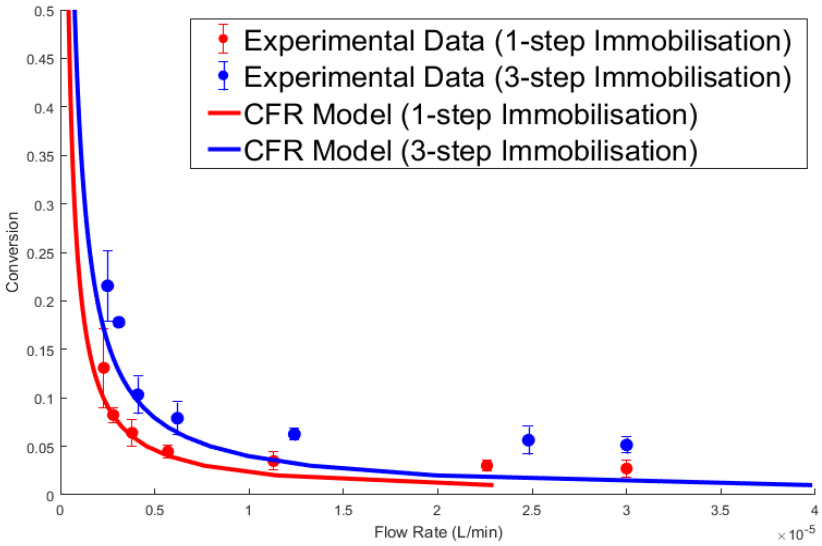

Figure 2: Conversion yield of GA/HPA to L-ERY at 4°C as function of flow rate in the poly(methyl methacrylate) (PMMA) microfluidic reactors. Experimental data - Comparison of product formation obtained from microreactors with transketolase immobilsed via 1-step and 3-step immobilisation techniques. The specific activities values derived from this data were 109±14 and 114±19 nmol·min^{-1}·mg^{-1} for 1-step and 3-step immobilisation, respectively. Error bars represent one standard deviation above the mean (n=2). Model (red and blue lines) - Data fitted for the steady state conversion at varying flow rates.

Overall, the 1-step immobilisation method requires less chemicals and time for preparation of the microreactor surface compared to the 3-step method while achieving similar enzyme binding efficiency, specific activity and conversion yields. The presented work is of wider interest as this method could potentially be applied for immobilisation of enzymes on to other polymer surfaces (e.g. polycarbonate, cyclic olefin copolymers) that have similar properties to those of PMMA and are commonly utilised for fabrication of microreactors, point of care and lab-on-chip devices.

Keywords: microfluidics, enzyme immobilisation, microreactor, histidine-tagged enzyme immobilisation, poly(methyl methacrylate)

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AP8

Substituent and catalyst effects on GAC lactonization of γ-hydroxy esters

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Intramolecular reactions affording cyclic compounds are key-transformations in organic chemistry. To better understand the factors that control the reaction rate of this important class of reactions, the acid catalysed ring closure of γ-hydroxyesters in a non protic solvent was investigated. First, a ^{1}H-NMR kinetic study of a set of monomethyl and/or gem-dimethyl substituted esters in CDCl3 was carried out. We evaluated the effect of the leaving group (ethyl vs. i-propyl ester) and the catalyst efficiency. We found that i) a monomethyl substitution produces a lowering of the energy barrier similar to that of a gem-dimethyl substitution (Thorpe–Ingold effect), ii) the ring closure of i-propyl esters is slower than that of ethyl esters, iii) strong acids are more efficient than weak acids according to the Brønsted relationship, and iv) the Thorpe– Ingold effect is not just an intrinsic feature of the linear precursor but depends on the catalyst as well.

With this purpose, we have studied the acid catalysed lactonization of several γ–hydroxyesters, *e.g.* **2a-d**, bearing methyl substituents at different positions. Interestingly, the lactone formation kinetic studies show that the observed reaction rate constant of **2b** is comparable to that of the *geminal* dimethyl ester **1c**, and both ring closures are faster with respect to that of the unsubstituted hydroxyester, *i.e.* **1a**.

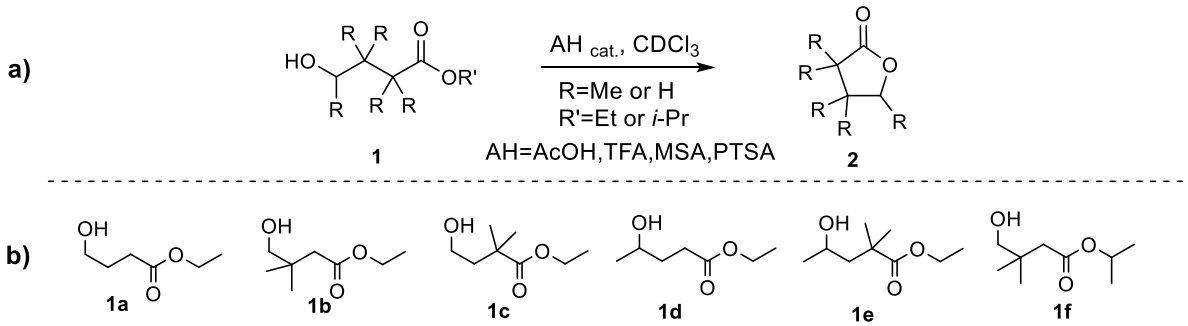

Figure 1: **a)** general ring closure reaction of γ-hydroxyesters; **b)** all the hydroxyesters synthesized

Quite surprisingly not so many reports about the effect produced by the presence of two substituents in a vicinal relationship are present in literature.

So, prompted by this strange "black hole" in literature, we would like to give an explanation and clarify two issues: i) how is the cyclization affected by the vicinal substitution? ii) is the effect stereospecific, that is to say, dependent on the diastereomeric relation (syn or anti) between the two vicinal substituents?

Preliminaries studies on model compounds indicate that the vic-disubstituent effect is not only stereospecific, since the anti linear precursors undergo the cyclisation reaction much faster than the corresponding syn diastereoisomers, but also much more efficient than the above mentioned Thorpe-Ingold effect. Indeed, the anti vicinal substituted adduct is faster than the gem-disubstituted of about 4 times.[3]

One of the most interesting application of this effect is the activation of prodrugs via a cyclization pathway. In Figure 2 is reported an analogous prodrug system based on a twice vic-disubstitutuent effect. A specific enzyme catalyses the cleavage of the ester group, then the free hydroxyl group attacks the carbonyl group to give the lactone and releasing the drug (Figure 2). The last step has to be very fast in order to release the drug instantaneously.

It allows a fine tuning of the rate of drug release through the appropriate choice of the functional groups involved in ring closure and stereoelectronic constraints in the course of the cyclization step.

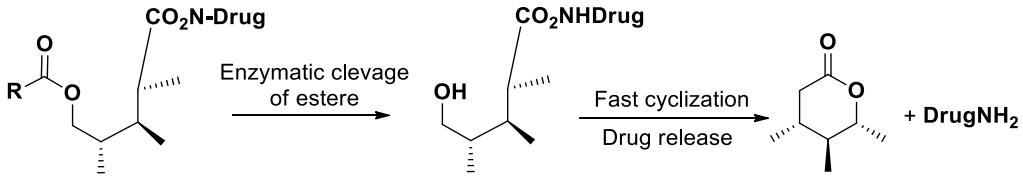

Figure 2: Prodrug system based on a twice vic-disubstitutuent effect

Keywords: lactones, *vic*-disubstituent effect, kinetics, prodrug delivery, γ-hydroxyesters

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COST ACTION CM1303- SYSTEMS BIOCATALYSIS

AP9

Magnetic cross-linked enzyme aggregates (mCLEAs) in microreactors towards glycocompounds production

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Magnetic cross-linked enzyme aggregates (mCLEAs) have emerged as interesting biocatalyst. The CLEAs technology present many advantages for industrial applications, as it is simple and amenable to rapid optimization, leading to low costs and short time to-market processes. One problem is the separation of CLEAs from reaction media. Centrifugation or filtration has been reported for separation of CLEAs from reaction mixture.

In this work the goal was the development of magnetic CLEAs of naringinase and □-D-glucosidase for the production of glycosides, in microreactors. To attain that goal, naringinase (NGase) and glucosidase (GLase) were obtained from different fungi (e.g. *Aspergillus niger* and *Penicilium* sp). Several parameters were studied in the production of enzymes towards activity optimization, namely: type of fungi, inducers, time, use of (non)specific substrates.

NGase_mCLEAs and GLase_mCLEAs were produced and biocatalysts activity was evaluated following the purification steps. The CLEAs and mCLEAs were evaluated in different pH, temperature, protein loading and substrates concentration. It was observed that mCLEAs enhanced the enzymes stability. Moreover, the mCLEAs retained almost 100% initial activity even after several cycles of reuse. These results were compared with commercial naringinase. The influence of different cross-linking agents and protein feeder (albumin) on comercial NGase cross-linked enzyme aggregates (CLEAs) and mCLEAs development was evaluated. Novel magnetic cross-linked enzyme aggregates of NGase were prepared by chemical cross-linking of enzyme aggregates with functionalized magnetite nanoparticles which was separated from reaction mixture using magnetic field. The mCLEAs were evaluated in a microreator (1 mL) with the production of different glycosides (naringenin, prunin, quercetin). Moreover, the mCLEAs retained almost 90% initial activity even after 7 cycles of reuse.

Key-words: Naringinase, Glucosidase, Magnetic CLEAS, Glycosides

This participation is in the frame of the COST Action CM1303 SysBiocat.

AP10

Use of silica nanosprings and electrospun nanofibers for the development of wall-coated enzyme microreactors

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Wall-coated enzyme microflow reactors have received significant attention towards the realization of continuous flow biocatalytic conversion.[1,2] However, the immobilization of sufficient amounts of enzymes into microreactors is a considerable challenge for development. To overcome this challenge, the enhancement of the binding capacity of the reactor's internal surface is necessary. The integration of high surface area nanomaterials into microchannels appears to be a solution.

In this work we suggest two approaches for increasing the internal surface area within microchannels: the integration of silica nanosprings (NS)[3] and electrospun-metal nanofibers.[4] NS-coated glass slide was integrated in a 2-plate microreactor and functionalized with sulfonic acid groups (NS-SO_3H). At the applied pH, the sulfonate groups act as a negatively charged linker for binding sucrose phosphorylase from *Bifidobacterium longum* (*Bl*SPase) fused to a strongly positively charged mini-protein called Z_{basic2}. This was used as the model enzyme. NS-SO_3H-microflow reactor showed excellent performance metrics with regard to the productivity and the operational stability compared with the untreated glass microreactor. Different mass of NS loadings were used for the enzyme immobilization. The effect of mass transfer in the internal structures on the performance of the microreactor was studied. Mathematical modeling was used to theoretically describe the system.

In the second approach, electrospun-metal nanofibers were integrated into a 2-plate microreactor. In this case, *Bl*SPase fused to His-tag was immobilized on the microchannel surface. The immobilization is based on the coordination bonds between Cu^{2+} ion embedded in the nanofibers and a His-tagged protein. The binding capacity for the enzyme was evaluated and tested for selected biotransformation.

Acknowledgement

Financial support of the EU FP7 project EUROMBR (Grant Agreement No. 608104), COST Action CM1303 Systems Biocatalysis, and of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia through Grants P2-0191 and BI-US/15-16-049 is acknowledged.

Keywords: microreactors, silica nanosprings, electrospun nanofibers, enzyme immobilization, sucrose phosphorylase

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Session B: CELLS WITHIN MICROREACTORS

BK

Parallel shaken bioreactor systems with advanced on-line measuring techniques partially replace lab-scale stirred tank bioreactors

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A problem in the past has been the absence of suitable on-line monitoring techniques for small scale bioreactors (shake flasks and microtiter plates). In screening projects, it is a very common practice that for the evaluation of the "best performers" only the final product titre is measured at the end of a somehow chosen culture time. However, with this approach there is no chance to obtain information on the specific peculiarities of the studied microbial system. In recent years several approaches were introduced to on-line follow microbial cultures also in shaken bioreactors like shake flasks or microtitre plates (MTPs). A precise and robust on-line monitoring technique (termed RAMOS) for oxygen and carbon dioxide transfer rate (OTR, CTR), and respiratory quotient (RQ) have been developed. In addition, the BioLector technique allows quantification of optical density (OD), pH-value, dissolved oxygen tension (DOT), and product (if this is a protein fused to a fluorescent marker). Applying these techniques it became obvious that the cultures can behave quite unexpectedly and counterintuitively. Most relevant and essential information is lost, if only the final product titre at the end of the cultures is utilized for evaluation.
The most valuable parameters from a bioreactor, oxygen and carbon dioxide transfer rate (OTR, CTR), and the respiratory quotient (RQ) were hitherto only available on shake flask level. Very recently a new technique has been developed which allows the assessment of the oxygen transfer rate in each individual well of a 48 well micro titre plate (μRAMOS). This opens completely new options for experimental strategies and bioprocess development. The approach can even be upgraded by combining the μRAMOS and the BioLector technique. In this way, essentially all the parameters which are currently only available from single well-equipped lab-scale stirred tank bioreactors can be provided in parallel from every well of a microtiter plate. The consistency of the data from these MTPs may even be higher compared to the standard fermenter technique as no bubbles are present which may interfere with optical measurements.
The utilization of the above described techniques also allows applying a systematic very efficient experimental strategy, which is, in contrast to the conventional (trial and error) methods, much less based on sampling and off-line measurement. These small parallel culture systems can be integrated into pipetting robots which allows completely automated experimental routines of complex operation sequences. The possibility of performing many tests in a small scale at the same time does not only increase the throughput, but, also enhances the quality of results compared to conventional methods. Experiments can now easily be carried out in comparison to standard references. Even small differences of metabolic behaviour can easier be detected than differences that show up in serial tests with conventional fermenters with their typical limited reproducibility.

BO1

Microbial single-cell analysis inside picoliter batch-cultivation chambers

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Microfluidics has opened a vast number of applications in the field of microbial single-cell analysis. In particular, devices based on polydimethylsiloxane (PDMS) combined with automated time-lapse imaging provide powerful tools to analyze microbial populations at the single-cell level with high spatial and temporal resolution. Furthermore, laminar flow conditions, efficient mass transport and large surface to volume ratios facilitate a high level of environmental control.

Till now, most microfluidic cultivation approaches comprise continuously perfused channels resulting in continuous nutrient supply.[1] Continuous media perfusion guarantees homogenous and stable environmental conditions throughout the cultivation, which is ideal for accurate perturbation studies. However, biotechnology is still dominated by fixed-volume batch cultivations using, for example, conventional shake flasks, multi-well plates, and bioreactors in which limitations and metabolite accumulation do affect cellular physiology and growth. Therefore, single-cell knowledge generated under continuous media supply can hardly be transferred to conventional scales.

In this contribution we present a PDMS-based device for microfluidic batch cultivations using picoliter sized chambers which can be reversibly isolated from the media supply. After the cell inoculation phase, the media supply channels are perfused with humidified air replacing any aqueous media residue, but due to the chamber layout and channel arrangement the liquid inside the chambers is preserved (Figure 1).

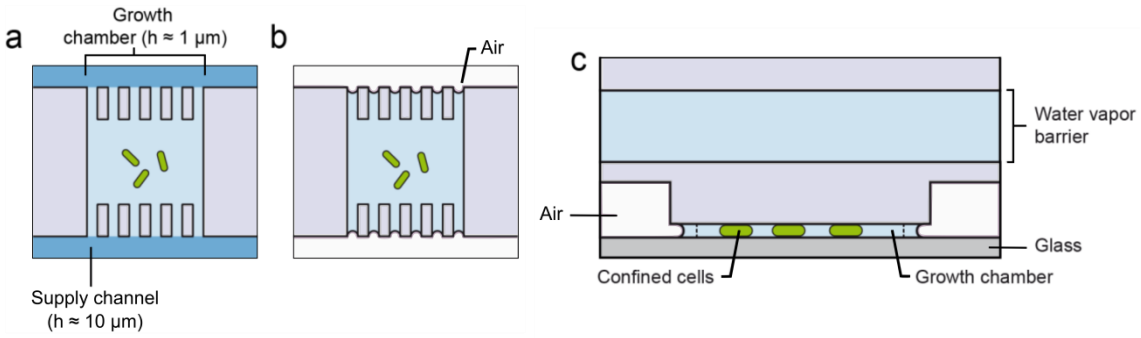

Figure 1: Microfluidic batch cultivation in air-isolated picoliter sized growth chambers: (a) cell inoculation, (b) chamber isolation by the infusion of air into the supply channels, and (c) cross-section of the isolated growth chambers.

As a proof of principle, we cultivated the industrially relevant organism *Escherichia coli* under batch conditions. Results clearly resemble growth behavior known from large scale cultivations including a significant exponential growth phase followed by a stationary phase with significant changes of cell morphology (Figures 2 and 3).

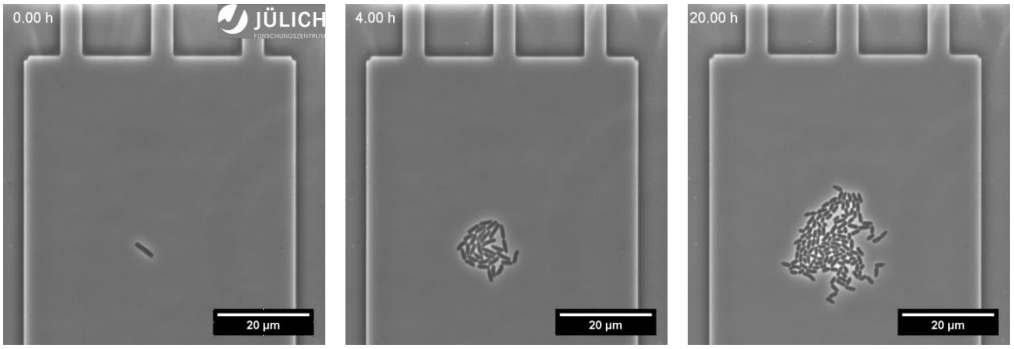

Figure 2: Phase contrast images of *E. coli* growth on LB medium in microfluidic batch reactors.

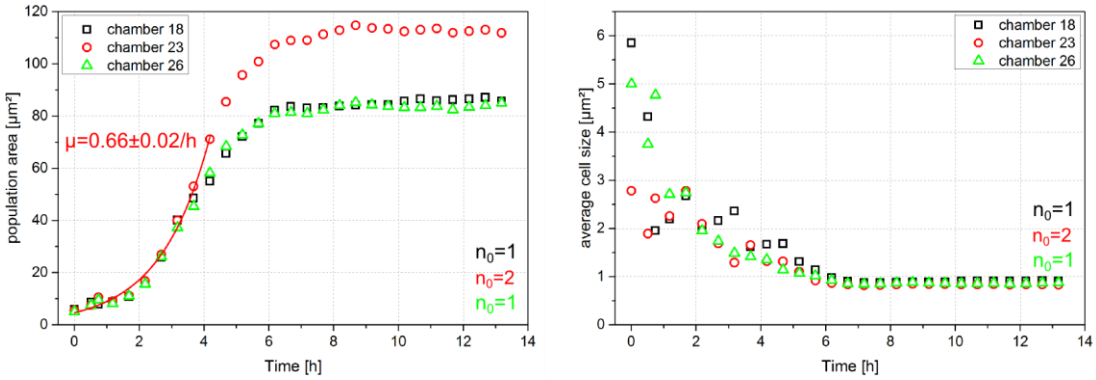

Figure 3: Projected population area (left) and average cell size (right) of *E. coli* cultivated inside isolated growth chambers. N denotes the initial cell number within the corresponding growth chamber.

We also confirmed the ability to perform repetitive cultivations within our device. Figure 4 shows the regrowth and associated cell size increase after a stationary phase of 16 hours inside the isolated growth chambers, due to the infusion of fresh LB medium through the supply channels.

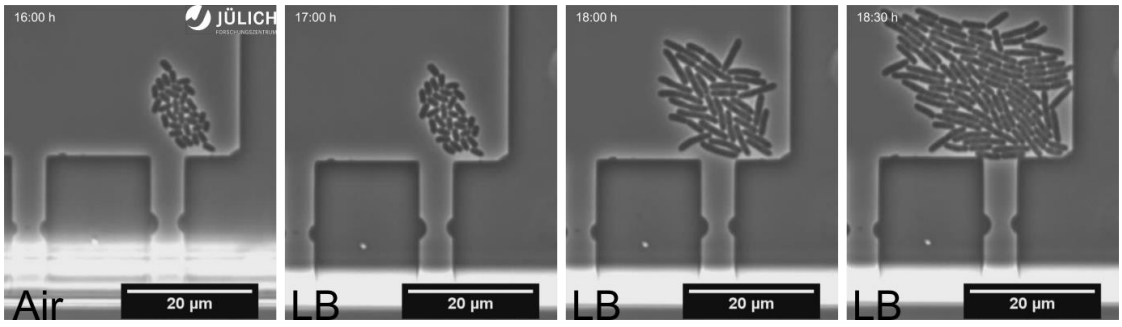

Figure 4: Repetitive growth of *E. coli* after remaining 16 hours at stationary phase in an air isolated growth chamber.

Keywords: Single-cell analysis, Microfluidics, polydimethylsiloxane (PDMS)

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BO2

Influence of scaffold microtexturing on cell cultures

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The recent progress in the field of microfabrication has allowed the development of engineered materials [1] and devices that can be used to monitor aspects of human health, and improve medical diagnosis and therapy. These novel materials characterized by micro- and nano- structured textures represent ideal substitute in the field of regenerative medicine for tissue repair such as muscle, bone, cartilage, skin and neuronal system. The polycaprolactone (PCL) [2] is a biocompatible and biodegradable polymer widely used for the realization of 3D scaffold [3,4] for tissue engineering applications [5]. In particular, we used Hot Embossing on silicon microstrutured molds for the fabrication of micro-textured PCL scaffolds. The fabricated scaffolds were used to carry out studies of cells/scaffolds compatibility [6] using the THP-1 and the HS-5 cell lines. THP-1 is a human monocytic cell line derived from an acute monocytic leukemia patient and HS-5 is a human bone marrow stromal cell line. Two types of micro-structured scaffolds were fabricated: with regular micropillars and -micro-structured scaffolds with irregular deformed pillars. Each scaffold was loaded with 50000 cells and, after 48 h of incubation the cells were stained for being observed by immunofluorescence analysis and scanning electron microscope (SEM) imaging.

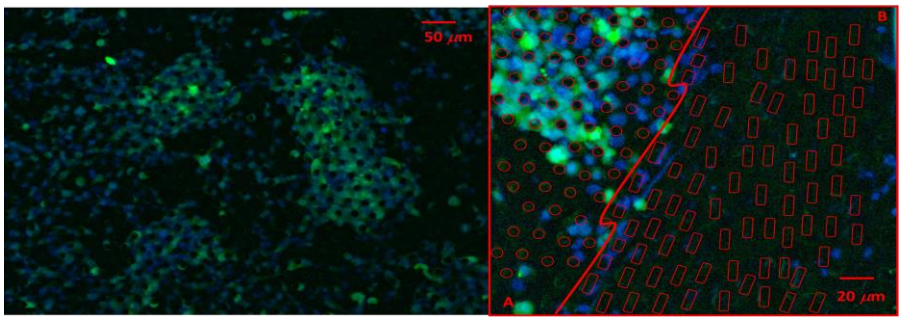

Figure 1: Blue and green superposed fluorescence microscope images of HS5 cells cultured on a PCL scaffold (left), magnification 10x; Zoom-in at the interface between areas with regular micropillars (A) and deformed micropillars (B) (right) where HS5 cells adhere and proliferate only in contact with regular pillars. The red lines are drawn around regular and deformed pillars.

These images show a scaffold's area of 820 x 780 μm^2 where it is possible to observe that the HS5 cells preferentially adhere in the presence of uniform and regular structures. The images at the SEM (Fig. 2) shows scaffolds with irregular deformed pillars and a scaffolds with regular micropillars, the image 2.C shows that M-THP-1 preferentially grow on non-regular micro-structures. The cells firmly adhere to the pillars straining toward their apex.

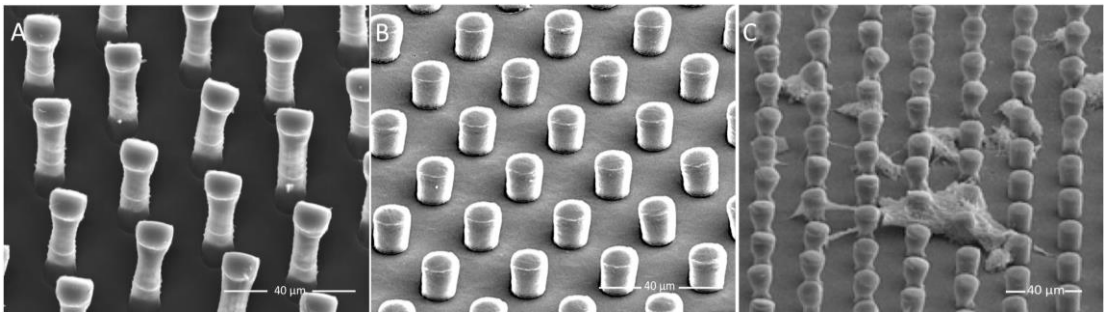

Figure 2: SEM images of irregular deformed pillars (A), regular micropillars (B), and M-THP1 cells cultured on PCL scaffolds

We hypothesize that this happens because the macrophages [7] (M-THP1) are cells involved in the cicatricial/inflammatory processes, and are recruited from peripheral blood in places where the tissue presents damages, consistent to the preference of M-THP1 for irregular surfaces, while the HS5 [8], can grow easier on regular periodic microstructures, according to their function to form connective tissue.
In conclusion, the mechanisms of cell proliferation appear to be influenced by the presence of specific micro texturing on solid supports. In fact, the cells look for the suitable substrate for their anchorage and grow adherent to this. Nano- and micro-topography of the substrate stimulate changes in the behavior of cells and play an important role in their proliferation, vitality and strength of adhesion to substrates.

Keywords: Polycaprolactone, Microtextured scaffolds, Cell adhesion, Cell proliferation

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BO3

Novel electrospun magnetized 3D nanofibers in microbioreactors: application to cell-based therapy

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Lysozyme is a highly cationic mucolytic enzyme present in nearly all living beings, capable of hydrolyzing the peptoglycan of bacterial cell walls, acting as a defense mechanism against bacteria. In the past, lysozyme was studies as a possible anti-cancer agent. Nano and micro materials have unique characteristics like a higher area/mass ratio and higher reactivity, among others. These properties have been exploited in controlled release of drugs, site specific targeting of biomolecules for different diseases treatments, as cancer.

The main goal of this work was to develop a magnetized nanofibrous system for the treatment of colon cancer patients. In fact, colon cancer is the third most common worldwide and the fourth most common cause of death. It affects men and women almost equally. Due to its invasive nature and evidenced poor patient adherence to chemotherapy post-resection, there is a need for alternative adjunct colon tumor therapies, especially ones which allow controlling tumor growth/recurrence. Research was directed for creating a containment system which could enclosure metastatic cells within the tumor mass region, slow down tissue's ECM remodelling and eliminate any altered cells in the system's vicinity.

The system envisaged, PVA electrospun fibers, loaded with lysozyme, cross-linked with fluorophenylboronic acid and functionalised with magnetic nanobeads, to be applied pre- and post-surgery on the tumor and ablation site respectively, since it will promote both tumor shrinkage, metastatic cells containment and cancer cells death. The system was successfully built and tested using a microscale approach. Evaluation of the morphology of nanofibers was carried out by SEM, thermal behavior by FTIR. The biological activities of the lysozyme loaded PVA nanofibbers were measured with an EnzChek lysozyme assay kit. To evaluate the success of the encapsulation process, in vitro lysozyme release, the ratio of adsorbed lysozyme on the fibbers, and lysozyme activity were determined in different pH and buffer, mimetizing the environment of cancer cells. Viability of cancer cells was evaluated, after being exposed to electrospun lysozyme CS-PVA.

The following conclusions were reached: i) Lysozyme activity was dependent of its concentration following a quadratic trend; ii) nanofibers with lysozyme encapsulated were produced using electrospinning,; lysozyme was successfully encapsulated in nanofibers, and the activity evaluated in microtiter plates (96 wells); iii) lysozyme release was tested from the systems and the results show that it was independent of the pH, although minor variations in the initial release may be explained by PVA degradation; iv) lysozyme encapsulated both in the fibers was able not only to retain its activity, but this was also enhanced and was independent on the type of modification induced to the system; v) Fibers containing lysozyme encapsulated, cross-linked with fluorophenylboronic acid and/or with IONPs adsorbed, were able to reduce viability of Caco-2 tumor cells seeded on them. A cell growth kinetic profile was established to evaluate cell proliferation on the fibers (MTT assay or Alamar Blue) along 10 days. Cell staining was evaluated for each of the timepoints used in the kinetics, using DAPI and phalloidin, to see the cells structure and distribution along the fibers. Cell death was investigated, using caspase-3 activity determination, TUNNEL assay and Annexin-V assay to confirm that cells dye through apoptosis.

Keywords: Lysozyme, Nanofibers, Microbioreator, anticancer activity, biologic activity

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BO4

Multiphase microreactors with intensification of oxygen mass transfer rate and mixing performance for bioprocess development

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Miniaturization of bioreactor technology to the microlitre-scale is a promising tool for screening and optimization of cultivation and biocatalytic processes. Microreactors have small working volumes (< 1 mL), are flexible, easy to handle in numbering up approaches, and enable *in situ* monitoring of important process variables[1].

This research is focused on microbioreactor development addressing two of the challenges that microfluidics is confronted with: poor mixing and mass transfer limitations. A rapid and efficient mixing is a fundamental requirement for a proper distribution and suspension of substrate and microorganisms during growth cultivation, to ensure an even temperature profile throughout the reactor, and guarantee the quality of the measurements of any bioprocess study, but many microfluidic systems are characterized by low Reynolds number (typically Re < 1) resulting in laminar flow conditions leading often to mass transfer limitation or poor mixing. Secondly, due to the low oxygen solubility in aqueous cultivation media and the high oxygen demand from aerobic bioprocesses, the oxygen supply is the most critical transport process and may lead to mass transfer limitations. To overcome these challenges a microbubble column-bioreactor (μBC) for biotechnological research is developed and characterized. A fine dispersion of air in the cultivation medium results in a very large specific interface (air/liquid), improving the oxygen mass transfer rate in comparison to other microfluidic systems. The buoyancy of the bubbles and the momentum exchange between air and liquid promote unsteady liquid flows, which contribute fundamentally to the mixing of the liquid phase.

Many studies have been focused on gas-liquid hydrodynamics and mass transfer in microreactors, but most of them consider flows in closed microchannels. Although it was possible to find hydrodynamic studies on horizontal micro-scale bubble columns[2], and in small scale bubble columns[3], there was a lack in the study of the hydrodynamic conditions in vertical micro-scale bubble columns.

This study focuses on a μBC with a reaction volume of 60 μL. The μBC was fabricated by wet etching and powder blasting technology in borosilicate glass (Micronit GmbH, Germany) and it is supported by a holder that facilitates the tube connections and allows the flow visualization through its inspection window. The air is supplied through a nozzle 26 μm in diameter at the bottom of the μBC. While supplying pressurized air through the nozzle, a continuous stream of microbubbles that rise through the vertical device is generated. The stream of ascending air bubbles contributes to a good homogenization of the reactor content, and an adequate oxygen mass transfer. The air flow can be regulated for the optimal performance, in this way the influence of the superficial air velocity on the oxygen transfer rate (OTR) and mixing time is investigated. The OTR characterization is carried out through the measurement of the concentration of dissolved oxygen in the bulk phase through a retractable oxygen microsensor (Pyroscience, Germany), and the mixing characterization is performed through image analysis of time-lapse pictures taken with a camera with 90 mm macro-lens and a house-made ultra short duration flash ($2 \cdot 10^{-6}$ s).

Additionally, computational fluid dynamics (CFD), performed with the software ANSYS CFX 17, is employed in this research as a supporting numerical tool to estimate the fluid behavior inside the μBC. Two-phase simulations are performed to model the fluid velocity field at different air flow rates. The mixing time experiments are combined with CFD simulations for a deeper understanding of the fluid dynamics.

Besides the development and characterization of the μBC, the focus of the investigation is on its application as platform for aerobic cultivation of different biological systems and for biocatalytic processes[4,5]. The integration of sensors for the real-time online monitoring of process variables like dissolved oxygen, optical density or CO_2 generation measured in the off-gas provides comprehensive knowledge of the system and the bioprocess.

The aim of the ongoing investigations is to demonstrate the advantages of these miniaturized devices in the field of biotechnology and to present the hydrodynamics and mass transfer properties of the μBC, and its considerable potential for aerobic cultivations and biocatalytic applications.

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Keywords: microbioreactor, bubble column, mass transfer, mixing, CFD

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BO5
Guiding efficient bioprocess development: one-step microbial synthesis of ε-caprolactone from cyclohexane

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Despite the potential of the biocatalytic process to operate at mild conditions and with excellent selectivity very few examples to date are operated on the industrial scale. Low turnover number (TN) and stability of biocatalysts, dilute product streams and high amount of waste generation contributes to high capital costs and operating costs making bioprocess difficult to meet economical metrics at industrial scale. To address these aforementioned issues, a systematic framework for using an integrated approach to address biocatalyst, reaction, and reactor engineering for high process performance is necessary(Figure) [1,2] We believe that such a systematic approach of integrating different process levels will play a key role in future bioprocess development. This approach is elaborated by designing a heterologous pathway in *Pseudomonas taiwanensis* strain VLB120 for the one-step conversion of cycloalkanes to lactones.[3]

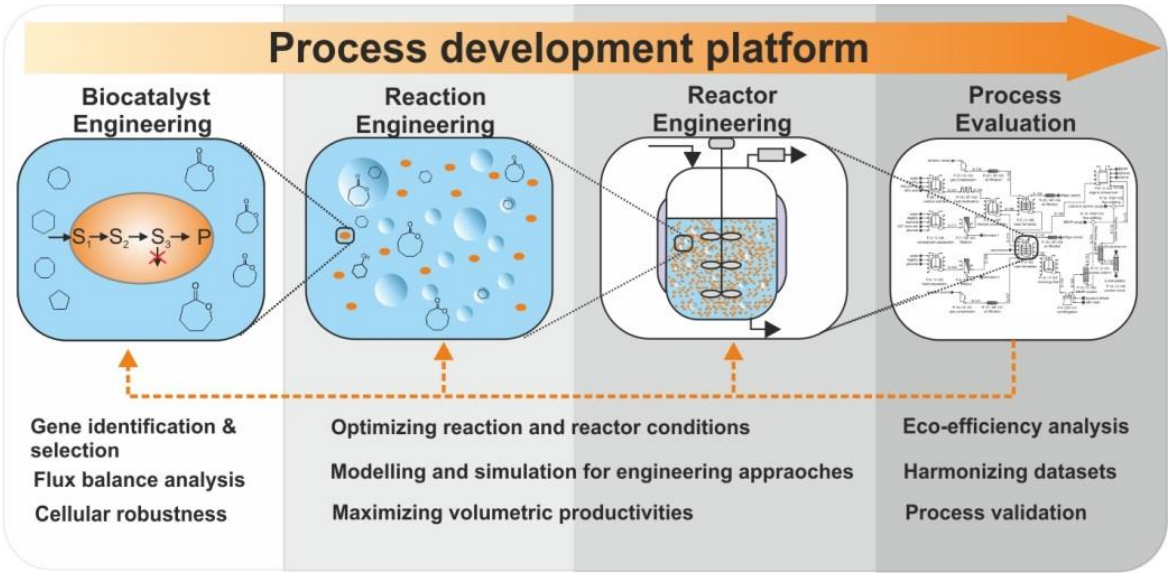

Figure 1: An integrated approach applied to bridge the different levels of process design for bioprocess intensification.

Biocatalyst engineering: Current industrial synthesis of ε-caprolactone from cyclohexane employs multi-step processes with several unit operations incurring numerous heat and pressure variations. Such process complexity very often results in high capital expenditures (CapEx) and operational expenditures (OpEx). Despite significant efforts to develop new chemical catalysts, high activities are accompanied by poor selectivity and harsh reaction conditions. In contrast, selective oxyfunctionalization is a key concept applied in Nature by microorganisms for hydrocarbon mineralization. Several microorganisms are reported to utilize cyclohexane as a sole carbon and energy source. By isolating novel cytochrome P450 monooxygenase, cyclohexanol dehydrogenase (CDH), and cyclohexanone monooxygenase (CHXON) genes from *Acidovorax* sp. CHX100 and their functional expression in recombinant *Pseudomonas taiwanensis* VLB120 enabled one-step conversion of cyclohexane to ε-caprolactone.[3] The whole-cell specific activities for the conversion of cyclohexane, cyclohexanol and cyclohexanone to ε-caprolactone are 22 U g_{CDW}^{-1}, 80-100 U g_{CDW}^{-1}, and 170 U g_{CDW}^{-1}, respectively. However, the poor stability of the biocatalyst coming from the toxic effect of cyclohexane was considered as a major problem for process development.

Reaction engineering: The biocatalyst stability was improved by adapting "gas-phase approach" and overcoming the toxic effects of cyclohexane in resting cell reactions. This approach enabled stable transformation rates of 20-22 U g_{CDW}^{-1} for several hours. It also indicated a beneficial balance between cyclohexane mass transfer from the gas phase to the aqueous phase and the cellular conversion rate. By exploiting multi-step reactions in one-pot circumvents several purification steps and provides opportunities to reduce waste, time, capital costs and operating costs. However, application of "gas-phase approach" was difficult to transfer on the reactor level because of the stripping of cyclohexane from aqueous phase due to a continuous air supply. Additional drawback was the high amount of waste generated, estimated from E-factor (3750). E-factor describes the actual amount of waste produced in relation to the desired product synthesized. A lower E-factor represents less waste and positive environmental impact. For bulk chemicals such as ε-caprolactone an E-factor of 1-5 are recommended because of high production volumes and lower product prices which restrict the use of reagents in a stoichiometric amount.

Reactor engineering: In order to reduce the E-factor, recycling and reusing water, reagents and biocatalyst in an effective manner is mandatory. One of the interesting concepts to retain whole-cells in the reactor format is to use them as biofilms. Biofilms are living functional reaction systems which have the ability to self-immobilize at a given interphase, constantly regenerating themselves. They are retained inside the reactor featuring a reaction system with an infinite TTN. Application of continuous biofilm reactors for production of bulk chemicals is an elegant approach to recycle product streams and consequently optimize waste efficiency. Therefore, an aqueous-air segmented flow biofilm membrane microreactor[4] was investigated for maximizing productivity. This reactor system ensured lower reactant toxicity due to membrane transfer, and continuous product formation at maximal volumetric productivity for several days. Finally, the strategy to scale-up such microreactor format will be addressed.

Overall, this work highlights an integrated approach on combining a powerful catalyst with beneficial microreactor designs for bioprocess development, which opens new opportunities for transformations of compounds which are toxic, volatile and low water soluble.

Keywords: Segmented flow, Biofilm microreactor, Whole cell biocatalysis; Cascade reactions; Cytochrome P450 monooxygenase; Baeyer-Villiger monooxygenase

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BO6

Miniaturized microbial fuel cell utilizing carbohydrate substrates

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Fuel cell is an electrochemical device generating electric energy directly from the chemical energy of a fuel, which is typically hydrogen or a hydrogen rich gaseous mixture, by its catalytic oxidation[1]. The microbial fuel cell (MFC) converts the chemical energy of a fuel (saccharides or other utilizable organic substrates) into electric energy by an action of the metabolic processes taking place in microbial cells[2]. In a biological fuel cell the fuel is oxidized in the anode compartment. The mechanisms of electron generation and of their transport to the anode differ depending on type of biological fuel cell. Electrons flow through the external circuit by an effect of a potential difference to the cathode. At the cathode, electrons are accepted by an oxidant. The final electron acceptor is oxygen that reacts with the protons forming water as a side-product[3].

In a MFC, metabolic processes generating electrons by oxidation of a substrate are isolated from the cell exterior by the cell membrane and a suitable mechanism of transport of the electrons from the cell to the electrode has to be provided. The electrons can be transferred to the electrode surface by means of artificial mediators, usually organic dyes (for example, methylene blue) or by covalent link of the cells to the electrode. In the former case, the molecules of the mediator diffuse to the cell surface, penetrate the cell wall and the cytoplasmic membrane, accept the electrons and the molecules of the reduced mediator diffuse out of the cell and are transported to the electrode surface, where they lose electrons and are oxidized back to the oxidized form.

This work focuses on design, fabrication and experimental testing of a microbial fuel cell prototype using common baker's yeasts *Saccharomyces cerevisiae* or *E. coli* cells and various carbohydrate substrates. The yeasts were cultivated for 20 hours at 25 °C in shaken flasks using medium containing phosphate buffer (2 g dm^{-3} NaH_2PO_4 and 2 g dm^{-3} Na_2HPO_4), glucose (25 g dm^{-3}), yeast extract (10 g dm^{-3}) and magnesium sulphate heptahydrate (0.1 g dm^{-3}), pH = 6. The cells were harvested by centrifugation at 4000 rpm for 20 minutes. The bacterium *Escherichia coli* was cultivated at the same conditions using the LB medium with 0.5% NaCl, pH=7. The cells were harvested after 20 hours (end of the exponential growth phase) by centrifugation at 2500 rpm for 8 minutes. The MFC consisted of two chambers (inner dimensions: 60×60×15 mm), separated by a sheet of the NAFION® N117 cation exchange membrane. The electrodes were fabricated of the platinized titanium mesh (59 x 59 mm, MAGNETO B.V., Schiedam, Netherlands). The mesh surface is 1.7 m^2 per 1 m^2 of its geometric area. The active surface of one electrode was therefore 118 cm^2. Each compartment was equipped with two ports for filling, sampling and for gas bubbling.

The fuel cells are mostly characterized by their operating curves, i.e., by dependences of their voltage or electric power output on the electric current or the electric current density. The operating curves of MFCs may be expressed in various ways. The open circuit voltage (OCV) value, i.e., the output voltage of unloaded cell ($R_{ext} \to \infty$), is very often used to briefly characterize the microbial fuel cell performance. The performance of the experimental MFC was evaluated from its voltage U and the electric current I measured at different external loads. The external load (resistance R_{ext}) was varied from 100 to 100 000 Ω.

The prototype microbial fuel cell was operated in three operating modes differing mainly in the ways of agitation of the electrode compartments: i) without electrolyte agitation in both compartments, ii) with agitation of the cathode electrolyte by air bubbling and the anode compartment unstirred, iii) with agitation within the anode compartment by nitrogen bubbling and with agitation of the cathode electrolyte by air bubbling. The anode compartment was filled with the suspension of yeast cells in the electrolyte with the substrate at chosen concentration and the methylene blue (5 mmol dm^{-3}). The anode compartment was

operated under anaerobic conditions. The cathode compartment was filled with the buffered solution of potassium ferricyanide (0.1 mol dm^{-3}) and was either bubbled with air or left unstirred.

The yeast cells are capable to utilize a wide spectrum of carbohydrate substrates. Glucose, fructose, saccharose and their mixtures and glycerol were therefore tested in the MFC to verify their applicability. Glycerol was used at two times higher concentration to keep the same number of moles of the metabolizable carbon. The maximum open circuit voltages achieved with various substrates varied within approximately 10% (see Fig. 1): glucose – 0.529 V; fructose – 0.545 V, saccharose – 0.523 V. Obviously lower maximum OCV value was observed with glycerol: 0.501 V. The maximum power output obtained under the resistance of 120 Ω reached 395 μW. The maximum generated volumetric power density was 3.7 W m^{-3}.

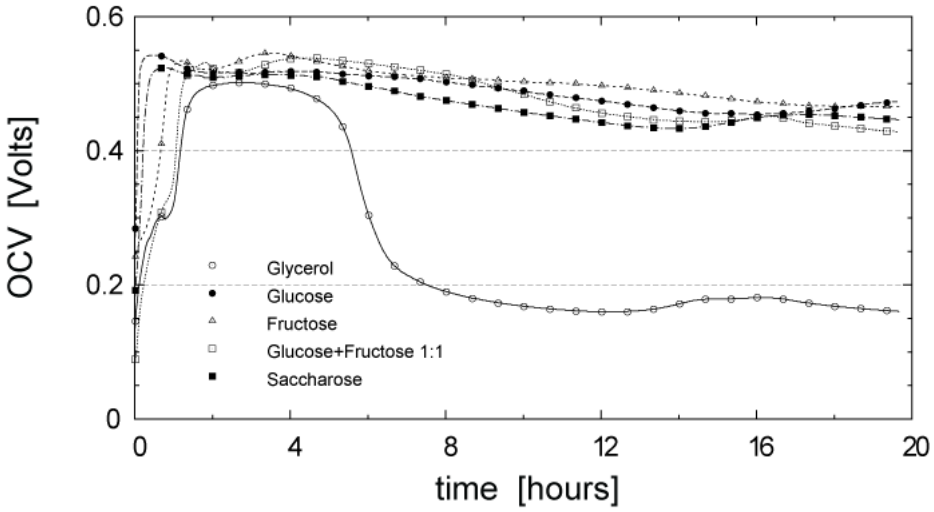

Figure 1: Open cell voltages (OCV) achieved in the yeast cells MFC with various substrates as functions of time of MFC operation.

Three different operational configurations of the MFC were employed and the influence of the compartment solution agitation was determined. In the most efficient configuration, the cathode compartment was bubbled by air to enhance the electron transfer from the oxidizer (potassium ferricyanide) to the final electron acceptor (dioxygen).

Although the MFC studied in this work was not primarily designed to use a wastewater as the fuel a municipal wastewater was also tested using the *E. coli* cells. The electrodes in the MFC designed for the applications of a wastewater are mostly fabricated from carbon or graphite materials, convenient for biofilm formation. The MFC prototype studied in this work employed platinized titanium electrodes. A water from the inflow to the municipal wastewater treatment plant in Suchdol, Prague was tested as a source of the fuel and microorganism. The maximum *OCV* reached 0.120 V only. In the second experiment, the cells of *E. coli* were added to the anode solution which contained the wastewater. *E. coli* naturally occurs in municipal wastewaters and *OCV* increased markedly, the maximum *OCV* generated by the MFC increased to 0. 220 V. Therefore, the municipal wastewaters may serve as a perspective source of the fuel for MFC technology.

Keywords: microbial fuel cell, yeasts, bacteria, open cell voltage, waste water

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BP1

Enantioselective desymmetrisation of achiral 2-substituted 1,3-diols by *Acetobacter aceti*: traditional approach and developments

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Among the wide range of microorganisms performing biocatalytic oxidations, *Acetobacter aceti* was selected as a valuable biocatalyst for the enantioselective desymmetrization of achiral 2-substituted 1,3-diols. The well-known oxidative potential of *Acetobacter aceti* [1] was employed with successful results in the production of (*S*)-3-hydroxy-2-methylpropanoic acid, an intermediate in production of Captopril, from 2-methyl-1,3-propandiol. [2] High conversion and high enantiomeric excess were observed. Therefore, the oxidative desymmetrisation activity of *Acetobacter aceti* was further investigated on a set of 2-substituted-1,3-propandiols, in order to obtain enantiomerically enriched hydroxymethyl alkanoic acids, which are interesting intermediates in the synthesis of α-substituted-β-lactones, currently under investigation as possible caseinolytic protease (ClpP) inhibitors. Aromatic and aliphatic substituted 1,3-propandiols were added directly to the culture broth, and the enantioselectivity of the reaction was found to depend on the steric hindrance of the substituent in position 2. Additional side-reactions were observed, leading to the formation of the corresponding α-methylenic and α-methyl alkanoic acids. [3] After considering the good results, questions about whether it was possible to improve productivity and selectivity were arisen. As possible improving technique, immobilization was taken into account, for simplifying work-up and reducing side reactions. [4] It was decided to immobilize cells by calcium alginate entrapment because of its feasibility and the high performances reported in literature. [5] The diol oxidations showing the highest amount of side products were tested with immobilized cells affording good results both for selectivity and conversion. Then, considering the advantageous employment of immobilized cells in a packed-bed column and the great benefits ascribed to flow technology, tests under continuous-flow conditions were performed with the most promising substrates. Although the reaction through *Acetobacter aceti* is not rapid and does not need intense heating (the most requiring conditions for the increased mass and heat transfer due to flow technology [6]), the high productivity and versatility of this technology were thought to benefit biocatalytic oxidations. In order to provide cells with the oxygen in the confined system of microtubes and reactor, an air-liquid segmented feed was provided, allowing increased mixing. [7] The increased productivity shown by using this technology has been considered of interest for future works by extending its application other substrates and by determining the best conditions for process intensification.

Keywords: *Acetobacter aceti*, 2-substituted-1,3-diols oxidation, immobilized cells, packed-bed reactor, air-liquid segmentation

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COST ACTION CM1303- System Biocatalysis

Session C: ANALYTICAL MICRODEVICES

CK

A new platform "Immuno-wall device" as a rapid diagnostics tool

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Microfluidic devices have great potential for medical and life science applications. Recent progresses in microfluidic devices have enable precise analysis of a small amount of a particular chemicals (e.g., protein, peptide, DNA, RNA, drug, etc.) in blood or urine. We have developed several immunoassay platforms using the microfluidic devices for rapid diagnosis of biomarkers[1-3] and detection of drugs[4-6] and enterotoxins.[7,8]

Recently, we developed a new platform for microfluidic-based immunoassay called an "immuno-wall device", which has the desired features for easy-to-use detection of biomarkers.[9,10] It has a wall-like structure inside a microchannel (Figure 1). The antibodies are immobilized onto photo-reactive polymer wall (40 μm width, 40 μm height and 4.0 mm length). We demonstrated it to several applications and confirmed our device provides rapid analysis (within 15 min) with high sensitivity, it is easy-to-use, and it uses small volumes of the sample and reagent.

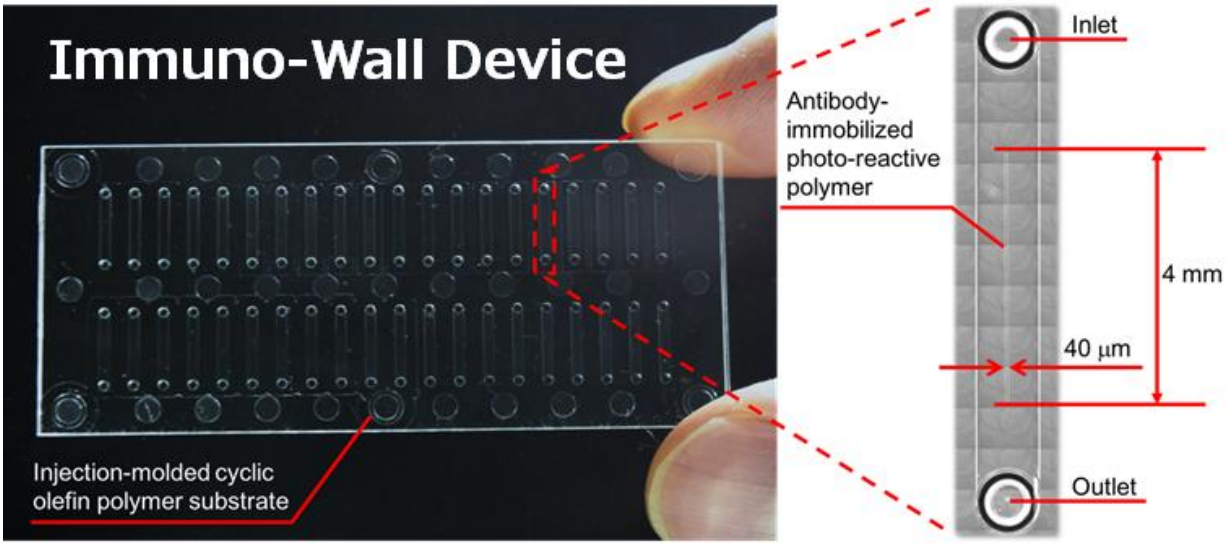

Figure 1: Photo and design of the immuno-wall device (adapted from reference 9)

We also developed a portable measurement system for the immuno-wall device in collaboration with a Japanese opto-electronics company (Hamamatsu Photonics K. K.). Figure 2 shows the portable measurement system. The dimensions (WHD) of the system are 130 mm × 135 mm × 220 mm. The weight is 1.2 kg. The system is suitable for Point of POCT (Point-of-Care Testing) applications.

In this presentation, I will present our recent progress on applications of the immuno-wall devices.

Figure 2: Portable measurement system for the immuno-wall device

Keywords: microfluidic device, immunoassay, immuno-wall device, photo-reactive polymer, diagnosis

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CO1

Development of a microfluidic platform mimicking cascading liver metabolic reactions

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To facilitate the rapid development of novel and more effective drugs, it is vital to have powerful tools at hand that enable the rapid evaluation of drug uptake and metabolism. This project aims at developing a microfluidic platform for mimicking cascading Phase I and Phase II metabolic reactions that take place within the human liver. The model enzymatic cascade consists of two reactions catalysed by cytochrome P450 (P450) and glucosyltransferase (UGT) enzymes (Figure 1). The metabolite product of the P450 chip is used as a substrate for UGT-catalyzed reaction in the next system.

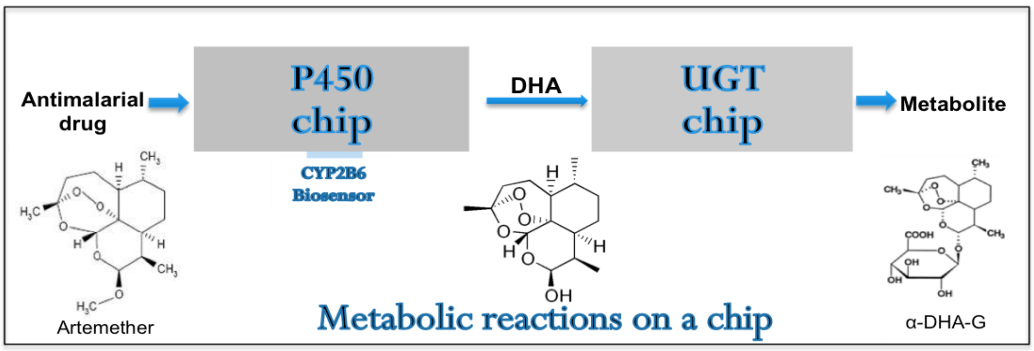

Figure 1: Schematic representation of a microfluidic platform mimicking cascading liver metabolic reactions. The model enzymatic cascade consists of two reactions catalysed by cytochrome P450 (P450) and glucosyltransferase (UGT) enzymes; these enzymes belong to Phase I and Phase II drug metabolism enzymes, respectively. Intermediate metabolite product (in this case, dihydroartemisinin (DHA)) of the first reaction in P450 chip acts a substrate for the second UGT-catalysed reaction in the cascading system

The first part of the cascade consists of a microfluidic chip integrated with a novel electrochemical cytochrome P450 biosensor.[1] A P450 enzyme is immobilized on the surface of biosensor, which allows running the reaction without the need of NADPH and co-enzymes. A microfluidic polymer-based chip was fabricated using stereolithography 3D printing technique and integrated with P450 biosensor. A P450 biosensor was fabricated by immobilizing P450 enzyme types, CYP2B6 or CYP3A4 (specific to test compounds), on DropSens gold electrodes. Substrate drug compounds (artemether, bupropion) were flowed through the biosensor chip using the setup shown in Figure 2.

Binding of test compounds to the enzyme in P450 chip was detected via electrochemical techniques. Responses of P450 biosensor to different concentrations of substrate and, consequently, calibration curves of substrate binding were successfully obtained. Results suggest that the biosensor is responsive to different substrate concentrations and can be used for online analysis in the P450 chip (Figure 3).

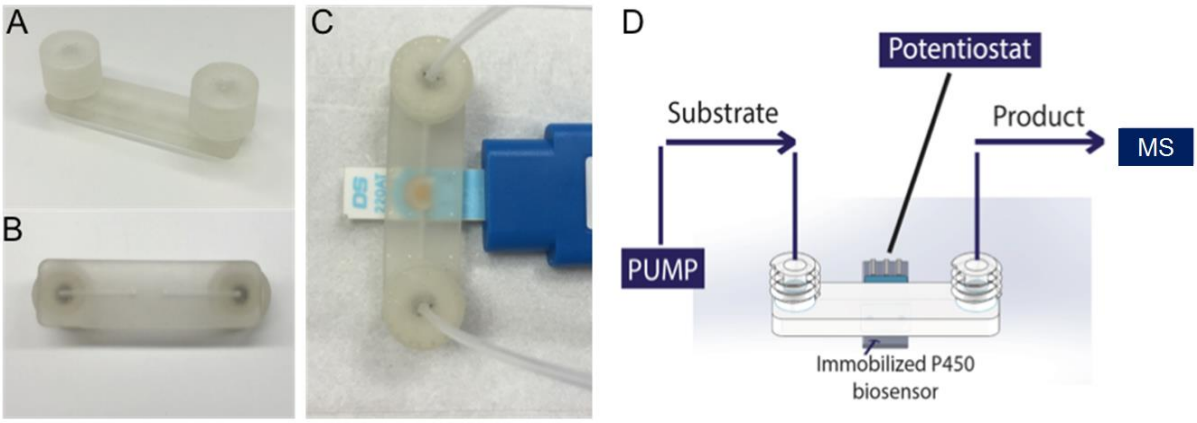

Figure 1: A, B) Photographs of 3D-printed P450 chip, isometric and bottom view. C) Assembled P450 chip with gold electrode biosensor connected to a PalmSens3 potentiostat for online measurements; D) Schematic representation of the P450 chip with a novel biosensor for the online detection of drug-enzyme interactions.

Additionally, surface coverage of a biosensor by the enzyme was determined and compared to literature values. To prove metabolite formation of reaction on P450 biosensor surface, product samples were analysed using LC-MS/MS. Methods for analyzing artemether and its product (dihydroartemisinin) were established and used in analysis of reaction samples. As a result, the expected product (DHA) was confirmed by ESI-MS/MS in which an MS peak corresponding to the molecular ion of dihydroartemisinin ammonium adduct was observed.

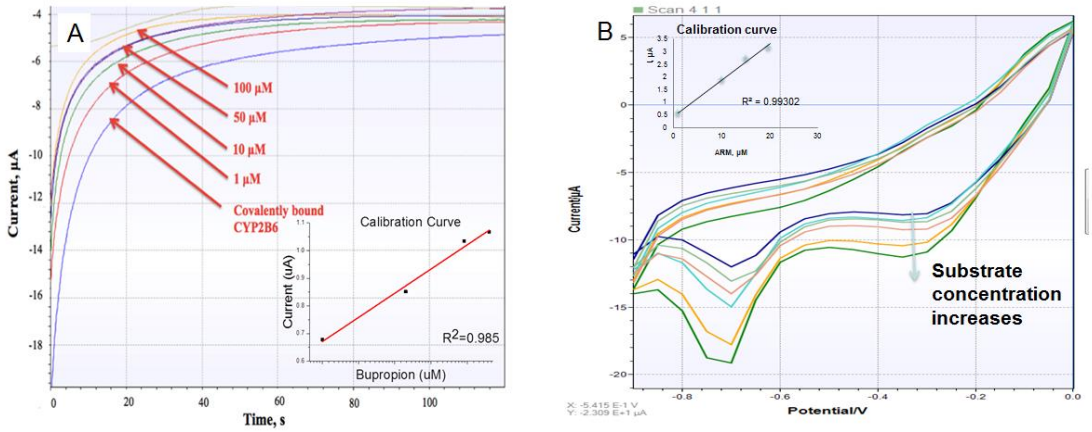

Figure 3: (A) Amperometric response of P450 biosensor to different concentrations of substrate, bupropion. Inset: Obtained calibration curve for the model drug. (B) Cyclic Voltammetry response of P450 biosensor to different concentrations of substrate, artemether. Inset: Obtained calibration curve for the model drug.

The ongoing work includes development of packed-bed reactor for the Phase II reaction of the liver pathway reaction and coupling of the two microfluidic devices.

Keywords: microfluidics, liver metabolism, cytochrome P450 biosensor

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CO2

Real-time determination of oxygen concentration and pH in droplet microfluidic culturing systems using optical sensor nanoparticles

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We show a method to acquire accurate and real-time information of oxygen concentration and pH in droplets during cell culturing by using luminescent sensor nanoparticles and a lifetime-based miniaturized readout device. In a microdroplet cell culturing system, which is adapted from microdroplet chemostats,[1] we monitored the concentration of oxygen, pH and optical density (OD) simultaneously in each droplet during the incubation of *Escherichia coli*.[2] Also, we monitored both pH and oxygen concentration in a group of pL droplets during the dynamic incubation of *Pichia pastoris* in a microfluidic incubator. The lifetime of luminescence from nanoparticles yields accurate measurement of oxygen concentration and pH, and allows one to monitor the metabolism of cells in droplet-based microfluidic systems.

We used poly(styrene-*block*-vinylpyrrolidone) nanoparticles (PSPVP NPs) as a versatile platform,[3] and entrapped different "sensing chemistries" to obtain oxygen sensitive or pH sensitive nanoparticles (Figure 1). We developed and synthesized the near-infrared (NIR) emitting dyes platinum (II) benzoporphyrins[4] and aza-BODIPY[5] for oxygen and pH sensing, respectively. The miniaturized fluorometer, in the footprint of a memory stick, is able to measure through tubing or other transparent chip materials. It was further adapted for measurements in droplets by equipping a lens to focus on an area about 80 µm in diameter.[6] The microdroplet cell culturing system was fabricated with customized Teflon connector and Teflon Fluorinated Ethylene Propylene (FEP) tubing (ID = 0.8 mm).[7] For pL-droplets, the incubation was performed in a microfluidic incubator, which was 3D printed with transparent resin.[8]

The online real-time information of oxygen concentration and pH in droplets can be used by microfluidic screening systems, e.g., cell analysis or sorting. It also provides better understanding of the system and mass transfer by monitoring the change of oxygen concentration or pH in microfluidic systems.

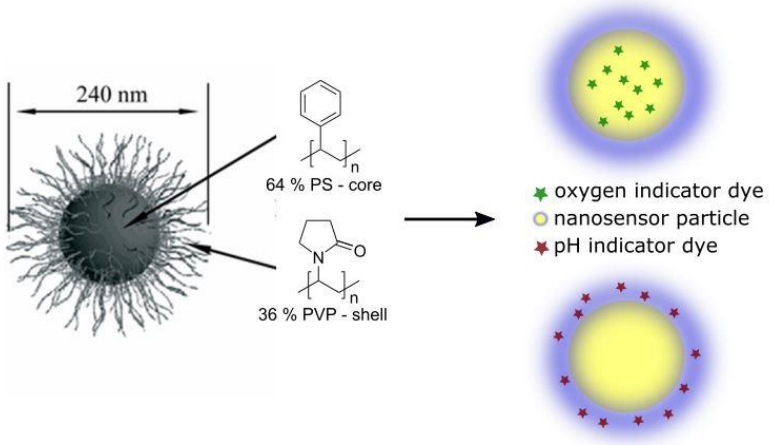

Figure 1: Scheme of PSPVP NPs for oxygen and pH sensing.

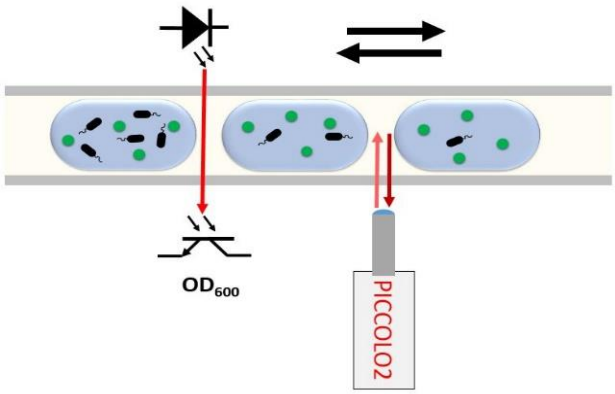

Figure 2: Scheme of on-line, real-time oxygen concentration, pH and OD measurements on microdroplets.

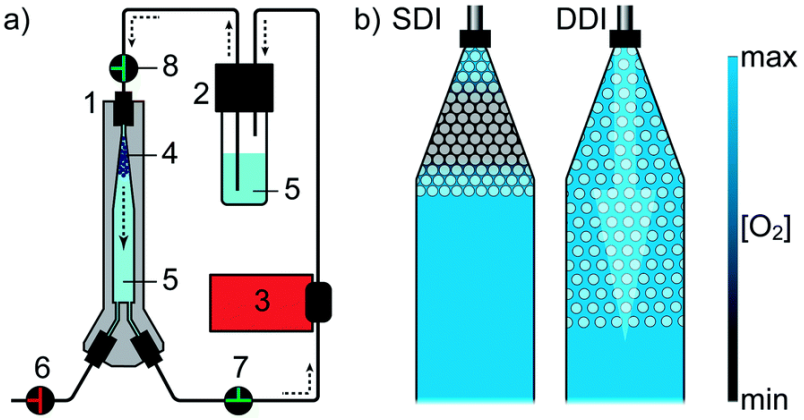

Figure 3: Scheme of 3D printed incubator for cultivation of cells in pL droplets.

Keywords: oxygen, pH, optical sensor, droplet microfluidics, cell culturing

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CO3

Multifunctional membranes based on co-electrospinning: new material for the development of microbioreactors and biosensors

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The development of advanced multifunctional platforms with sensing properties and based on new nanomaterials has attracted great interest in the past few years due to their tunable physical, chemical, optical, mechanical and electronic properties, versatility, and applicability in many different fields[1].

Among the wide diversity of novel functional nanomaterials, nanofibres membranes prepared by electrospinning technique offer several advantages [2]. In addition, co-electrospinning technique allows one-step fabrication of multifunctional coaxial micro- and nano- fibre membranes which generates two different chemical environments that increase the functionality of these nanofibres (see Figure 1) as well as their applicability, including sensing applications[3]. This core-shell morphology allows obtaining two different chemical environments (inner hydrophobic environment and outer hydrophilic environment) that permits the incorporation of compounds with different hydrophilic properties in the same matrix maintaining their best chemical and analytical features.

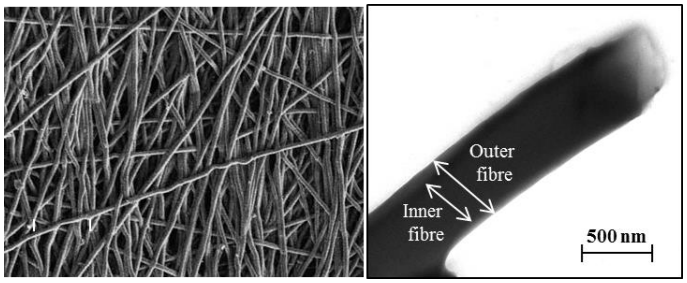

Figure 1. TEM pictures of the coaxial nanofibre membrane.

On the other hand, the use of optical oxygen transduction is useful in many fields including biosensing and motorization of an enzymatic reaction in microbioreactors [4].

In this work, we have developed and characterized a multifunctional coaxial material prepared from two different polymeric solutions by using co-electrospinning, which originates nonwoven nanofibre membranes with very large surface area, excellent mechanical properties (resistance to abrasion, high tensile strength, flexibility, easy handling) and high chemical resistance.

To develop this kind of membranes, it is necessary to develop a material with 2 functionalities: 1) it must allow the immobilization of a biomolecule on its surface to catalyse the oxidation of the analyte and 2) it must contain an oxygen sensitive dye to measure the consumption of oxygen during this oxidation. In addition, due to the different character of the biomolecule (hydrophilic) and the oxygen sensitive dye (hydrophobic), these bi-functional materials have to contain two different chemical environments: a hydrophilic environment for the enzyme and a hydrophobic environment for the oxygen-sensitive dye.

In order to obtain the hydrophilic environment, PolymBlend® was selected for the outer polymer solution because it is hydrophilic and oxygen permeable, it contains OH groups that can be easily functionalized to allow the covalent immobilization of the enzyme, and it has been formulated with an optimum mixture of two high molecular weight statistic copolymers to provides excellent mechanical and chemical properties of non-woven mats produced by electrospinning.

For the hydrophobic environment to incorporate the oxygen-sensitive dye, PMMA was selected because it is hydrophobic, compatible with the oxygen sensitive dyes, oxygen permeable, transparent and it was probed that it is a good polymer to develop oxygen sensing films by electrospinning [3]. As oxygen sensitive dyes we have selected two commercially available metalloporphyrins (PtTFPP and PdTFPP), one modified Pt(II)–benzoporphyrin (PtTPTBPBr), and one cyclometalated Ir(III) complex ($[Ir(npy)_2(bpy\text{-}pyr_2)]PF_6$), due to their exceptional luminescence properties and compatibility with PMMA.

The percentage of polymers, kind of solvents, injector and collector voltages and outer and inner flow rates were fully optimised to obtain a homogeneous compound cone in the steady state for long time and to produce a regular and homogeneous formation of the fibres, during the co-electrospinning procedure.

In order to demonstrate its applicability, two different oxidase enzymes have been evaluated (glucose oxidase and uricase), in addition, we have evaluated four different ways for the enzyme immobilisation: one physical adsorption, and three covalent immobilisations; one via glutaraldehyde crosslinking, other via maleimide-PEG-succinimidyl ester linker and the last one via biotin-streptavidin interaction. After the immobilization of the enzyme, the material preserves the oxygen sensing properties of the embedded dye and can specifically react with the substrate of the enzyme, thus it allows monitoring the consumption of oxygen during the enzymatic reaction.

The resulting materials have been used to develop biosensors with optical oxygen transduction, showing good analytical features and good correlations (closed to 1) when the obtained results are compared with those obtained by a reference laboratory. Thus, coelectrospinning led to the preparation of a multifunctional material with an outer high capacity for immobilizing biomolecules and an inner ability for entrapping optical indicators, resulting in a material with excellent versatility, functionality and applicability in many fields, including optical sensing applications and microbioreactors.

This work has received funding from the People Programme (Marie Curie Actions, Multi-ITN) of the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 608104 (EUROMBR) and the Spanish Ministry of Economy and Competitiveness (AP2012-0944, PTQ-11-04904 and CTQ2014-53442-P). The authors also thank the research groups of E. Baranoff and I. Klimant for providing the IrBPY and PtTPTBPBr complexes, respectively, for this work.

Keywords: coelectrospinning, oxygen transduction, multifunctional material, microbioreactors.

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CO4

Integrated biosensors as tools for online microbioreactor analytics

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Micro-bioreactors and microfluidics is a field of technology that can reduce the amount of valuable reagents, accelerate reactions, and allow for parallelization by miniaturizing various chemical reaction platforms, which are applicable within pharmacy, chemistry, medicine and biotechnology. One branch in biotechnology where these advantages are appreciated the most is in fermentation sciences as it enables a great amount of strain screening and bioprocessing work to be made economically relevant[1]. In comparison with standard practice, micro-bioreactors can also address low data density acquisition and lack of control over cultivation conditions [2]. Biosensors are highly specific and selective analytical devices that lend themselves easily to miniaturization and parallelization[3] and their integration into micro-bioreactors and microfluidics can enrich the research with online acquired data on nutrient consumption, fermentation conditions, target molecule production, as well as cell growth and physiology. The major challenges for biosensor integration into micro-bioreactors is their high sensitivity (linear range), sterilization and sensitivity decay caused by the degradation of the biological component. Our aim is to present developed biosensors, their characterization, designs and solutions for their integration into micro-bioreactors and microfluidics in model bioprocessing applications.

Enzymatic electrochemical sensors for glucose and lactate have been developed and fully characterized. A novel protocol for rapid determination of ageing characteristics have been developed; based on the linear dependency of sensitivity decay on temperature. The glucose oxidase biosensor was found to have good shelf life stability; however, needed to be changed on daily bases if operated in continuous use at elevated temperatures (beyond 30°C). In order to perform online measurements in micro-bioreactors, linear range of the biosensor was required to meet the high concentrations present in fermentation broth. A laser-engraved PMMA micro flow chip was designed to accommodate a diffusion membrane that forms a barrier between the fermentation broth on one side and a circulating buffer on the other side, separating the sterile environment of the bioreactor and semi/non-sterile carrier buffer and sensor. The micro-bioreactor with integrated micro flow chip can be sterilized and the electrochemical biosensors can be installed just before use without compromising the sterility of the bioreactor. As only a reduced small amount of the analytes can diffuse through the membrane, the concentration in the carrier buffer is significantly reduced and hence addresses the linear range of the integrated biosensors. In order to test this technological approach, a special micro-bioreactor was constructed using stereolitographic 3D printing technique, featuring a designated space for the integration of the integrated biosensor microfluidic chip; the transparent walls of the bioreactor allowed for the attachment of an optical density sensor (λ = 650 nm) for cell growth monitoring (Figure 1).

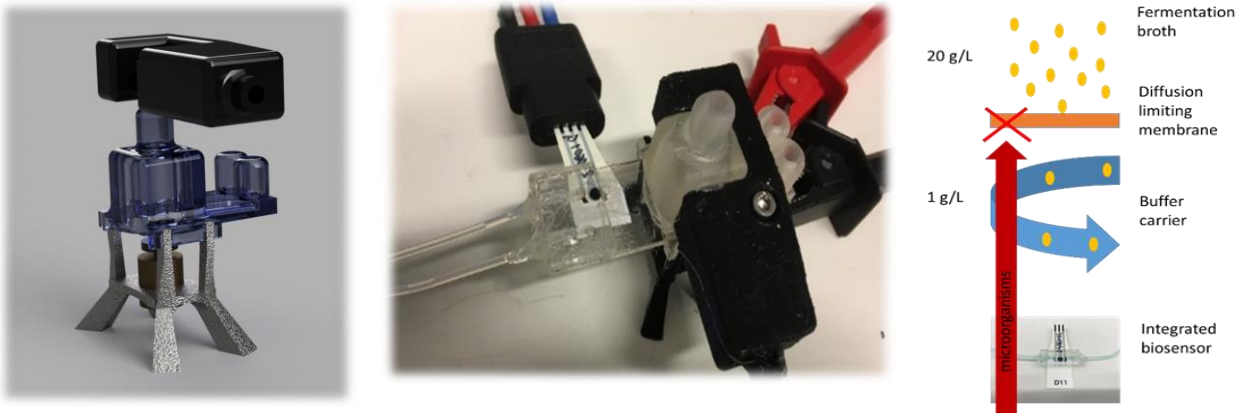

Figure 1: Left: A picture of the micro-bioreactor with integrated OD optical sensor and stand;center: A 3D-printed micro-bioreactor with integrated glucose biosensor and OD optical sensor. -right: Diffusion membrane and sensor scheme.

Due to its favorable stability, sensitivity and quick response times, the glucose biosensor was utilized in microfluidic devices where glucose was used as an inert tracer for reactor performance evaluation. As a model example, the glucose oxidase biosensor was integrated at the outlet of a packed bed bioreactor with Novozym® 435 particles containing immobilized lipase B used for esterification. The residence time distribution data obtained with a glucose tracer and monitored with the developed sensor could serve for validation of fluid flow simulation results. The advantage of this online system is that glucose can be monitored rapidly and selectively without the need to undergo lengthy sampling and measurement procedures typical for standard laboratory analytics (e.g. HPLC). Besides offering the advantages of a high-throughput analytic arrangement, the glucose oxidase biosensor was found to have significantly lower detection limit and greater selectivity than a HPLC, making it the tool of choice for further implementation in a continuously operated enzymatic microreactor.

The great strength in biosensor technology is the specific and selective nature of the biological component; equally the great weakness of biosensors is in its biological component due to denaturation, decomposition and deactivation processes related to environmental conditions (e.g. temperature, solvent, pH, oxidation, etc.…). To address these problems, synthetic polymer receptors have been investigated in order for them to be applied as substituted biosensor receptors and used in micro-bioreactor model applications.

The collective work to be presented was funded by the People Programme (Marie Curie Actions, Multi-ITN) of the European Union´s Seventh Framework Programme for research, technological development and demonstration project: The European network for innovative micro-bioreactor applications in bioprocess development (EUROMBR, Grant No. 608104).

Keywords: biosensors, micro-bioreactors, biosensor integration, online analytics, microfluidics, 3D printing, synthetic receptors

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CO5

Sensor integration in microbioreactor systems for high-throughput bioprocesses

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Enzyme and strain screening, as well as optimization of bioprocess operating conditions are key steps in the development of a biotechnological process. They involve a reliable and scalable study of all process parameters, mapping their influence and interactions for each of the possible enzymes/strains. The final process target determines the different requirements and conditions which are in focus during the development, thus further increasing the number of variables to consider during optimization, as well as the variability of the process development as a whole. The high number and variety of enzyme/strain candidates and corresponding optimal conditions, thus result in an expensive and time-consuming development process (1). Often it is not possible to test all the desired options. Therefore, dependent on the application, high throughput methods (HTP), mainly performed on microtiter plates and in shake flasks, are the preferred experimental test platforms. HTP allows the reduction of costs and time for industrial bioprocess development and scale-up, since several process parameters can be simultaneously tested in a controlled environment (2). Microtiter plates and shake flasks, however, offer limited control and information on the process conditions, which can lead to suboptimal process conditions due to differences between initial screening experiments and the final production process (3). Microbioreactors (MBR) offer the potential to find a balance between process control and throughput through the integration of new sensor technology and the development of devices tailored to the desired process by combining several MBR devices in a "plug-and-play" approach to better mimic or optimize a process.

The main objective of this research project is to develop a commercially viable HTP microbioreactor design capable of performing multiple biocatalytic reactions simultaneously in a controlled manner. Three microfluidic systems were individually tested and then combined in order to achieve such goal: a meander microfluidic channel (iX-factory, Germany) with integrated oxygen sensors (TUGraz, Austria), a microvalve system (Microfluidic ChipShop, Jena, Germany), and a meander channel for continuous thermal inactivation of enzymes (Figure 1). The model reaction chosen to test these systems was a glucose oxidation cascade reaction. The integrated oxygen sensors have a fast response to dynamic changes in substrate (provided by the valve system), while allowing to accurately monitor glucose indirectly. The continuous thermal inactivation meander channel is capable of denaturing both enzymes in less than 1 min at 80 ^{0}C, thus being able to be used as an intermediate step in a more complex modular system. This "plug and play" system can be used as a simple and fast enzymatic screening platform for reactions involving consumption or production of oxygen. Coupling of the system with a kinetic model may allow the monitoring of further parameters using only the oxygen sensors, and can further be used as a platform for optimizing either an enzyme or an enzymatic process.

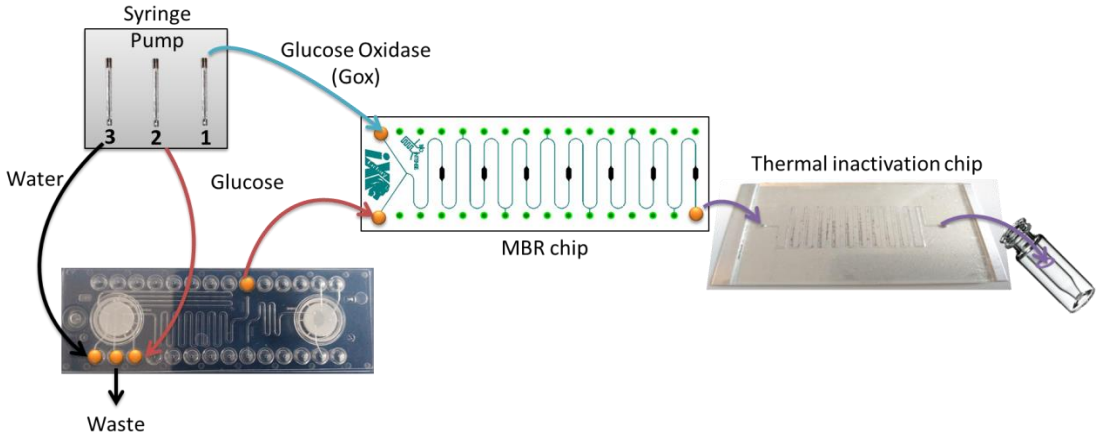

Figure 1: Schematics of "plug and play" assembly of the three microfluidic systems used.

Keywords: Screening, "plug and play", microbioreactor, continuous, oxygen sensors

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CO6
Miniaturized high throughput ecotoxicity test for microorganisms growth

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The proposed subject aims to develop miniaturized ecotoxicity tests with millifluidic devices. The regulatory context at both European and International level evolves rapidly thus relevant assessment methods are needed for rapid acquisition of possible toxicological determinants for industrial substances.
Ecotoxicity tests are a major challenge because they use animal testing to a limited extent and should be limited as much as possible. However, these tests are essential in order to determine the acute and chronic environmental toxicity of substances to different living organisms: microalgae, daphnia, fish (for the aquatic compartment), bacteria, worms, or plants (for the terrestrial compartment).
We propose here to develop and adapt some of these tests in miniaturized fluidic systems in order to benefit from the screening capacities of these tools[1-3]. In particular, we would like to focus on testing with microorganisms which size is compatible with our tools. For these reasons, we have adapted the chronic toxicity tests from microalgae (standard NF EN ISO 8692) in our millifluidic design.

We use a millifluidic approach in which the microorganisms are confined within a drop containing the chemical substance. The droplets are transported in a continuous immiscible phase. The use and the interest of the microdropplets to investigate microalgae or bacteria have already been demonstrated in the literature[4-9]. Drops generated in the millifluidic systems can circulate in the flow channels but also be stored. Since each of the drops represents a replicate, it is possible to generate several drops of identical or variable composition; it becomes possible to access a large amount of information. This makes it possible, for example, to have a high statistical power for a given condition or to screen several compositions in parallel and thus accelerate the data acquisition phases.

Figure 1: Air-liquid droplets in a 2.2 inner diameter tubing (high purity fluoropolymer)

Preliminary tests have been performed to follow the growth of Pseudokirchneriella subcapitata in microdroplets with various type of tubing. The droplets are generated thanks to two co-axial tubing and are separated by air plug. We observe that algae growth is higher in PTFE tubing, probably due to higher oxygen permeability if we compare it to glass tubes (figure 2,A). The device reliability has been evaluated from three different inoculums. The results shown in figure 2,B are similar and allow to confirm the reliability of this miniaturized test.

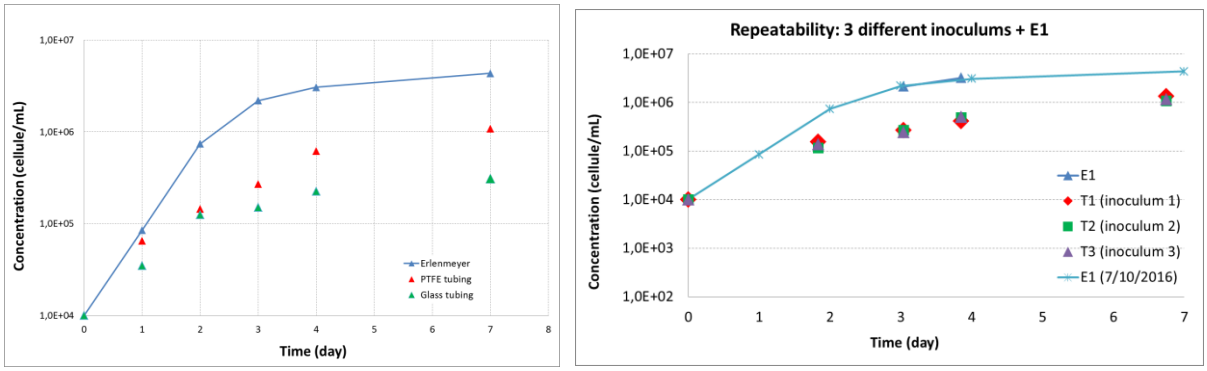

Figure 2: Algae growth evolution comparison within two different tubing (glass and fluoropolymer), and from 3 different inoculum

Other tests have been done at various concentrations of a reference substance (3-5 dichlorophenol) and show the inhibition of growth of algae in comparison to the control. Relation between the classical tests in Erlenmeyer has also been investigated. Other concentrations will be performed in order to estimate the LC 50.

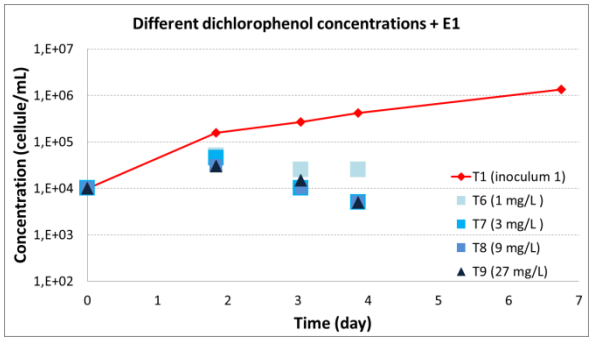

Figure 3: Algae growth evolution with various polluant concentration (3-5 dichlorophenol)

We plan to adapt this non-intrusive quantitative online growth measurement to follow automatically the Algae growth in several miniaturized droplet tubing to evaluate the toxicity of various products.

Then we will evaluate the possibility to miniaturize other toxicity and biodegradability tests.

Keywords: miniaturization, millifuidic, ecotoxicity test, automatization, algae microorganism

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CP1

Glucose sensor employing optical oxygen transducer with tunable dynamic range for applications in microreactor

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One of the most widespread methods for glucose sensing is based on the enzyme glucose oxidase. The overall reaction of glucose oxidation can be simplified as following:

$$C_6H_{12}O_6 + O_2 \rightarrow C_6H_{10}O_6 + H_2O_2 \qquad \text{Equation 1}$$

One approach to determine the concentration of glucose is to measure the amount of oxygen consumed during the glucose oxidation under steady-state conditions by employing optical oxygen transducers.

Previous study shows that the flow rate effects the response of the sensor considerably.[1] The consumption of the oxygen during glucose oxidation results in the decrease of the concentration of oxygen in the matrix where the oxygen sensor is measuring. The amount of the oxygen supplied to the sensor is influenced by the flow rate. A kinetic equilibrium is established by the rate of oxygen consumption and supply. For the application of an optical glucose sensor in microfluidic systems and microreactors, i.e. rhomic chamber chip, the flow rate influences the amount of glucose and oxygen that can diffuse into the sensor layer and potentially reacts with glucose oxidase resulting in the consumption of oxygen.

We investigate the flow rate dependency of an optical glucose sensor in a rhombic chamber chip (volume of 250) with both numerical method - computational fluid dynamics (CFD) simulations, and experimental method. By adding a diffusion-limiting layer, we could reduce the concentration of glucose on top of the sensor to extend the dynamic range of the sensor. Therefore, we present a rational design of an optical glucose sensor with tunable dynamic range by varying the thickness of the diffusion layer with CFD simulations. We demonstrate the glucose sensors with a dynamic range up to 30 mM, which makes them suitable for applications in clinical diagnosis as well as for applications in biotechnology industry.

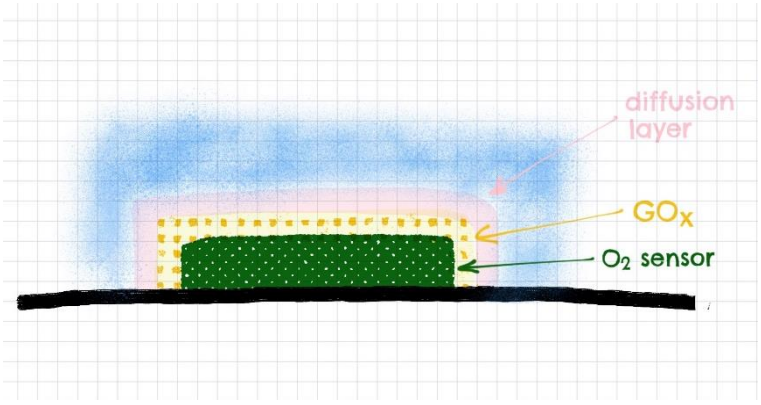

Figure 1: Scheme of glucose sensor employing optical oxygen transducer.

Keywords: optical glucose sensor, flow rate dependency, computational fluid dynamics

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CP2

Miniaturization of the *Lemna minor* toxicity test: application for testing of metals

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For last decades many chemicals were put on the market with limited information about their environmental effects and fate. Gathering information on all these chemicals dramatically increase laboratory toxicity testing and thus there is a need for adaptation of current testing methods. This can be achieved by miniaturizing the test systems, resulting in a reduced amount of organisms and chemicals, which also increases the cost-efficiency. The miniaturization and simplification of test systems has been already demonstrated by using microtiter plates in static toxicity tests with bacteria, algae but also for various invertebrates[1]. However, miniaturization of testing vessels for higher plants has not been introduced so far.

Therefore the aim of our study was to transfer the standard test design of duckweed *Lemna minor* to 6-well microtiter plates. Differences of the toxicity test using standardized OECD system[2] and the miniaturized microtiter plate were evaluated by determination of the plant specific growth rate and 168hEC_{50} values for standardized compound KCl. Furthermore, the miniaturized system was used for toxicity testing of Zn in the form of $ZnCl_2$. Because various metals are important environmental pollutants and many of them are also adsorbed on borosilicate glass beakers during static toxicity test affecting the overall toxic impact and thus also usage of plastic microtiter plates could bring many advantages in metal toxicity testing.

Comparison of 168hEC_{50} values showed no significant difference in KCl toxicity when test was performed in 100 mL glass beakers according to standardized OECD (ST) and in microtiter plates (MP); 7.333 ± 0.903 g/L (± SD, n = 3) and 7.933 ± 0.330 g/L (± SD, n = 3), respectively. Similarly, specific growth rate of *Lemna minor* was not affected by miniaturization of the system. In the ST test the specific growth rate was 0.258 ± 0.026 d^{-1} (± SD, n = 6) and in the MP test 0.253 ± 0.014 d^{-1} (± SD, n = 6) and thus MP test was assessed as comparable replacement of the ST test design. Results of toxicity test with $ZnCl_2$ showed a very good dose-response relationship; 1, 10 and 100 mg/L caused 2, 48 and 69% inhibition of specific growth rate of *Lemna minor*. Plants were also visually checked and in 100 mg/L of $ZnCl_2$ chlorosis occurred. The concentration of Zn measured at the beginning and at the end of the experiment did not differentiate more than 0.1%.

Our results showed that such toxicity test adaptation may reduce culturing of *Lemna minor* by 40%. Similarly, volume of tested compound is considerably reduced from 50 mL in ST test to 10 mL in MP test saving up to 80% of substances tested. Metals are less adsorbed on the plastic walls and thus microtiter plate seems to be more suitable test system than glass beakers for metal toxicity testing. Another advantage is reduction of spaces needed for illumination of test vessels and thus more experiments can proceed at once and save energy required for climate chambers and light sources.

Keywords: duckweed, metals, test miniaturization, toxicity testing

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CP3

The development of a biosensor with optical oxygen transduction based on a new multifunctional material made by coelectrospinning for determining uric acid in serum

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In this study, we have successfully used a multifunctional material developed by NanoMyP® to develop an optical biosensor for determining uric acid in human serum by using optical oxygen transduction following this recognition mechanism:

$$Uric\ acid + 2\,H_2O + O_2 \rightleftarrows Allantoin\ +\ CO_2 + 2\,H_2O_2$$

This material is formed by nanofibres with a coaxial morphology, which originates two different chemical environments: an inner hydrophobic environment containing and oxygen indicator dye (PdTFPP) and an outer hydrophilic environment specialized designed for immobilizing biomolecules (see Figure 1).

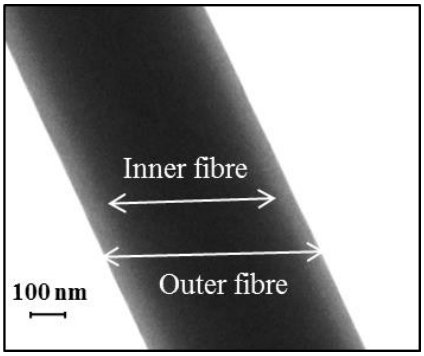

Figure 1: Structure of the coaxial material used for the development of the biosensor. The inner fiber is formed by meso-tetra(pentafluorophenyl)porphine (PtTFPP) solubilized in poly(methyl methacrylate) (PMMA), and the outer fiber is based on PolymBlend® (a polymer blend which has been formulated with an optimum mixture of two high molecular weight statistic copolymers to produce materials by electrospinning).[1]

In this work, several chemical parameters (glutaraldehyde concentration, pH and buffer for the immobilisation, concentration of the enzyme and reaction time) have been optimized in order to optimize the immobilization of the uricase as well as to achieve the best analytical figures of merits. The obtained biosensing film provides good analytical performances (detection limit of 15 µM and linear range of 50-500 µM) which allow determining uric acid in serum samples. The obtained results showed a good correlation (0.991) with those obtained by an external laboratory, which indicates that this material can be used as a promising sensing film. Moreover, it could also be used for the immobilization of other enzymes and for hosting other dyes, which could originate interesting applications in the field of biosensing.

This work has received funding from the People Programme (Marie Curie Actions, Multi-ITN) of the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 608104 (EUROMBR) and the Spanish Ministry of Economy and Competitiveness (Ramon-Marquez's grant reference AP2012-0944, Medina-Castillo's Torres Quevedo contract reference PTQ-11-04904 and project CTQ2014-53442-P).

Keywords: multifunctional material, nanotechnology; oxygen transduction; optical biosensor; uric acid

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CP4

Application of the biotin-streptavidin interaction to improve the immobilization of uricase on the development of optical biosensors

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In this study, we have modified a multifunctional nanofiber membrane produced by coelectrospinning by NanoMyP® to improve its ability in the development of an optical biosensor for determining uric acid in serum.

The base material is formed by nanofibres with a coaxial morphology, which originates two different chemical environments: an inner hydrophobic environment containing an oxygen indicator dye (PdTFPP) and an outer hydrophilic environment functionalized with NH_2 for immobilizing biomolecules. In this work, we have used these NH_2 groups to covalent bond streptavidin (SAv) to the surface of the coaxial membrane and use the biotin-streptavidin interaction to develop a biosensor for determining uric acid in serum (see Figure 1).

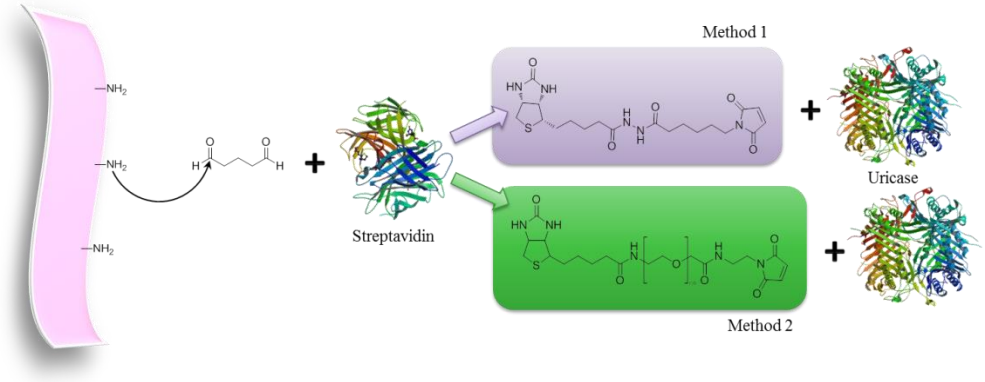

Figure 1: Strategies employed for the immobilization of uricase on the coaxial membrane.

The immobilization of the uricase was carried out by two different strategies using biotin-streptavidin affinity interaction. The amount of immobilized enzyme and its relative activity were determined for each immobilization strategy, obtaining the best response with the uricase immobilized using the biotinylation reagent with polyethylene glycol. The presence of streptavidin on the surface of the membrane improved the uricase immobilization and the chain of polyethylene glycol improves the solubility of the reagent. It increases the yield of the biotinylation and acts as a long linker, ensuring that the enzyme is kept at an adequate distance from the surface of the membrane that does not affect its biological activity.

The resulting proposed biosensor provides good analytical features (detection limit of 0.9 µM, quantification limit of 3.0 µM, and linear range from 5 to 300 µM) and can be used to determine the uric acid concentration in serum samples without any sample treatment. The obtained results were compared with those obtained by

an external reference laboratory, achieving a good correlation (0.997) which demonstrates its applicability as biosensor with optical oxygen transduction.

Compared with a previous work in which uricase was directly immobilized on the surface of the coaxial material without SAv modification, it is possible to conclude that the use of biotin-streptavidin affinity interaction increases the activity of the immobilized enzyme and improves the analytical figures (the limit of detection is improved more than 15 times).

This work has received funding from the People Programme (Marie Curie Actions, Multi-ITN) of the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 608104 (EUROMBR) and the Spanish Ministry of Economy and Competitiveness (Ramon-Marquez's grant reference AP2012-0944, Medina-Castillo's Torres Quevedo contract reference PTQ-11-04904 and project CTQ2014-53442-P).

Keywords: optical biosensor, oxygen transduction, multifunctional material, biotin- streptavidin, uric acid.

CP5

Fabrication and characterization of a microfluidic device to ultrapurify blood samples

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Often, modern diagnostic techniques necessitate the separation and purification of components of particular interest, with different morphologies from patients' samples[1], blood, saliva or stool. Here we provide a successful microfluidic integration of ultra-purification of blood samples, including capability of sorting whole blood from erythrocytes and debris exhibiting 97% capture efficiency. The innovative device is fabricated in three layers of Poly(methyl methacrylate) (PMMA) of thickness 0.8 mm, through milling. A filtering membrane 0.01mm thick is made out of PMMA through photolithography and plasma etching, later bonded together by a solvent or UV light assisted bonding process. The membrane separates upper micro-chamber from a bottom micro-chamber connected to input and output channels[2] (0.25mm wide; 0.20mm deep). The membrane is composed of an array of rectangular holes ranging 0.05mmx0.2mm, allows cell sorting by morphological differences (Figure 1). Once the blood sample containing different cell populations is injected into the upper chamber either by pipette or external syringe pump, the cells of interest, in this case erythrocytes and debris are separated because only one type of cells can pass through the micro holes of the membrane. The cells are forced to cross the membrane holes through gravity[3]. The filtered samples are then recovered from the upper chamber by flushing it with a syringe connected to it at high flow rate (1ml/min) and clogging the inlet and outlet of the bottom chamber. The yields of the number of filtered cells are calculated according to the following formula:

$$\%yield = \left(1 - \left(\frac{(Ci - N)}{Ci}\right)\right) * 100$$

Where Ci is the initial concentration of fluxed erythrocytes in the upper micro-chamber, N is the medium value of erythrocytes collected and calculated by Burker chamber.

1 2

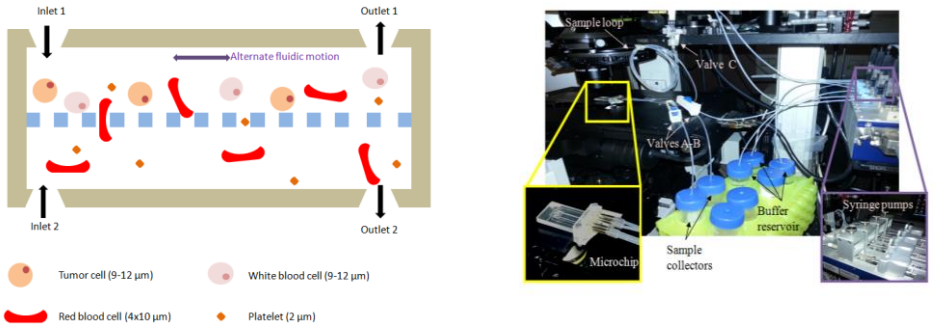

Figure 1: Working principle of the filtering membrane (blue): sample is injected from Inlet 1. Outlet 1 recovers the non-filtered cells; Outlet 2 recovers filtered cells. Inlet 2 is used to inject buffer; Figure 2: Experimental set-up of the microfluidic device

The results show majority of the erythrocytes injected in the upper chamber (Figure 3a); after alternate flow, the cells of larger size (10-15 µm) remain in the upper chamber (Figure 3b), while the erythrocytes (Figure 3c), having dimensions (6-9 µm)) collected in the lower micro chamber. Three devices are used to perform the experiments with different membrane hole size, 5-6-7 µm and mixing flow rate, 300-400-600 µl/min, that resulted in varied cell sorting percentages (Figure 4). The experimental results have proved to be very

satisfactory, not because it accounted to high capture efficiency but because it overcomes a great challenge related to the microfluidic methods that use the filtration mechanism; "clogging of the filter".

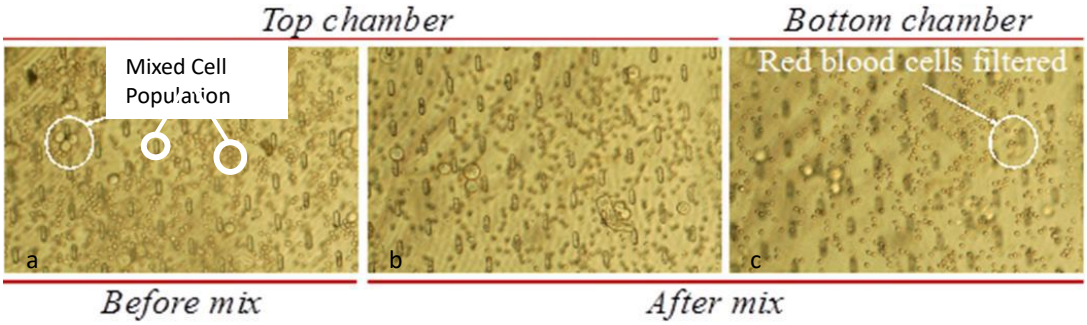

Figure 3: Top Chamber pre- and post-alternating motion; bottom chamber after mix

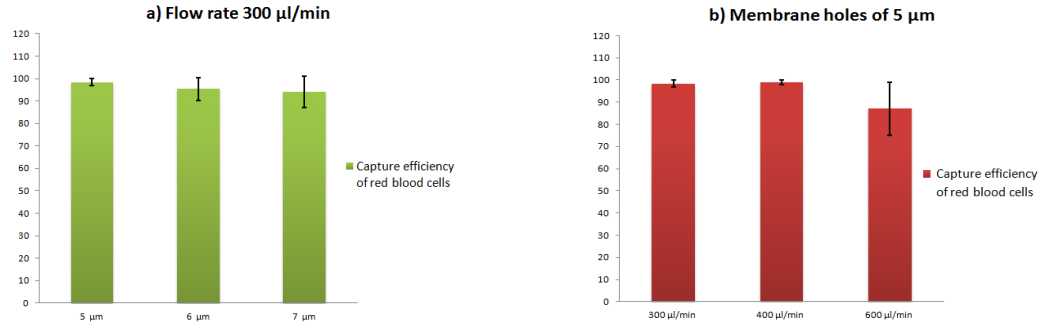

Figure 4: Graphs showing different sorting capabilities with respect to membrane hole dimensions and flow rate – a) Capture efficiency of red blood cells at fixed flow rate and different microdevices; b) Capture efficiency of single microdevice with different mixing flow rate;

The micro device developed has numerous advantages, of which, are: 1) Purification of cell samples that are not reachable with conventional instruments; 2) Possibility to recover the isolated sample for downstream analysis; 3) High purity sorting in smaller sample volumes; 4) A simplified set- up; 5) Use of inexpensive single use devices and instruments; 6) Compatibility with mass production technologies; 7) Inexpensive and robust device. The device compatibility and ability to ultra-purify cells from populations allows this technique to be used experimentally in a broad range of potential advanced analytical instrumentation.

Keywords: Microfluidic Cell sorting, Microfluidic Device, Ultra-purification;

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CP6

Immobilization of microbial transglutaminase on carboxyl-functionalized magnetic iron oxide maghemite (γ-Fe_2O_3) nanoparticle clusters for application in microfluidic flow injection analysis of biogenic amines

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Microbial transglutaminase (MTG) is an enzyme deriving from a variant of *Streptomyces mobaraensis* which catalyses an acyl transfer reaction between the γ-carboxylamide group of a peptide-bound glutaminyl residue and a primary amine. Due to its mass production, low molecular weight, and Ca^{2+} independency as well as good stability under a wide range of pH values and temperatures, this enzyme has been widely applied in food processing. It is used for gelling food products, improving the solubility, water-holding capacity or thermal stability of food proteins and restructuring food products in order to improve their flavour, nutritional value, appearance or texture [1]. In addition, MTG can also be applied in determination of biogenic amines (BAs), which are the products of amino acid decarboxylation in food fermentation. These nitrogenous compounds show toxicity at high levels of intake and can indicate food spoilage. MTG is able to catalyse the amine incorporation reaction between a primary amine (e.g. BA) and an acyl donor substrate (e.g. Z-Gln-Gly) in which ammonia is released [2]. Selecting a proper immobilization approach, which leads to enzyme-catalysed reactions where substrates are immobilized on solid surfaces, is essential for ensuring and/or improving enzyme properties, such as stability, activity, specificity, as well as recovery. Furthermore, the possibility to reuse the biocatalyst has not only the economic advantages, but it also offers user-friendly handling of biocatalyst and related devices. All these advantages indicate that immobilization of the enzyme is suggested, when applied as industrial biocatalyst [3]. Miniaturization of the flowing system provides the improvement of reagent consumption, shortening the time of analysis and high sample throughput, as has been shown for various microfluidic analytical platforms [4]. In such systems the controlled immobilization of enzymes is important for the reliability and sensitivity of the analysis [5].

The immobilization of microbial transglutaminase (MTG) was performed on carboxyl-functionalized magnetic iron oxide maghemite (γ-Fe_2O_3) nanoparticle clusters (f-COOH@NP) with the size of 120 nm. First, the nanoparticles were activated using carbodiimide chemistry in order to activate the carboxyl groups at the surface of functionalized nanoparticles, which then react with amino group (-NH_2) of the amino acid on the enzyme. Next, the MTG (440 μg/mL) was added and the solution was incubated for 1 hour at room temperature. The remaining carboxyl groups were blocked with amine-containing TRIS buffer. After 30 minutes of incubation, the suspension of MTG-bioconjugated nanoparticle clusters was washed three times with phosphate buffer (1 mM, pH 7.4). For the purtpose of BA determination, the efficiency of the immobilization was determined after 90 minutes of BAs incubation with MTG-bioconjugated nanoparticle clusters at 37°C where released ammonia was quantified. The released ammonia was introduced into a microfluidic device for indophenol blue formation and further detected in a glass microchip by thermal lens microscopy (TLM) [5] (Fig.1). To exclude false positive results control solution (Test) was prepared in parallel where only TRIS buffer was added to the reaction mixture instead of MTG.

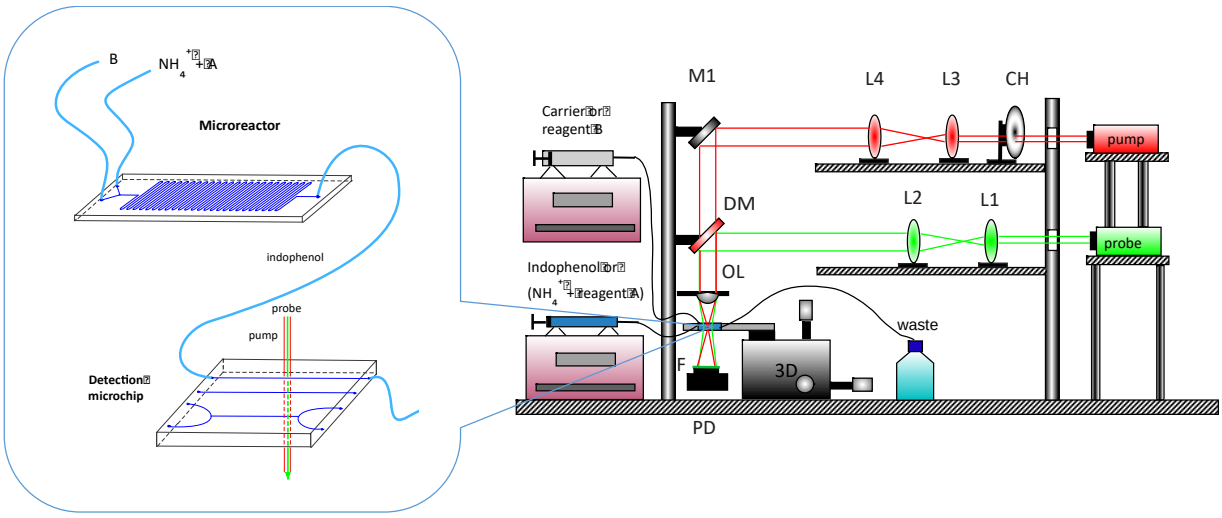

Figure 1. µFIA-TLM system and microchip connection for reaction and detection of indophenol blue.

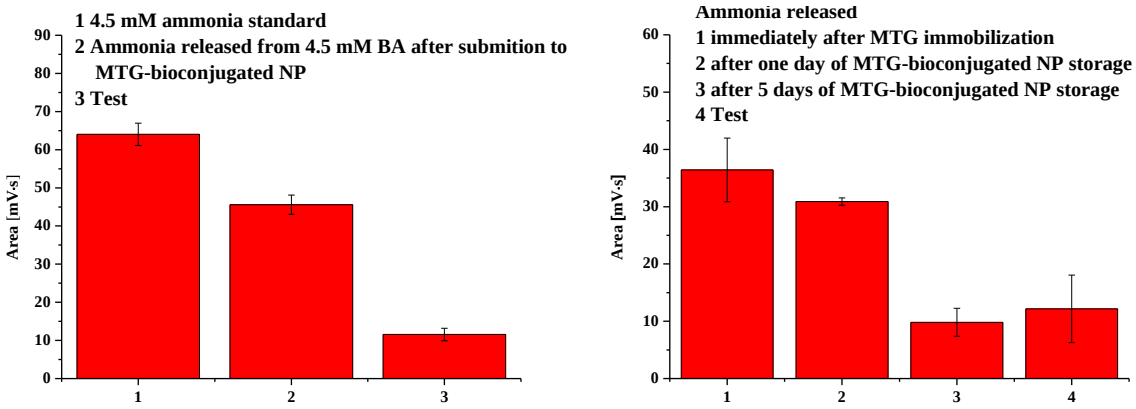

Figure 2. TLM determination of ammonia released from BAs as a result of immobilized MTG activity.

According to Figure 2, 70% of ammonia was released in the catalytic reaction from BAs subjected to the MTG-bioconjugated nanoparticle clusters. The enzyme also retained 80% of the activity after one day of storage at 4°C, but has completely lost the activity after 5 days of storage under same conditions. Nevertheless, the achieved results show a promising start of a bioreactor design for the indirect determination of BAs through the detection of ammonia in a microfluidic system.

Keywords: immobilization, microbial transglutaminase, biogenic amines, microfluidics, thermal lens microscope

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CP7

A microfluidic device with integrated coaxial nanofibre membranes for optical determination of glucose

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In this work, a multifunctional material with a core-shell structure containing an inner optical oxygen transducer (PdTFPP) has been successfully used for the immobilization of glucose oxidase on its outer surface and the subsequent determination of glucose. The material was fabricated by co-electrospinning and immobilizing the enzyme by physical adsorption. The sensing mechanism is based on the oxidation of glucose by glucose oxidase following this reaction:

$$Glucose + H_2O + O_2 \rightleftarrows Gluconic\ acid + H_2O_2$$

This oxidation produces a localized decrease in the dissolved oxygen amount and consequently originates a measurable increase in the luminescence intensity of the inner oxygen transducer, which can be used to determine the concentration of glucose.

The coaxial material was introduced into a microfluidic chip and the determination of glucose was successfully carried out with higher sensitivity, detection limit of 35 μM (0.0063 mg mL^{-1}) and quantification limit of 105 μM (0.019 mg mL^{-1}), and a large calibration range, from 0.1 to 140 mM (0.02 to 25.00 mg mL^{-1}); the sensing response for the coaxial membrane inside the chip was over 300 times higher than the response for the coaxial material outside of the chip.

The results show that the combination of this coaxial material, that is formed by polymers with optical clarity/transparency, permeability to oxygen and oxygen-sensitive dyes with long luminescence lifetimes and high photostability and oxygen sensitivity, integrated into microfluidic chips can create promising optical biosensors with high sensing features.

This work has received funding from the People Programme (Marie Curie Actions, Multi-ITN) of the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 608104 (EUROMBR) and the Spanish Ministry of Economy and Competitiveness (Ramon-Marquez's grant reference AP2012-0944, Medina-Castillo's Torres Quevedo contract reference PTQ-11-04904 and project CTQ2014-53442-P).

Keywords: multifunctional material; microfluidic; optical biosensor; glucose; oxygen transduction

CP8

Shaping of lipid membranes in a microfluidic diffusion chamber

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Lipid membrane compartments with a large area-to-volume ratio, such as the mitochondria or the chloroplast, harbor many fundamental cellular processes and also represent an essential framework for synthetic biology. Yet, despite significant advances of biochemical techniques in synthetic biology, methods for controlled shaping of synthetic lipid membranes remain scarce. The main challenge of membrane shaping is that flaccid membranes are extremely soft, undergo noticeable thermal fluctuations and can irreversibly warp under the slightest hydrodynamic flow. Here we present a novel approach for controlled shaping of giant lipid vesicles by regulating osmotic conditions and the concentration of membrane-shaping molecules. The method is based on the microfluidic diffusion chamber[1], where the solution can be repeatedly exchanged solely by diffusion, without any hydrodynamic flow that could deform the membrane.

The diffusion chamber is designed as a simple dead-end microchannel extending from the main microfluidic channel (Fig. 1). A systematic experimental and theoretical analysis revealed that the flow properties in the chamber depend decisively on its depth/width ratio[2]. Notably, if the chamber aspect ratio is higher than approximately 0.51, counter-flow vortices emerge even at vanishing Reynolds number. In the vortex-free regime below the threshold aspect ratio, the flow velocity decays exponentially away from the chamber entrance, with a decay length that scales with the width of the chamber and depends also on the aspect ratio of its cross section. In a typical chamber used for membrane shaping (depth = 40 μm, width = 100 μm, length = 250 μm) the decay length is approximately 23 μm, rendering the back end of the chamber effectively flow free.

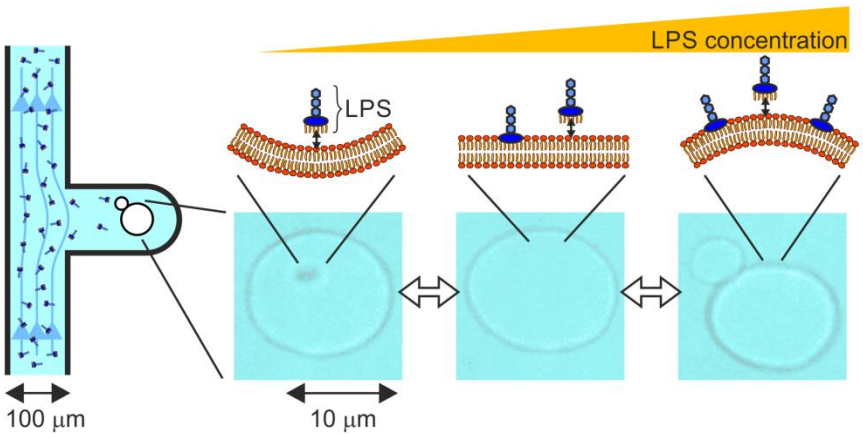

Figure 1: A schematic representation of vesicle shaping in a microfluidic diffusion chamber. A spherical vesicle is first transferred into the diffusion chamber by optical tweezers. The vesicle is then osmotically deflated and its shape is regulated by adjusting the concentration of LPS molecules which intercalate into the membrane and drive membrane bending. The method provides a universal framework for fast, controlled and reversible shaping of lipid membranes at moderate area-to-volume ratios.

In the membrane-shaping experiments, a spherical, undeformable lipid vesicle is transferred into the diffusion chamber by optical tweezers. The vesicle is then osmotically deflated into a flaccid state and its shape is controlled by adjusting the concentration of membrane-interacting molecules in the main channel. Specifically, we use lipopolysaccharide (LPS) molecules, which partition from the solution into the outer leaflet of the membrane, do not flip to the inner side and thus drive the membrane bending by the bilayer-couple mechanism (Fig. 1). We demonstrate controlled and reversible shape transformations across three shape classes at intermediate area-to-volume ratios, from invaginated to evaginated flaccid vesicles, which are in excellent agreement with the theory of equilibrium vesicle shapes. More extensive shape transformations remain reversible, but often comprise non-equilibrated shapes. The results indicate that controlled shaping into highly convoluted membranes resembling cell organelles will require additional membrane shaping mechanisms, such as macromolecular scaffolding[3], and new techniques for membrane equilibration, such as electroporation-on-a-chip.

To the best of our knowledge, the microfluidic diffusion chamber provides the first universal approach for fast, controlled and reversible shaping of lipid vesicles that could lead to controlled fabrication of synthetic membrane compartments harboring efficient membrane-bound biochemical processes.

Keywords: lipid membranes, synthetic biology, synthetic organelles, diffusion chamber

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Session D: BIOPROCESS INTENSIFICATION AND INTEGRATION

DK

Manufacturing Development Strategies for Bio-Lamina-Plate Technology and Other Process Intensification Technologies

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Methane or natural gas (CH_4) is a common low-value or waste stream from industrial and biological processing, as well as agriculture and landfill operations. These streams are usually flared, resulting in wasted energy and increased CO_2 emissions. Conventional technologies for conversion of methane gas to fuels or other high-value products require large capital investments for commercial-scale plants. Further, these processes are inefficient at small scales, and so cannot be used to harness the energy contained in remote and dispersed natural gas or waste CH_4 resources throughout the United States. Microbial biocatalysts can be integrated within microchannel process technology as a means for converting methane sources into liquid fuels at small scale under ambient conditions. These micro-scale bioreactors may be deployed directly at the methane source, eliminating the need to collect, recompress, store, or transport methane gas to a costly, centralized plant. They also offer the ability to produce a wide variety of products from methane streams, using the same basic hardware.

In this manner, the integration of microchannel process technology with bioreaction technology has great potential for reducing the size and capital costs of chemical processing facilities making distributed fuel production a reality. However, cost and technical risk remain barriers for acceptance of this technology within chemical industries. This presentation will highlight efforts over the past 15 years to commercialize process intensification technologies through the Microproducts Breakthrough Institute at Oregon State University. Key to these commercialization efforts has been the ability to model the cost-of-goods-sold for embodiments of intensified chemical unit operations. Examples will be discussed showing how these cost analyses have informed decisions for advancing intensified technologies to the marketplace.

Specifically, preliminary cost analyses for a Bio-Lamina-Plate (BLP) microreactor will be discussed. BLP microreactors are currently under development at Oregon State University for the production of liquid fuels. To explore the scale-up of fuel production using BLP technology, a plant simulation has been developed in Aspen Plus (Figure 1) for the production of butanol at a capacity of 500 barrels per day (BPD). A requisite techno-economic analysis was performed to determine the economic feasibility of using BLP microreactors for this application. A major finding from this study was a trend in reactor material cost savings with each additional technology innovations (see Figure 2). This results in the potential to develop a BLP microreactorbased plant with capital costs below the current industrial plant limit of $100k/BPD as identified by the U.S. Department of Energy. The use of these cost analyses have influenced the design of the micro-scale bioreactor as we have shifted R&D priorities towards major cost inhibitors such as hardware material selection, process consumables, and downstream processing efficiency. The cost-of-goods-sold analysis has served as an initial step in evaluating the cost of a fully modular industrial-scale bio-chemical processing plant. A novel microscale liquid-liquid separation unit, developed in parallel the BLP microreactor, has been incorporated into this analysis, with further plans to replace other traditional operation units with higher efficiency micro-chemical processing technologies under development at Oregon State University.

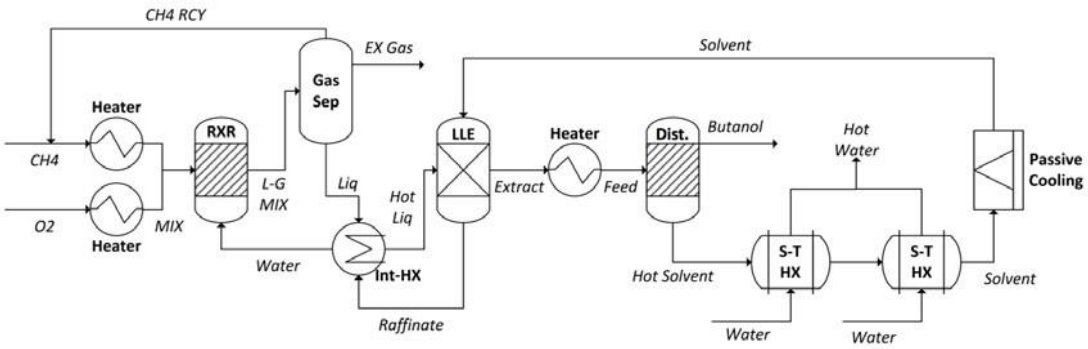

Figure 1. *Process flow diagram for a butanol production plant utilizing BLP reactors and micro-scale liquid-liquid separation technology. Simulated with Aspen Plus.*

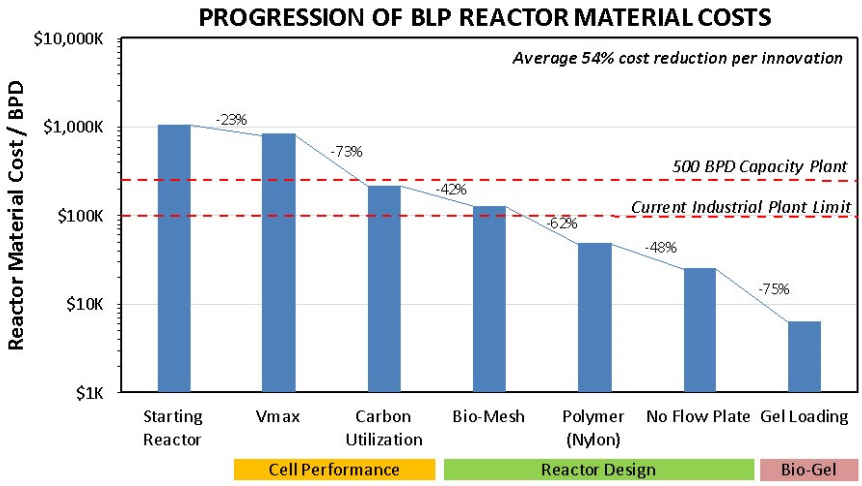

Figure 2. *BLP reactor material costs with each additional major technology innovation for a 500 BPD capacity plant for butanol production.*

More recently, efforts at Oregon State University have shifted toward injecting new manufacturing technologies into the upstream chemical equipment supply chain through the Rapid Advancement of Process Intensification Deployment (RAPID) Institute in partnership with the American Institute of Chemical Engineers. In RAPID, we are working with downstream chemical manufacturers to pool resources and combine markets to enable the upstream capital investments needed to drive down manufacturing costs in the supply chain. RAPID presents an opportunity to breakthrough cost and reliability barriers that stand in the way of advancing these types of bio-based process intensification technologies to market.

DO1

Sustainability of process options for enzymatic packed bed flow reactors at relevant scale and future role of 'Spaciants'

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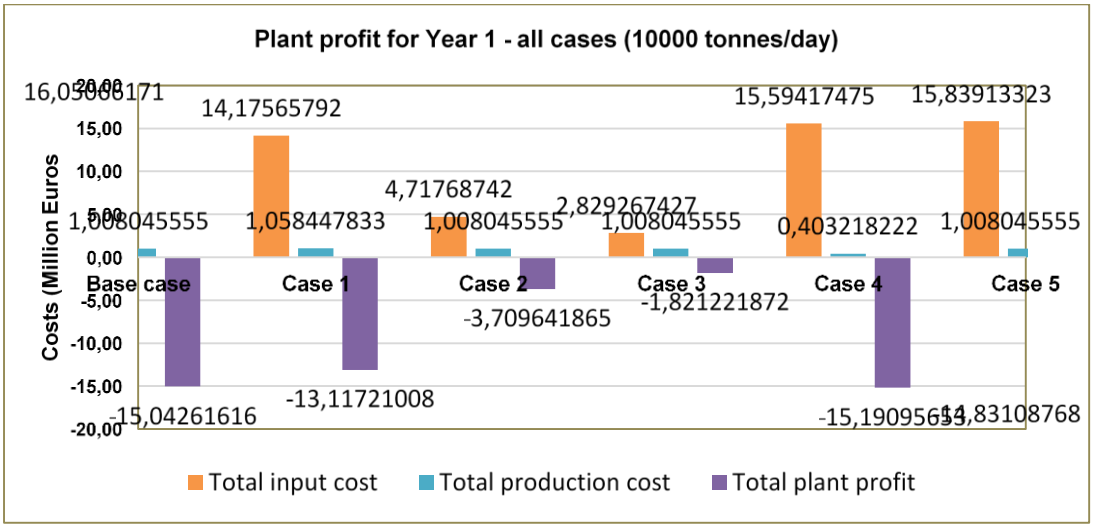
Biodiesel production technology is a good test case for enabling technologies. It is commercial, yet far from being a breakthrough business. It can still be further intensified by faster mass transfer across larger specific liquid phase boundaries (between the biodiesel reactants, alcohol and oil) and smart catalyst can enable the use of more sustainable base materials / feedstocks. Microreactors make the first happen, whereas enzymes (and cells) as biocatalysts open door concerning the last issue. So far mainly, isolated approaches with either microreactors or enzymes were reported. To our best know-ledge, no commercial transfer has been reached and no demonstration of sustainability was achieved. Question thus is what the combination of the two – the bio flow chemistry – offers as intrinsic potential.

Starting from an overview of today's industrial biodiesel production, the advantages and disadvantages of the lipase catalyzed transesterification process in microreactors as chosen case of study are discussed and a cost analysis of biodiesel production in enzyme packed-bed microreactors is given.[1] It is shown that the enzyme fixation, i.e. the choice of cost-efficient polymeric supports to which the enzymes anchor, is the most decisive point in the sustainability profile. This outperforms even the otherwise so relevant parameters as activity retention after immobilization and enzyme stability in immobilized state (cycle time). This clearly shows that the supporting materials commonly in use, such as the Eupergit polymeric resin, are far from commercial use at relevant scale.[1] Thus, a pretty cost-efficient polymer is proposed, SEPABEADS EC-EP - Oxirane support, which is only available since recently and thus could not receive wide attention for enzyme fixation.[2] Own experiments with Thermomyces lanuginosus lipase confirm a successful immobilization strategy (immobilization efficiency, retention) with that polymer and ability for efficient reaction (yield). This enables to use cheaper starting material, i.e. sunflower and waste oil as opposed to the more expensive commonly used rapeseed oil. The cost profile of various intensified process options will be shown.

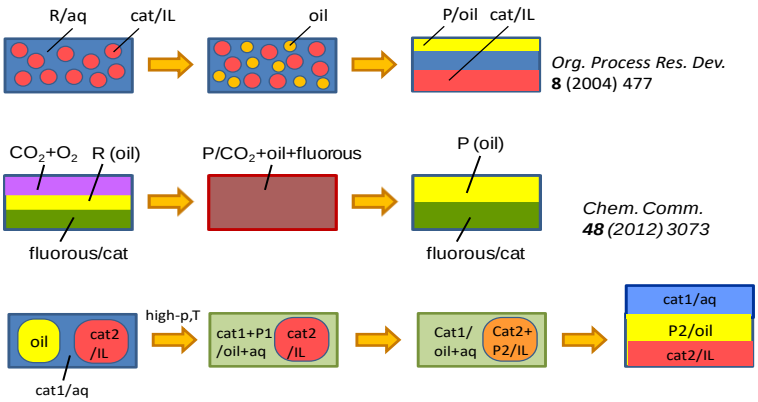
A parametric sensitivity analysis was carried out next to study the effect of the above mentioned key variables on the cost of producing biodiesel in flow. Five cases were studied where one parameter was changed at a time, while maintaining the others constant. The cases were as follows:

Case 1 *Activity retention - 60%*
Case 2 *Days before new enzyme needs to be immobilized: 39*
Case 3 *New SEPA beads support (SEPABEADS EC-EP - Oxirane support 85% cheaper the Eupergit CM)*
Case 4 *Raw material – Waste sunflower oil (instead of refined sunflower oil)*
Case 5 *Raw material – Rapeseed oil*



Above Figure shows the total plant profit for the first year for each of the cases mentioned above. We can see that the vase case has a large negative total balance. Case 1 shows also a high negative annual economic balance, but a reduction in negative balance is noted. Especially case 3 has potential, after some further process improvements, to result into a commercial case. The number of reactors needed in order to achieve the 10000 tones biodiesel/year has been reduced to 14.

The just started (01/17) FET Open project ONE-FLOW could mark a further dimension of intensification hereabout, by providing an entirely new systemic innovation on a process-design scale.[3] 'The Green-Solvent Spaciant Factory' is here proposed. Green functional master solvents allow to open and close interim reaction spaces alike reactors do, but more elegantly; thus, being named spaciants. Some generic solvent phase switching functions are shown in the Figure right.



In this way, the reaction is intensified and, more importantly, the separation as well. With the concept itself being presented, some thoughts will be given of useful process functions for the enzymatic continuous biodiesel case.

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DO2

Continuous lipase B-catalysed isoamyl acetate synthesis in a two-liquid phase system using Corning® AFR™: optimization and scale-up

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Biocatalysis is more and more looked at as a key component of sustainable processes for the production of commodity chemicals, pharmaceutical intermediates, flavour and fragrances.[1,2] Lipases (triacylglycerol lipases, EC 3.1.1.3) are being widely used as biocatalysts for "natural" short chain esters synthesis, which are widely used in food, cosmetic and pharmaceutical industry.[2] Lipase-catalyzed reactions in two-liquid phase systems benefit from increase of product and substrate solubility, shift of reaction equilibrium in the direction of esterification, increase of selectivity towards a desired product by taking advantage of the conformational role of solvents upon lipases, and significant reduction of the number of downstream separation operations and process streams.[3] Two-liquid phase reaction/separation systems are usually operated in batch or semi-batch modes, even though continuous process operations are known to offer several advantages.[1,3-5] Process-intensifying equipment such as novel reactors, and intensive mixing, heat-transfer and mass-transfer devices leads to a substantially smaller, cleaner, more energy-efficient, cheaper and sustainable technologies. [1,6] Besides, by exploiting similarity effects along the development chain and the modular nature of the small flow units, an easy implementation to modern modular plant environments (Future Factories) can be achieved. [1,7]

Among the flow reactors available on the market, the ones developed by Corning are easily scalable and can be customized to specific needs. The reactors are made by connecting in series fluidic modules made in a specialty glass or ceramic and have hydraulic diameters in the range of 0.3 up to few millimeters. The fluidic modules have volumes between 0.5 and 260 ml allowing operating flow rates between 2 g/min up to about 46 kg/min. The family of designs based on chains of identical cells – HEART – provides efficient mixing for homogeneous systems, and also creation and maintenance of fine dispersions for multi-phase applications, enabling excellent mass transfer performance.[7-9] This product line enables seamless scale-up: despite the increase of the internal dimensions, mixing quality, specific energy, heat and mass transfer coefficients are kept at equivalent levels.[8]

The low flow (LF) Corning fluidic module was successfully tested for *Candida antarctica* lipase B (CaLB)-catalyzed isoamyl acetate synthesis in a two-liquid phase system comprising of 100 mM sodium phosphate buffer with pH 8 and *n*-heptane.[5] The achieved productivity was the highest reported so far for this reaction. Furthermore, the chosen two-phase system enables *in-situ* extraction of the produced isoamyl acetate to the *n*-heptane phase, which allowed the reuse of the dissolved biocatalyst in several consecutive biotransformations performed within the LF Corning fluidic module.[5]

The aim of this study was twofold: the optimization of CaLB-catalyzed isoamyl acetate synthesis in a two-liquid phase system in a LF Corning fluidic module with 0.5 mL volume, extended with tube to give final volume of 1.1 mL, and then the scale-up of the process in a reactor at G1 scale with 70 mL volume.

The influence of enzyme concentration, flow phase ratios, isoamyl alcohol/acetic anhydride molar ratio and temperature were studied in order to find the operation conditions giving the highest productivity. Increasing the concentration of reagents and decreasing the aqueous/organic flow ratio led to a significant improvement. Enzyme precipitation and a strong (67 %) decrease of activity were observed at temperatures higher than 50°C when using high concentrations of acetic anhydride (2 M) and isoamyl alcohol (6 M). The best operating point corresponded to an aqueous/organic ratio of 15/85 (% vol.), with initial concentrations of acetic anhydride and isoamyl alcohol in *n*-heptane being as high as 2 M and 4 M, respectively. These conditions significantly reduce catalyst and solvent consumptions, making this process cheaper and sustainable.
The optimum operating conditions were applied in a LF reactor, as well as in a G1 scale reactor. The results proved the scalability of the processes brought by Corning AFR, since the same outstanding productivities were obtained at both scales (Figure 1).

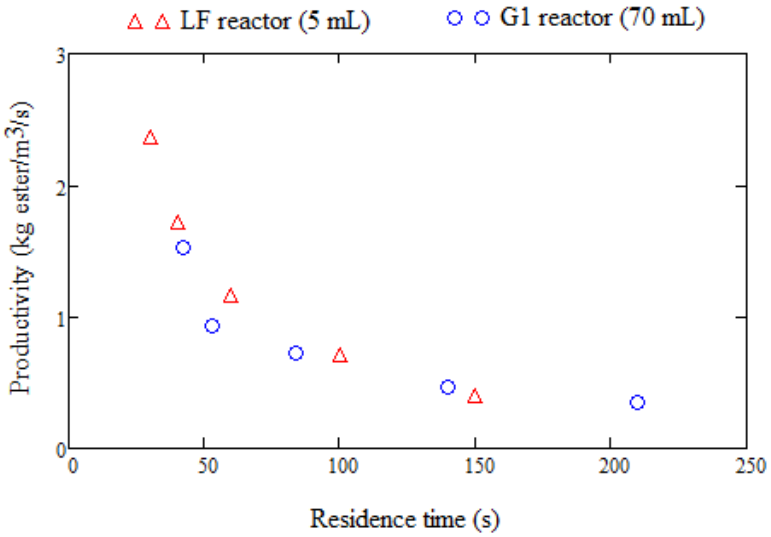

Figure 1: Comparison of the obtained productivity in LF and G1 reactor. Inlet concentrations: CaLB 0.24 g/L, 2 M acetic anhydride and 4 M of isoamyl alcohol in *n*-heptane; flow rate ratio of aqueous phase (0.1 M potassium phosphate buffer with pH 8) and *n*-heptane: 15:85 (% vol.); 30°C

Keywords: Corning AFR, esterification, Lipase B, isoamyl acetate, scale-up

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DO3

Development of a microbioreactor system for biopharmaceutical applications and analysis of scale down effects

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Particularly in early stage process development microbioreactor (MBR) systems are gaining increasing importance due to their capability of generating large quantities of data while reducing the amounts of substrates and utilities applied for each experiment. Hence, bioprocess development is accelerated, which is mostly accompanied by decreasing costs. Smaller reaction volumes allow for higher parallelization, enabling multiple reactions simultaneously, which in turn makes it possible to examine greater numbers of process variables. Therefore, miniaturization is a key factor in improving the performance of MBRs, making it economically and practically feasible[1-3]. MBRs are widely used for investigating of various biological systems from industrial and pharmaceutical industry, aiming for replacing experiments in shake flasks. Besides cell free experiments, bacterial and cell culturing was performed in different cultivation systems with reaction volumes ranging from µL to mL^2. Here, µL-scale bioreactors can be used among others for mammalian cell line screening, investigation of metabolic pathways, selecting of recombinant protein expressing clones and *in vitro* toxicity tests. The wide range of application explains its great benefit for many process developments[1,2].

Online measurements methods allow for monitoring of crucial cultivation process variables *in situ*, such as pH, dissolved oxygen, fluorescence and optical density without affecting the running experiment, providing process data through in-process real time monitoring. As a result, MBRs enable conclusive scaling up / down of pilot or large scale bioprocesses and are hence capable of mimicking bench-scale reactors, since transferability of data obtained in systems operated at volumes in the µL-range to reactors in industrial relevant capacities was shown for several MBRs. Nowadays, innovative technologies allow for fabrication of increasingly smaller shapes and devices, what on the one hand opens new opportunities in research and application of MBRs. Downsizing on the other hand is also accompanied by several difficulties and issues, which have to be addressed. Despite of the minimized dimensions, MBRs have to provide all features of a laboratory-sized reactor. Mandatory requirements of MBR systems for most bacterial and cell culturing cultivations are among others supply of sufficient oxygen and nutrients. Therefore, developing a suitable method of mixing is being of prime importance[3-5].

Here, first investigations of a novel design of a MBR system will be presented with a reaction volume in the lower µL-range entirely fabricated of glass, which is offering key advantages over commonly applied organic or hybrid materials in terms of hydrophobicity and surface condition as well as reduced amount of manufacturing steps. The considerably small working volume requires an appropriate mixing method to overcome capillary and adhesive forces. Therefore, electrical exciter attached to the glass plate set cultivation media into defined high-frequency movement. Adjusted to a frequency at which a certain fluid exchange pattern is excited, complete homogenization can be realized to ensure sufficient supply of cells with nutrients and oxygen. Each cavity will be equipped with non-invasive optical sensors and waveguides, for rapid generation of reliable process data without affecting the current cultivation. To further enhance automation and parallelization the reactor system will be implemented into a liquid handling system for defined dosage of cell cultivation media and active substances. Using piezo-based inkjet technology, volumes less than a µL can be dispensed discontinuously. Thus, cultivations in fed-batch or even continuous mode in minimized format are getting feasible.

The MBR will subsequently be applied for *in vitro*-cytotoxicity studies. Furthermore, throughout ongoing development of lab-on-chip technologies it offers future potential in the field of individual therapy, where the effect of active pharmaceutical ingredients on cells and on the formation of cellular tissue can be determined.

The presentation will give a brief insight into the development and characteristics of the MBR system and its application for biotechnological and pharmaceutical research. The aim for near future is to fully integrate automated and parallelized MBR systems into daily laboratory operations.

Acknowlegdements. LF, DR, AD, SM, and RK gratefully acknowledge the financial support from the German Research Foundation (DFG) within the project Development of microreactors for biopharmaceutical applications (DI 1934/9-1, KR 1897/5-1). RK and SLM gratefully acknowledge the financial support provided by the People Programme (Marie Curie Actions, Multi-ITN) of the European Union's Seventh Framework Programme for research, technological development and demonstration within the project EUROMBR - European network for innovative microbioreactor applications in bioprocess development (Project ID 608104).

Keywords: microbioreactor, scale down, in vitro toxicity test, mixing, lab-on-chip

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DO4

Mechanistic models in the development of microfluidic screening technologies

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The interest in downscaling within industrial biotechnology has increased significantly in the last decade[1]. The design, development and further implementation of small scale reactors, such as microbioreactors (MBRs), with integrated sensors and parallel operation is an adequate solution addressing the needs of biotechnology and process development for rapid, high-throughput, and cost-effective screening and synthesis of valuable chemicals[2],[3]. However, the successful application of MBR technology will only be possible if it can rely on appropriate software and automated data interpretation of the MBR experiments. Thus, the software and data interpretation tools should allow maximizing the exploitation of the flexibility and the capabilities of the MBR platform to deliver information-rich experiments on the one hand, and on extracting as much information as possible from the obtained experimental data on the other hand.

Applying mathematical models to experimental data will allow a better understanding of the bioprocess and the reactor performance[4]. In this project a mechanistic model was used for the description of the enzymatic reaction of glucose oxidase and glucose in presence of catalase inside a microfluidic platform (Figure 1).

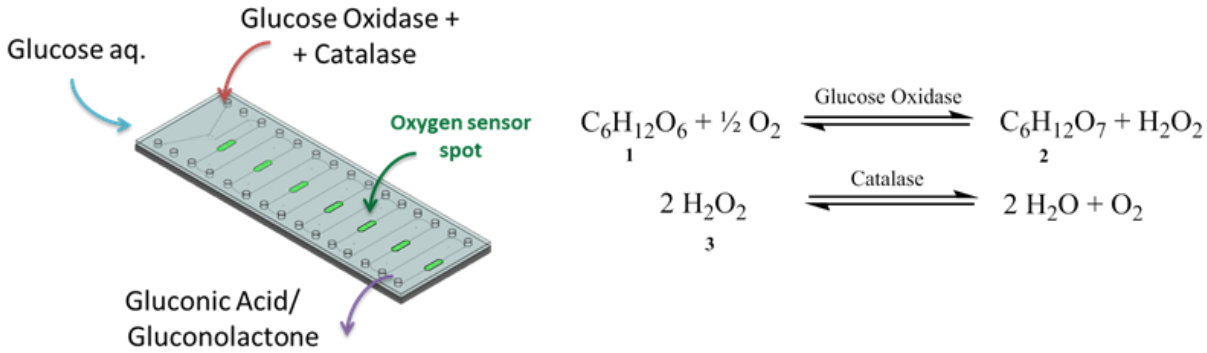

Figure 1: Schematic representation of the enzymatic reaction of glucose oxidase and glucose (1) in the presence of catalase.

The presented model is based on solving non-linear ordinary differential equations, which combine the biocatalytic kinetic term, as well as the reactor performance. The model parameters in this model are estimated based on the experimental data produced by integrated into the microfluidic platform optical sensors. The on-line monitoring of the reaction was possible by quantifying the oxygen production during catalyzed decomposition of hydrogen peroxide (3) (Figure 1). The developed model shows a good agreement with the obtained experimental data (Figure 2).

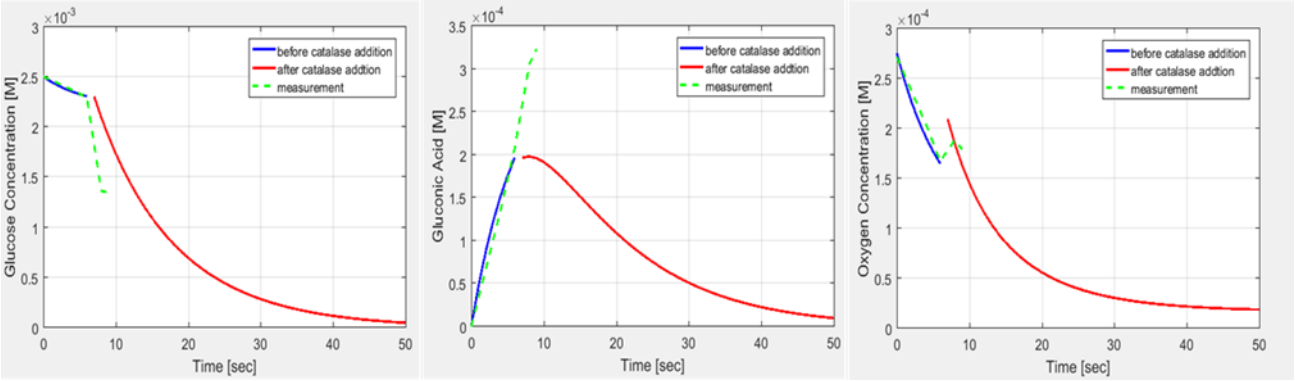

Figure 2: Comparison of concentration profiles for glucose, gluconic acid and oxygen in MBR: simulation results (solid line) vs. experimental data (dashed line).

Modelling the bioprocesses in a MBR together with the sensor response modelling will lead to the development of a very powerful tool. It will combine the determination of the transfer phenomena, physical response of the integrated sensor systems and the kinetics of the bioprocess together with the exact mechanism. Therewith, it will open up possibilities for subsequent design of improved experiments on the MBR platforms.

Keywords: mechanistic models, bioprocess modelling, biocatalysis, microfluidic platforms.

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DO5

Multiphase separation in microscale-based systems using capillary pressure gradients

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This research & development effort focuses on the development of flexible and easily distributed multiphase separation technology. The technology utilizes the concept of Microscale-based Processing Technology (MPT) with potential for significant gains in process intensification by accelerating mass transport and decreasing energy input per liquid volume compared to conventional methods. The power input per liquid volume for microscale-based separation is 3 to 4 orders of magnitude lower compared to conventional extractors. Mass transfer coefficients are up to 500X larger versus packed bed columns. Beyond energy consumption, the scalable and distributed nature of MPT makes it amenable towards utilizing renewable energy sources. To the best of our knowledge (although lab-on-a-chip-scale micro-contactor separation has been investigated by various groups) no large-scale, economically feasible MPT separation applications exist. This design approach focuses on addressing the overall cost of separation process intensification, while considering manufacturing of microchannel lamina plates that can be scale-out in the x-y-plane (larger plate area) and z-plane (numbering up) to increase processing throughput with dramatically decreased unit manufacturing costs.

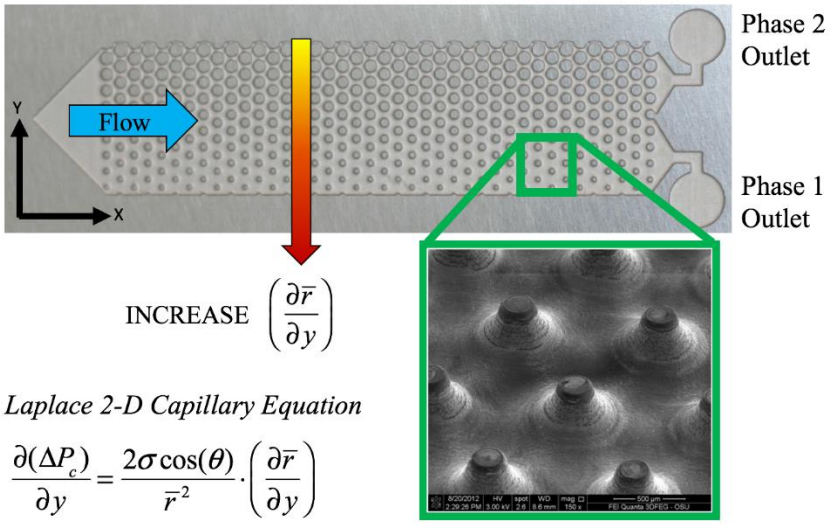

Figure 1: Microscale-based flow system with "micropost" features creating a capillary force gradient.

This multiphase separation in microchannel systems directs flow of each phase by creating a capillary force gradient via size and spacing of micro-scale architectural features, thereby controlling interfacial curvatures. The 2-D Laplace capillary equation shows the relationship between curvature and capillary pressure and an example of an implemented gradient in micro-post features are shown in ***Figure 1***. With a proper choice of surface properties, the system is designed so that a particular phase cannot overcome capillary forces in one direction of the gradient with inertial and viscous forces, guiding the fluid towards a particular outlet stream. The two phases are introduced to the separator as microdroplets or microbubbles of Phase-I in a continuum of Phase-II via imbedded micromixers, resulting in intensified extraction/absorption from the point of formation until exiting the separator.

solvent phase, Phase-I. ***Figure 3*** shows the phase separation efficiency as a function of the inlet volumetric flowrate ratio (Organic/Aqueous)

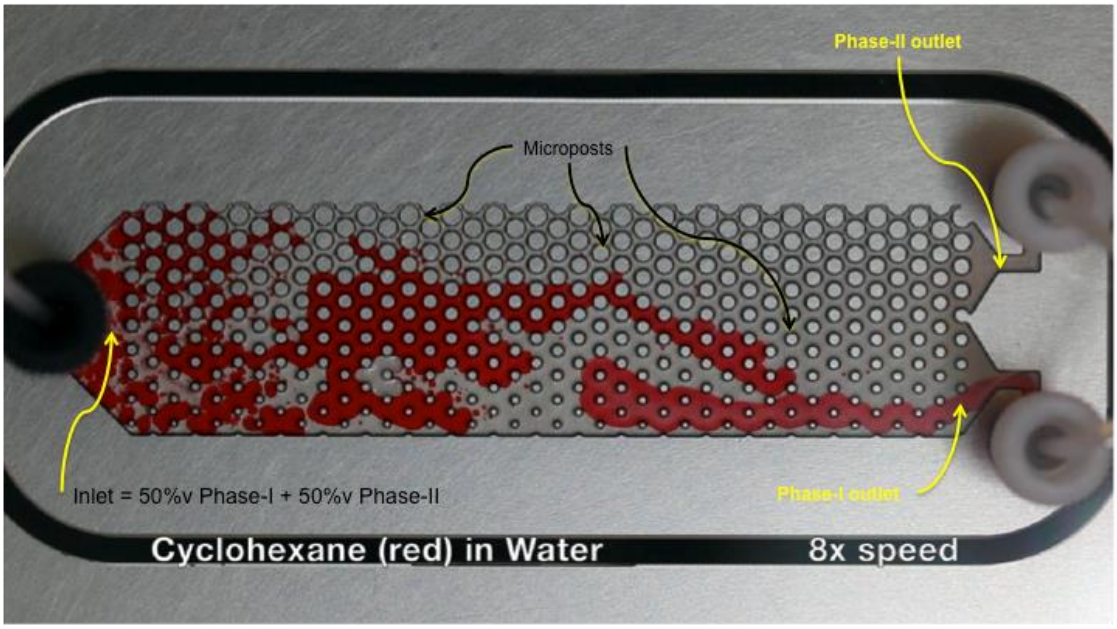

Figure 2: A photograph of a phase separation experiment.

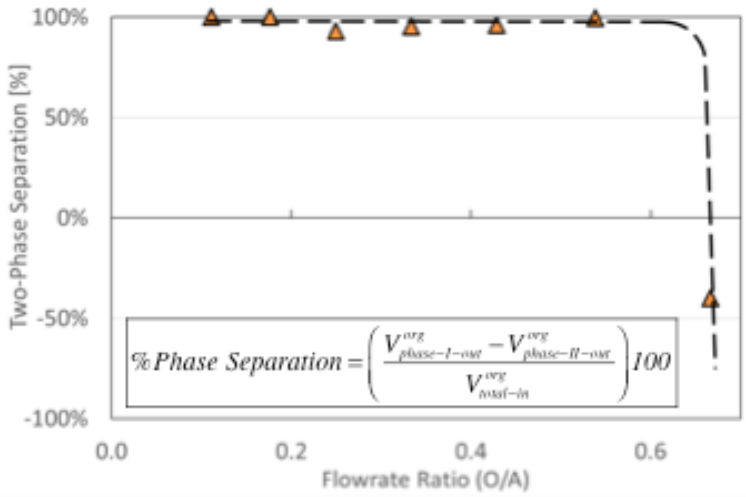

Figure 3: Phase separation efficiency as a function of the inlet volumetric flowrate ratio (Organic/Aqueous)

We have demonstrated functional prototypes for gas-liquid (CO_2 adsorption into ionic liquids) and liquid-liquid extraction of butanol from aqueous solution using various extracting solvents. Additionally, a numerical study was performed on gas-liquid separation in a Pin-Plate separator based on Lattice Boltzmann modeling approach. Experimental findings in identical system were extremely positive, with almost all bubbles directed towards a single outlet stream, exceeding expectations set by numerical simulation results. Observed bubble velocity was staggered reflecting events of bubble interface disconnecting from micro-posts on the tail end of the bubble, and formation of a new interface line between posts in front of the bubble. We are currently investigating liquid-liquid extraction and separation for methane-based liquid fuel products as part of the US Department of Energy ARPA-E REMOTE program. The focus of this work is to define the operational range for the current system design that produces 100% solvent separation and maximum extraction.

DO6

Study on aqueous/organic two-phase flow in nanochannel for femto-liter solvent extraction

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Microfluidics has realized miniaturized and high efficient chemical devices for chemical synthesis, medical diagnosis and cell analysis. Our group has established a general method of integration using parallel multiphase flows in microchannels.[1] Chemical unit operations such as mixing, reaction, separation, are achieved in parallel multiphase flows (micro unit operations: MUOs), and MUOs are combined in parallel and series in a microchannel network (continuous-flow chemical processing: CFCP). By this method, various complicated chemical processing for synthesis and environmental analysis has been integrated in microfluidic devices. Recently, we have furthered the field to nanofluidics exploiting 10-1000 nm space to develop novel chemical devices such as single cell/single molecule analysis.[2] In order to extend our integration method (MUO and CFCP) to nanofluidics, we have developed a method to form parallel multiphase flow in nanochannel considering dominant surface effects in 10-1000 nm spaces.[3] Surface of nanochannel is partially modified to hydrophobic using focused ion beam, and aqueous/organic parallel two phase flow is formed by controlling immiscible liquid-liquid interface. In addition, we verified femto-liter solvent extraction of lipid by using parallel two-phase flow in nanochannel for the first time. However, condition of stable formation of parallel two phase flow has not been revealed. The objective of present study is to investigate properties of parallel two phase flow in nanochannel.

Figure 1 illustrates schematics of the partial surface hydrophobic modification method in nanochannel. When the 100 nm channel is partially modified hydrophobically, organic phase remains in hydrophobic region, while aqueous phase in hydrophilic region, because of 100 kPa Laplace pressure by dominant surface tension FIB has 6 nm resolution and enough energy to break hydrophobic material. Modified hydrophobic material on inclined substrate is partially broken by FIB. Hydrophobic material only at shade area made by the channel wall remains. In this way, the nanochannel can be partially and uniformly modified. The method was proved using a 150 nm channel. Octadecyltrimethoxysilane (ODS) was used for hydrophobic modification. The result of SEM observation showed that ODS was uniformly modified as designed. When the glass substrate of nanochannel partially modified with ODS was bonded to another glass substrate to construct fluidic channel, low temperature bonding method,[4] which we previously established, was employed to keep the ODS function.

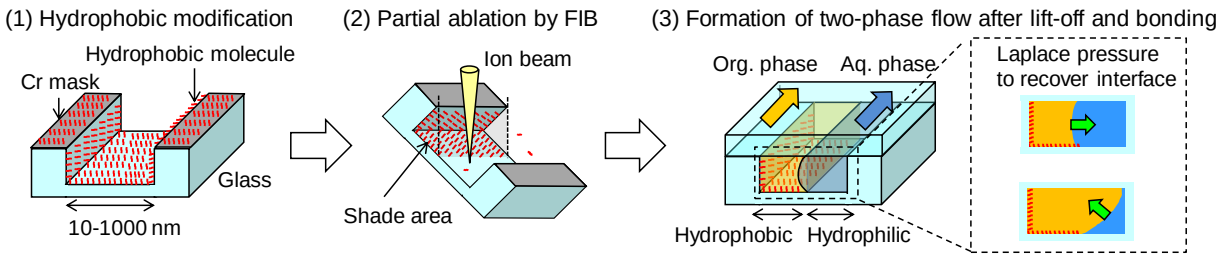

Figure 1: Schematic of partial surface hydrophobic modification method in nanochannel.

We verified formation of parallel two-phase flow in nanochannel partially modified by ODS. Water and dodecane were used as aqueous and organic phases, respectively. As shown in Figure 2, the parallel two-phase flow was stably formed in the modified channel. A volume of the two-phase flow is only 25 fL, which

is much smaller than single cell. Furthermore, we verified formation of parallel two-phase flow by water and chloroform, which is a solvent generally used in solvent extraction for bio analytical applications. By changing pressures to drive aqueous and organic phases, we estimated conditions to stably form parallel two-phase flow. The results suggest different property of the parallel two-phase flow in nanochannel from that in microchannel, due to dominant surface tension and unique liquid properties, which we previously reported.[1] This work greatly contributes to design and integration in nanofluidic devices exploiting parallel multiphase flows for analysis and synthesis.

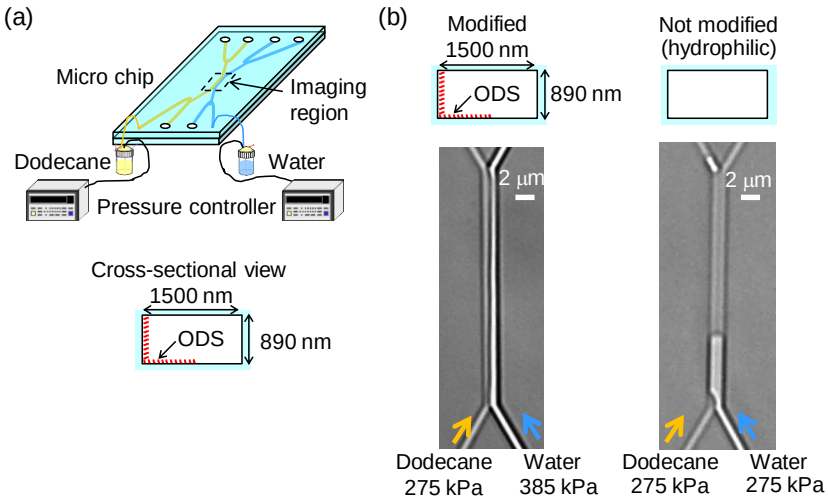

Figure 2: Verification of formation of parallel two-phase flow in nanochannel. (a) Schematic of experimental apparatus. (b) Microscopic image of parallel two-phase flow in 800 nm channel.

Keywords: nanofluidics, nanochannel, surface modification, parallel multiphase flow

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DP1

Study of plastics for 3D printing of microreactors

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Additive manufacturing, also referred to as 3D printing technology, is an automated inexpensive and customizable process that will make possible for laboratories and small companies to use chemical engineering tools usually used only in large-scale industrial settings. Additive manufacturing comprises of manufacture of three-dimensional physical objects from a digital model. The 3D printer takes the virtual design from computer-aided design (CAD) software and reproduces it layer-by-layer until the physical definition of the layers gives the designed object. The major advantage of this technique is that the architecture can be controlled and new iterations to the design can be made in matter of hours.

Depending on the technology, materials used for the production of an object can be in powder, liquid and solid form. The most commonly used are polymeric materials (ABS - acrylonitrile/butadiene/styrene, PLA - polylactide, PET - poly(ethylene terephthalate), etc.), but also, less frequently metals and their alloys, ceramics and composite materials are used. The selection of materials for microreactor manufacturing greatly depends on the desired application and on several of the parameters: the type of reaction that is carried out, chemical compatibility with solvents and reagents, the type of microreactor, the mechanical requirements of the microreactor, price, availability on the market, ease of production etc.

In this paper, thermal characteristics and the effects solvents (water, ethanol and acetone) of the polymeric materials used in selective laser sintering – SLS, fused deposition modeling - FDM and stereolithography – SLA, were studied by a differential scanning calorimetry and by using swelling tests.

This work has been supported by Croatian Science Foundation under the project entitled “Development of materials for 3D printing of microreactors” (UIP-2014-09-3154).

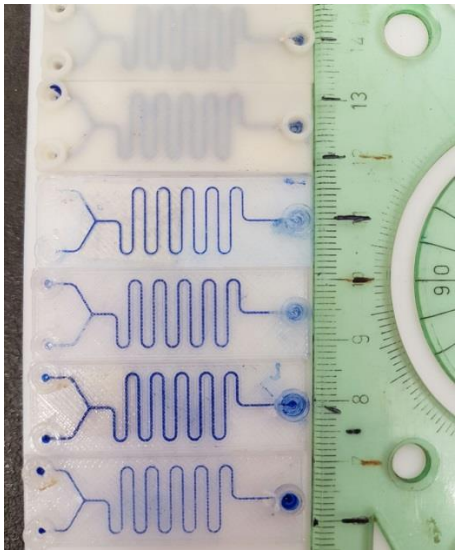

Figure 1: 3D printed microreactors

Keywords: 3D printing, ABS - acrylonitrile/butadiene/styrene, PLA - polylactide, PET - poly(ethylene terephthalate)

DP2

Theoretical and experimental evaluation of the Corning® AFR™ module for liquid-liquid extraction of ω-transaminase-catalyzed reaction product

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ω -transaminases are recently gaining attention as promising biocatalysts allowing for sustainable production of enantiomerically pure compounds that possess a chiral amine moiety and thereby are essential building blocks of intermediary metabolites and pharmaceutical drugs. Their immobilization in microreactors has shown to enable process intensification and the possibility to perform efficient cascade reactions.[1-3] In order to develop an efficient biocatalytic process, further integration with the downstream units is inevitable. The aim of the present work was to set-up an efficient system for the separation of components in the outlet from the continuously operated (micro) flow reactor with immobilized ω –transaminases, which could be used also on different scales. Based on the model reaction system[3], separation of the remaining substrate methylbenzylamine (MBA) from the product acetophenone (ACP) using liquid-liquid extraction was investigated.

The continuous flow reactors developed by Corning® using process intensification paradigm, are conveniently scalable and customizable, with hydraulic diameters in the range of 0.3 up to few millimeters and internal volumes between 0.5 and 260 ml, ensuring wide range of operating flow rates.[4] The HEART cell design used in the Advanced-Flow ™ Reactors (AFR) is based on chains of identical cells – HEART – with variable cross-section and internal elements, which force the fluids to split and recombine, while creating and maintaining fine dispersions in heterogeneous chemical processes.[4] Furthermore, the dispersed phase, subjected to coalescence/breaking phenomena, constantly renews its active interfacial area.[4,5]

The Advanced-Flow™ Reactor with 0.5 mL volume (low flow, LF module) has been evaluated regarding the MBA and ACP transport from aqueous (Tris buffer) to organic phase (*n*-heptane). In order to achieve phase separation at the exit, the LF module was integrated with the membrane separator as previously described.[6] The system was operated at various flow rates and inlet concentrations and characterized by inspecting fluid flow regime and outlet concentrations of MBA and ACP, measured in both aqueous and organic phases using HPLC and GC analyses. A short segment of the reactor system was modelled in terms of transport phenomena in selected two-phase system, using the geometrical data provided by the manufacturer. The dynamics of droplet generation and two-phase flow stabilization in a single cell were theoretically described, thus enabling the prediction of the behaviour of interfacial area under various operating conditions. The two-phase flow was simulated using the OpenFOAM® open-source computational fluid dynamics software, with addition of the code for solute transport to the stock multiphase solver, which is based on the volume of fluid (VOF) method. Using this complex velocity profile, estimated for averaged interfacial areas, a mathematical model, incorporating convection and diffusion was developed. The numerical results have shown a good correspondence with experimental measurements and the optimal working parameters for maximum extraction efficiency were determined.

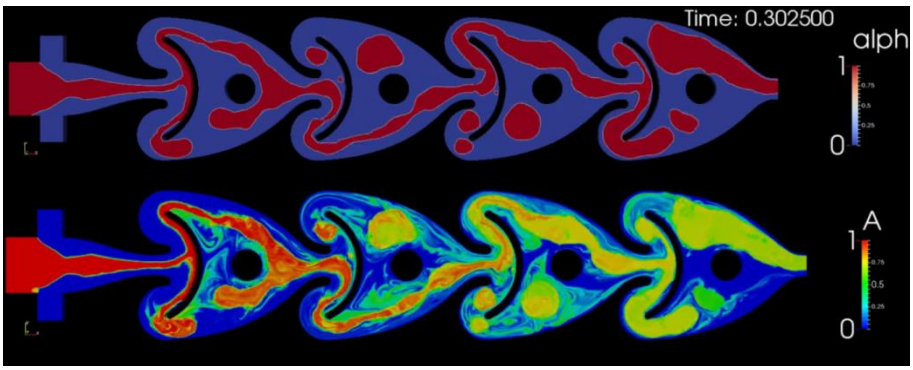

Figure 1: Advanced-Flow™ Reactor CFD simulation results: alph presents aqueous phase/*n*-heptane ratio (aqueous phase is blue, while *n*-heptane is red) and A is normalized concentration profile of MBA.

Keywords: liquid-liquid extraction, ω-transaminase, Corning® Advanced-Flow™ Reactor, mathematical modeling

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DP3
Simulations of immobilised enzyme microreactors with the lattice Boltzmann method

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The need for process intensification and the growing availability of microstructured devices in recent years has led to the development of numerous types of microreactors. Among these are also enzymatic microreactors that combine the operational and mass transfer advantages of microfluidic systems with biocatalytic reaction processes, known for their high reaction rate and selectivity. In many cases these systems are designed to be operated continuously requiring the immobilization of catalyst - enzymes on the inner walls of the reactor to avoid separation procedures downstream. The enzyme immobilization can be achieved in various ways, e.g. by surface group functionalization or by loading the enzyme onto a support. Some of the possible supports include porous monoliths, beads, droplets and gel layers. These various design options should be studied and compared prior to moving into production through a combination of scale out and numbering up. To better assess, compare and quantify the microreactor performance for different designs and parameters, numerical simulations based on verified mathematical models represent an invaluable tool. In this work we will present numerical simulations of reaction-diffusion-advection processes in immobilized enzyme microreactors. The simulations will be based on the lattice Boltzmann method. Special attention will be devoted to the various options in implementing the enzymatic reaction, either as a volume reaction or as a surface reaction. Based on the simulation results we will look at the possibility of determining effective dispersion and reaction rate coefficients to develop simple models for the reactant conversion.

Keywords: immobilized enzyme microreactor, Michaelis-Menten kinetics, lattice Boltzmann method

DP4
A low cost 3D DLP stereolithography printer suitable for microfluidic features production

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Stereolithography (SLA) is a liquid-based additive manufacturing (AM) process, from which 3D parts are made by curing a photosensitive polymer kept in vat, thus the term vat photopolymerization process is often used. Controlled light irradiation induces a curing reaction, forming a highly cross-linked polymer. SLA process can produce parts with fine features and good accuracy using various polymers[1].

In the last years, the resolutions of digital light processing (DLP) projectors have been significantly improved due to the use of new cost effective Digital Micromirror Devices (DMD). DLP SLA is used in various fields including medical applications, where biocompatible and biodegradable materials ought to be used[2,3].In this work, the development of a desktop stereolithographic printer using a DLP projector as a light source is presented. At first, the equal illuminance over all projection area was achieved by developing an appropriate software mask, then the influence of illuminance from bottom and top of the photopolymer vat was examined, and at last the optimal process parameters were defined by Taguchi based surface response methodology and used to produce a part with micro features.

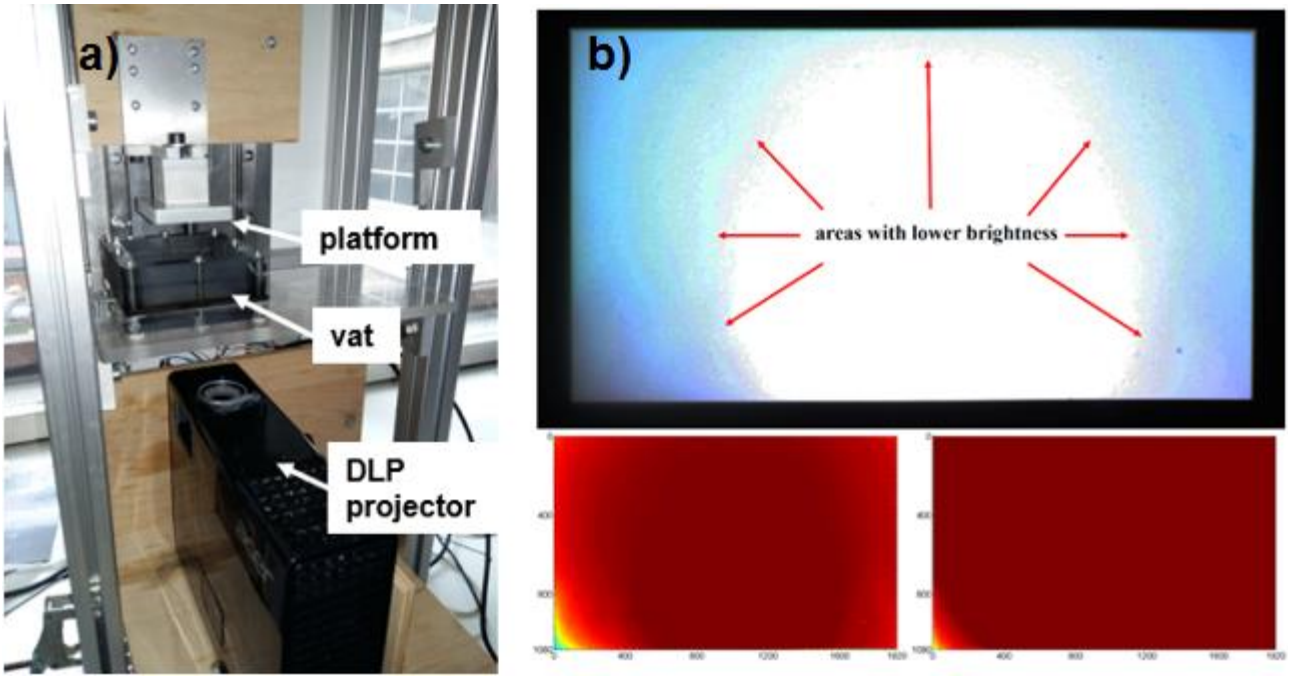

Figure 1. a) Custom made DLP SLA printer with illumination from the bottom arrangement. b) Captured image of the DLP projector illumination. Below: (left) image without mask and (right) image with applied mask. Dark red color represents pixel intensity level of 255.

Materials and methods

All experiments were performed on a custom built DLP stereolithographic printer (Fig. 1.a). Photopolymerisation was initiated by a DLP projector Acer P1500 having 1920 x 1080 pixels and 3000 lumen. The photopolymer used in this research was Deep Black produced by FunToDo. 3D printer control software Creation Workshop was used.

To build a software mask to compensate uneven illumination, a testing site to capture the DLP projector image was build. The site consists of a computer connected to the DLP projector, a screen on which a white image was projected and a camera used to capture the image. The camera was a Canon 30D with 50 mm lens, 1/125 s shutter speed and /4.5 aperture.

Design of experiments (DOE) was executed for SLA process parameters optimization. Based on preliminary tests exposure time, layer thickness and time between the consecutive exposures were determined as most influential factors. Thus, three parameters (factors) on three levels were used for the design of experiments. Larger features of the sample workpieces were measured by a micrometer. Microchannels were measured with Alicona InfiniteFocusSL focus-variation microscope.

Results

A problem using a commercial DLP projector is uniformity of illumination. To improve evenness of illumination we applied a software mask. A mask is applied over an image of projecting layer, thus the luminance of each pixel can be altered.

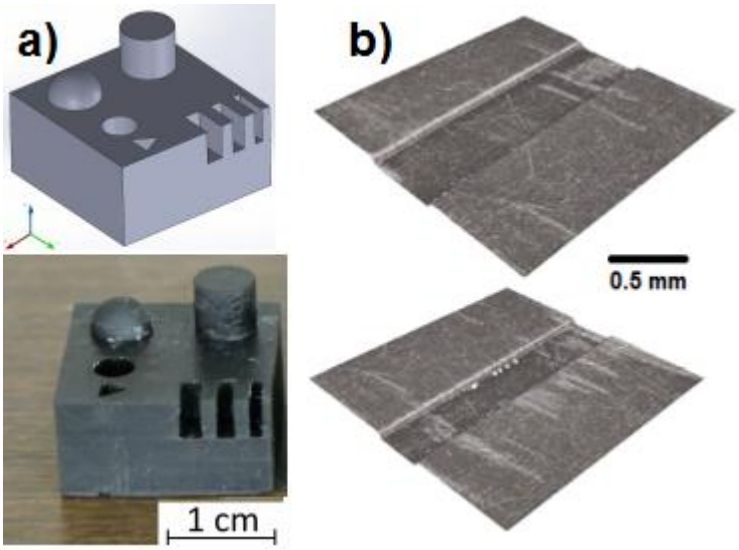

Figure 2. a) 3D model of a test specimen and a printed part. b) 3D scan of low aspect ratio printed microchannels with nominal width 0.6 mm (above) and 0.4 mm (below).

The results showed that illumination from the bottom of the vat yielded better results. Standard deviation of the lateral dimensions of the features was ±0.05 mm and dimensional accuracy in comparison to 3D model was within ±0.07 mm. The optimal input parameters, obtained with numerical optimization are: exposure time 16.5 s, layer thickness 0.06 mm, time between two consecutive exposures 4 s.

By reducing the thickness layer to 0.01 mm we were able to accurately print microchannels with aspect ratio below 1. The smallest microchannels printed were 0.4 mm in width. Lateral dimensions were within the tolerance of ±0.03 mm and depth within ±0.02 mm with a draft angle of 5°.

Conclusions

The implementation of the mask successfully reduces the unevenness of the DLP projector's illumination and thereby the parts of the same quality are built in entire printing area. Building a part on the bottom of the vat is recommended. The results on printing microchannels show good enough dimensional repeatability and accuracy for the developed printer to be used for microfluidic prototyping.

Future work will be oriented into using a better resolution DLP projector with improved collimation. Also lowering the wavelength of the light source and better photosensitive polymer should improve printing resolution.

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DP5

Monitoring of lactose hydrolysis in a microfluidic packed bed reactor using an integrated glucose oxidase biosensor

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Enzymatic hydrolysis of lactose is one of the most important biotechnological processes, widely used in diary industry for lactose-free products, as well as for lactose derivatives as bioactive ingredients in human nutrition[1]. β-galactosidase (EC 3.3.1.23), also known as lactase, catalyzes hydrolysis of lactose yielding glucose and β-D-galactose, as well as transglycosylation reactions used for the synthesis of di-, tri- or higher galacto-oligosaccharides (GOS)[2]. In order to establish economically feasible and sustainable lactose hydrolysis process, continuously operated reactors using immobilized lactase have been applied for decades[1].

One of the most efficient ways to retain biocatalysts within the reactor and at the same time preserve their activity is to entrap them within the porous matrices. In this study, biocatalytic material was immobilized in polyvinyl alcohol (PVA)-based lens-shaped hydogels LentiKats®, which possess excellent physico-mechanical characteristics, such as high elasticity and low abrasion that provide for robustness and long lifetimes[3].

A synergy between biocatalysis and reactor/unit operation miniaturization has recently been shown to be a powerful tool for the rapid development of continuous biocatalytic processes, as well as for process intensification[4]. Miniaturization of packed bed reactors with immobilized enzymes include more controlled fluid flow, high biocatalyst load, extended operational life of the catalyst, small pressure drop relative to conventional systems, as well as the possibility for an efficient increase in the production capacity[3,4]. One of the key challenges for industrial application of this promising technology is the establishment of an efficient bioprocess monitoring by (bio)sensors integration.

In this study, a microfluidic packed bed reactor for lactose hydrolysis with LentiKats® carrying β-galactosidase has been integrated with an electrochemical glucose oxidase biosensor as schematically presented in Fig. 1a. A two-plate microreactor system[3] with a single 0.4 mm deep layer of LentiKats® in a rectangular shape of 4 mm width (Figure 1a) has been assembled. Various concentrations of lactose solutions in 0.1 M acetic buffer (pH 4.5) were fed in the channels by means of a syringe pump at various flow rates and thereby residence times. In order to test the reactor performance, a microfluidic chip of 4 μL internal volume with immobilized glucose oxidase electrochemical biosensor has been integrated at the outlet of the packed bed reactor. At the same time, eluent samples have been taken and analyzed by an HPLC (15 min method using Rezex® column at 85°C, deionized water as the mobile phase and refractive index detection).

The amperometric measurements of glucose concentration using PalmSens3 potentiostat were found to be directly proportional to the quantity of hydrolyzed lactate, measured by HPLC, and the biosensor had considerably higher sensitivity and selectivity than the HPLC. Furthermore, the biosensor has been integrated online, so data of high density (10 Hz readouts) were instantly available. The accuracy of results has been assured by the characterization of the calibrated glucose biosensor regarding sensitivity, selectivity and linearity.

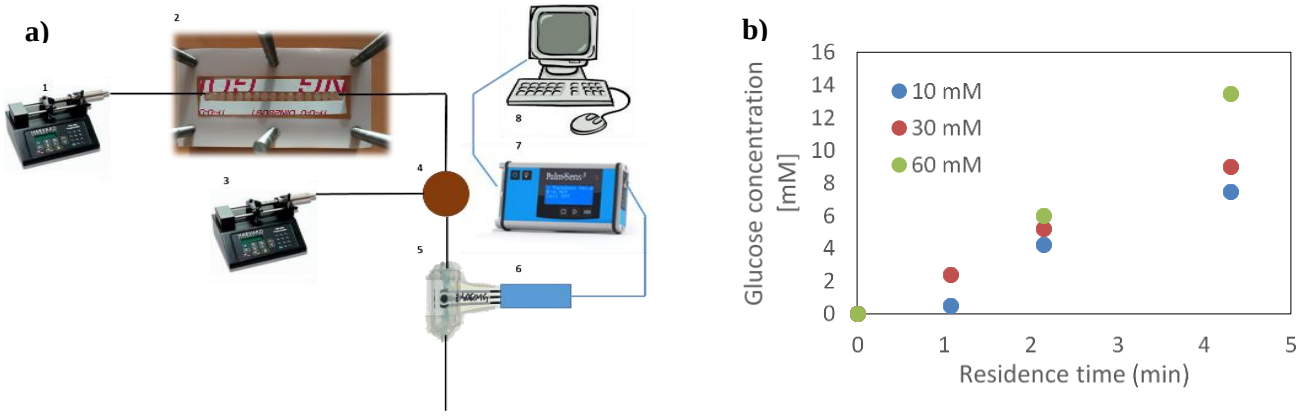

Figure 1: a) Experimental setup scheme and b) glucose concentration at the outlet of the microreactor packed with LentiKats® at various flow rates yielding different residence times and with various inlet lactose concentrations stated in the legend.

As evident from Figure 1b, the glucose concentration at the microreactor outlet increased with the residence time and with the inlet lactose concentration. However, the reaction rate is not proportional to the inlet substrate concentration, which might be attributed either to enzyme inhibition by both products, or to the formation of oligosaccharides. Further work to confirm this conclusion is planned, as well as the systematic study of the increase in reactor size on its performance and the production rate. Figure 2 presents the larger microreactor with wider channels in hexagonal geometry enabling also larger enzyme loads while preserving favorable hydrodynamic conditions.

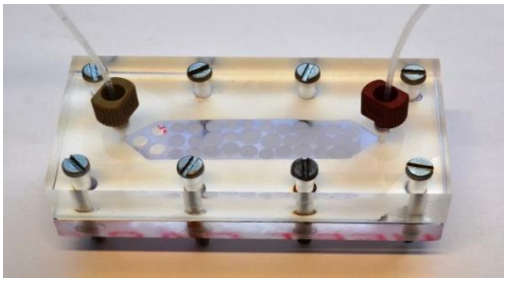

Figure 2: A hexagonal packed bed microreactor with LentiKats®.

Keywords: lactose hydrolysis, packed bed reactors, biosensors, β-galactosidase, glucose oxidase biosensor

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DP6

The development of easy-to-make continuous flow micro-reactors for biological and chemical purposes

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The manufacturing of continuous flow micro-reactors involves numerous steps and expensive materials, and in a large-scale production this will strongly affect the final commercial price. Moreover, the quality of high precision lab-on-a-chip devices (in terms of geometry, size, high reproducibility etc.), has a crucial impact in both research and process. Therefore, there is no doubt that the development of *easy-to-make* and less expensive construction procedure is nowadays an important goal to achieve.

Compared to the numerous steps of the conventional photolithographic process,[1] many of which are extremely expensive or can only be performed within a particular environment such as clean rooms, soft lithography has taken hold in recent years by offering great alternatives like micromilled replica mould (μMi-REM),[2] and 3D printed replica mould (3DP-REM)[3]. In particular, it is possible to skip all the photolithographic steps entirely by using 3D printing to design and create the master, followed by the bonding procedure (Figure 1).

Here we present our results in creating microfluidic devices using cost effective, reproducible and easy to design methodologies. The high-resolution 3D printer (OBJET Connex), and the less expensive "desktop" 3D printer (Ultimaker2+) were used to print moulds, and the final devices obtained by 3DP-REM were tested. The investigation was carried out on channel size, 3D printing resolution limitations, oxygen plasma bonding issues, leakage, flow focusing capability and procedure costs of the manufacturing processes. Moreover, the 3DP-REM micro-reactors were tested for the synthesis of organic and inorganic colloids.

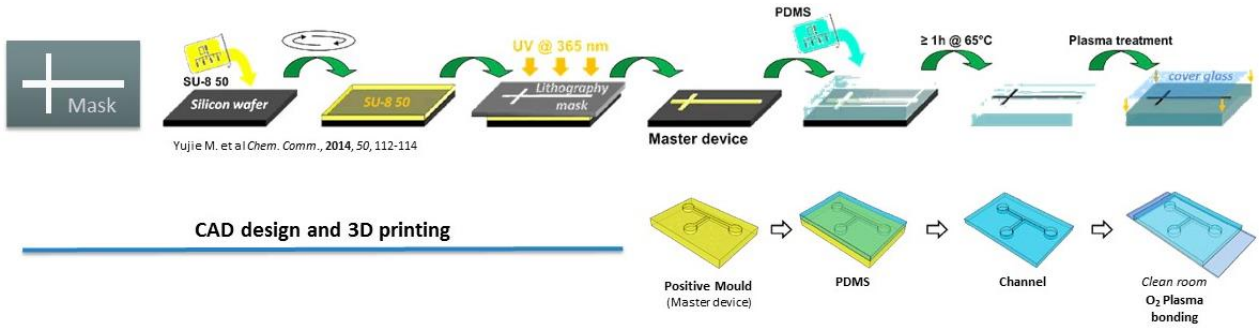

Figure 1: Stepping down the photolithographic process by 3D printing replica mould (3DP-REM).

Solidworks© and ANSYS ICEM© were used for the design of the shape and size of the channels, number of inlets and junctions for several moulds, based on the final purposes of the device. In particular, for the inorganic chemistry test, two 10×8 cm microfluidic devices, having the same 1 m length but different channel widths (0.25 mm and 1.00 mm), were 3D printed in order to be able to compare diffusion and advection dominated mixing regimes. The high-resolution 3D printer (OBJET Connex 350) can be used for the production of both types of reactors with different size of the positive mould of the channels, while the Ultimaker2+ has a notable size limitation for channels under 1 mm size. For comparison similar sized (1 mm) devices were produced with the alternative μMi-REM methodology.

For organic colloid production, the highest resolution of the OBJET Connex 350 (0.1 mm channel size) was tested, designing a 0.1 x 0.1 mm channel with three inlets having a 45-degree junction and the total length of 3 cm.

Finally, for all the devices, Polydimethylsiloxane (PDMS) was poured on to the mould, cured and bonded to glass following a recently developed procedure.[2] The mixing capacity of the device was then tested using dye containing solutions (Figure 2).

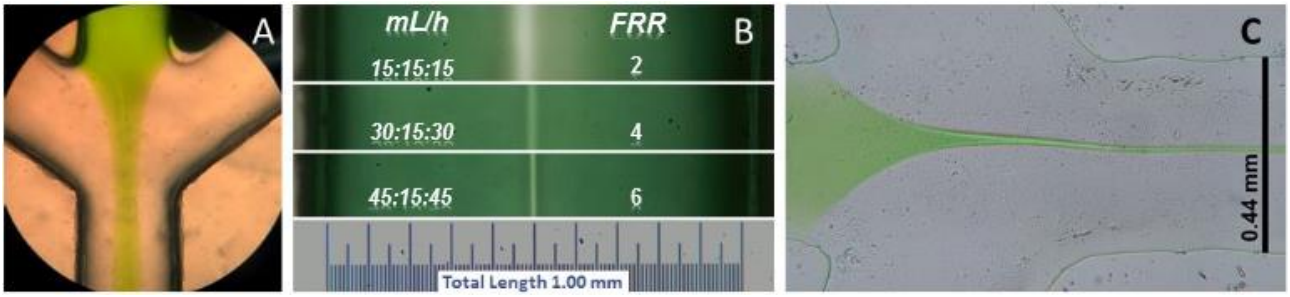

Figure 2: A) Flow focusing in the junction of the 1 mm device used for the synthesis of inorganic colloids (center channel: green dye, side channels: water). B) Different diffusions regimes of water (center channel) and green dye (side channels), for several flow rates in the 1 mm device. C) Flow focusing was also achieved for the junction of the designed 0.1x0.1 mm device, for biological synthesis (center channel: green dye, side channels: water).

Maintaining the same advantages of the well-known PDMS/glass Lab-on-a-Chip, although some limitation in the small-scale fabrication, the 3DP-REM devices are quick and easy-to-make and moreover extremely cost effective compared to other techniques. This characteristic is extremely important in prototyping, and for industrial uses, where a balance between results and investment is crucial. 3DP-REM would be definitely suitable for large-scale synthesis in chemical or biological fields.

Further results with such fabricated devices will be presented on the conference for continuous flow synthesizing nanomaterials towards biological and chemical applications, together with a discussion on scopes and limitations of our approach.

Keywords: Lab-on-a-chip, Continuous-flow, 3D printing, Cost effective, Large-scale.

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DP7

Biodiesel purification in a microseparator: deep eutectic solvents vs water

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Introduction

Once biodiesel is produced it could be used in internal combustion engines if fulfil the quality parameters prescribed by the American standard ASTM D 6751 and the European standard EN 14214. The purity of biodiesel is achieved through the purification process that involves the removal of excess alcohol (methanol), unreacted triglycerides, glycerol and catalyst and the resulting soap the share of which in biodiesel depends on the raw material from which biodiesel is produced and on the used catalyst. The conventional purification methods include water washing of biodiesel (also known as wet washing), dry washing and membrane separation. However, water rinsing generates large quantities of wastewater that must be properly disposed and represents a great economic and environmental burden[1]. In addition, it results in a loss of biodiesel during the washing phase. Recently, among those "alternative" methods, deep eutectic solvents (DES) have gained a lot of attention among researchers because they resemble various unique characteristics such as low volatility, non-flammability and non-toxicity. Another big advantage is that a large diversity of them can be prepared easily using affordable raw materials[2]. When talking about biodiesel purification, it has been shown that a DES synthesized using choline chloride salt and ethylene glycol was able to efficiently remove all free glycerol from biodiesel[3-5].In this study application of microseparator for biodiesel purification was explored using choline chloride ethylene glycol and water as a purification medium. A system with continuous recirculation of water or DES was proposed.

Results

Biodiesel was first synthetized by free Lipolase 100L in a batch reactor (V = 250 mL) according to the procedure described by Budžaki et al.[6] using edible sunflower oil and waste cooking oil (WCO). The maximum fatty acids methyl esters (FAME) content for both substrates after 6 h was around 96%. After the biodiesel was produced, it was placed in a separation funnel (most common method) to separate glycerol from biodiesel. Samples were taken and analysed before and after separation. After 24 h it was determined that 90% of the glycerol was removed therefore the procedure was repeated once more. After second separation additional 6.19% of glycerol was removed. According the quality parameters prescribed by the American standard ASTM D 6751 and the European standard EN 14214 the percentage of glycerol in biodiesel has to be less than 0.02% therefore additional purification techniques had to be applied. As a next step centrifugation at 14000 rpm and 4 °C for 20 min and filtration trough a 0.20 μm pore size filter (Chromafil Xtra, Macherey-Nagel, Germany) were performed leading to insignificant improvement in glycerol content. As a next purification step microseparator (glass microchannel, length:width:depth = 330 mm: 250 μm: 50 μm with the internal volume of 6 μL, Micronit Microfluidics B.V., Netherlands) was used. Choline chloride ethylene glycol or water was used as a purification medium in experiments performed in a microseparator. Results are shown in Figure 1a and 1b. As expected, the ionic liquid provided a much more efficient transfer over water and almost all glycerol was extracted from the biodiesel in experiment performed with ionic liquid. The separation efficiencies were determined to be 75.19% for water and 98.35% for DES. On the other hand, due to much lower viscosity, maximal efficiency was achieved at a much lower residence time for water (τ = 8.2 s compared to 174 s for the ionic liquid). Reason why separation efficiency wasn't higher than 75% for experiment performed with water is probably water capacity. Therefore, wet

washing is repeated several times to ensure sufficient glycerol removal when biodiesel is produced in macrosystems. When glycerol separation was performed within microseparator using DES in combination with separation funnel as first step, the overall 99.98% of glycerol was removed that corresponds to ASTM D 6751 and EN 14214 standards. In second experiment performed in a microseparator and with water as purification agent overall 99.19% of glycerol was removed. According to the biodiesel quality standards for experiments performed with water additional purification is needed. Since in experiment performed with DES the amount of glycerol still present in biodiesel after purification (around 0.02%) was at maximum allowed level some additional optimization was necessary. For that purpose mathematical model was proposed and developed.

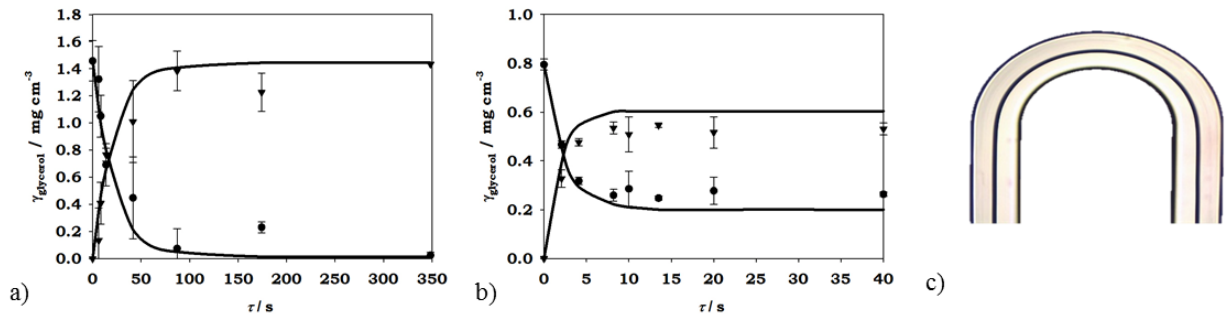

Figure 1: Influence of the residence time on the extraction of glycerol from the biodiesel using a) DES and b) water and c) flow profile for DES:biodiesel system observed under the microscope (▬ mathematical model)

In order to minimize waste stream a continuous system with recirculation of purification media was proposed. After the preliminary results were obtained it became obvious that that system will not be sufficient for water as purification medium since it has low capacity towards glycerol and additional fresh water has to be fed in to a microseparation system. Based on preliminary experimental data and mathematical model predictions two different approaches were proposed for biodiesel purification: a single microseparator unit with recirculation for DES as purification medium and three microseparators connected in to the series in case of water as purification medium.

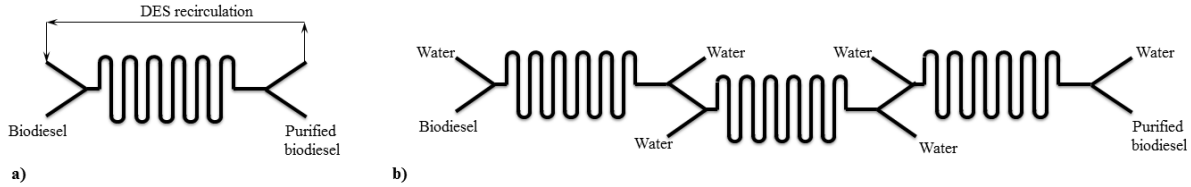

Figure 2: Scheme of proposed microseparation system for glycerol removal using a) DES and b) water

Conclusion

Comparing the extraction efficiency of both investigated systems results go in favor of DES, which has the advantage to require much less quantities than water, resulting in less waste to be treated together with better overall efficiency. Based on mathematical model predictions some additional optimization was made going towards biodiesel with less than 0.02% of glycerol.

Keywords: biodiesel, glycerol, microseparator, DES, water

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DP8

Experimental and theoretical evaluation of residence time distribution in miniaturized packed bed reactors with Novozym® 435

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Microfluidic devices offer numerous advantages for biocatalytic processing such as small size and high surface-to-volume ratio leading to very efficient mass and heat transfer, continuous operation mode and improved safety, among others.[1] Novel microreactor designs, such as miniaturized packed bed reactors (MPBRs) aim to harness these advantages and lead to faster process development and intensified production. Furthermore, they offer simplicity regarding assembly and scale-up/-out.[2]

Here we report on experimental and theoretical evaluation of an MPBR randomly packed in one layer with Novozym® 435 beads containing lipase B from *Candida antarctica* (Fig. 1), which was used for transesterification. In order to characterize the reactor performance, residence time distribution using pulse test was measured in two MPBRs with rectangular channels of different lengths: the shorter (23.93 mm × 3.12 mm × 0.40 mm), and the longer (81.00 mm × 2.94 mm × 0.40 mm).

The experimental apparatus comprised of a syringe pump, HPLC-style 6-port injection valve with 10 µL sample loop, the MPBR, and a glucose oxidase biosensor, which measured the tracer (glucose) concentration at the output. The devices were interconnected with PFA tubing of inner diameter 0.5 mm. The electrochemical glucose oxidase biosensor was integrated into a microfluidic chip with 4 µL. It was developed and fabricated by the Measurement Technology Unit of the University of Oulu, Finland. PalmSens3 was used for amperometric detection of glucose.

The pulse tests were carried out by injecting 10 µL of 50 mM glucose solution in 0.1 M phosphate-buffered saline (PBS, Sigma Aldrich) as a tracer using the HPLC injection valve. The results recorded with the biosensor were then processed.

Figure 1: MPBR of dimensions 81.00 mm × 2.94 mm × 0.40 mm, randomly packed with Novozym® 435 beads.

The MPBR was also theoretically described by simulating the pulse test with the lattice Boltzmann method (LBM). The developed computational program generated random packings of the shorter MPBR with the

corresponding fluid flow distribution using at the beginning the D3Q19 lattice model. When the velocity field reached the equilibrium, the program started to use the D3Q7 lattice model to simulate the tracer transport in the device. The latter was simulated based on the passive solute transport assumption. In order to achieve realistic Schmidt numbers, a modification of the LBM as proposed by Sullivan *et al.*[3] was used.

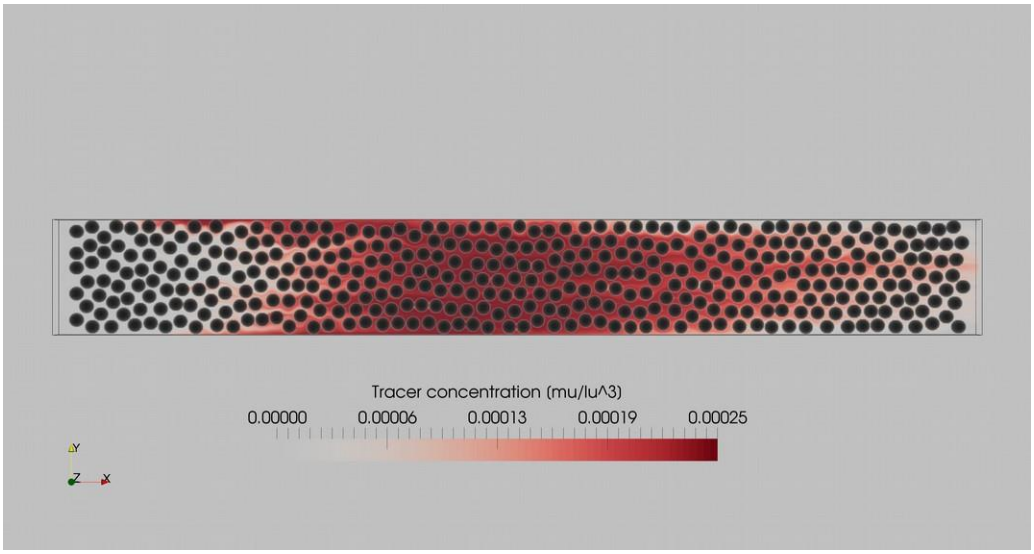

Figure 2: A snapshot of the LBM simulation of the pulse test.

The computational program developed calculated the tracer concentration at the outlet of the MPBR. These results were then processed by taking into account the pulse injected at the inlet. Theoretical results showed good agreement with the experimental data, obtained with the integrated glucose oxidase sensor.

Keywords: miniaturized packed bed reactor, Novozym® 435, pulse test, lattice Boltzmann method

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DP9

Implementation of Aqueous Micellar Two-Phase Systems within a Microfluidic Device for Protein Purification

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Microfluidics have shown great industrial potential in the past two decades especially in the fields of chemistry, engineering, and biology. It involves the controlled fluid manipulations along with the design of devices or systems that can consistently execute such tasks. Its role in chemical/biological processes is mostly focused on enhanced system performance and functionality by integrating different components into individual devices[1].Among the variety of uses, liquid-liquid extraction (LLE) is the one that especially benefits in performance when compared to standard large-scale equipment, owing to the short molecular diffusion distance and very large specific interfacial area[2,3]. LLE has been applied commonly within microfluidic devices for both (bio)molecules purification and reactions with integrated *in-situ* extractions[1-4].

Usually, LLE is accomplished using organic solvents and water to create the two-phases platform of extraction[3], however, these compounds are hazardous to the environment and towards biomolecules. Therefore, a new approach was considered, namely the use of aqueous two-phase systems (ATPSs) aiming at the increase of the water content, providing mild operational conditions that more easily allow the (bio)molecules to keep their native conformation and biological activity. In fact, ATPSs are considered highly flexible, since a considerable array of compounds can be used to achieve high purification effectiveness, selectivity, and yields. Hence, there has been an increased interest in microfluidic extractions applying different types of ATPS for the extraction of different cell types and several (bio)molecules[2]. Though, these systems display some drawbacks, namely the high viscosity and cost of some polymers and the use of corrosive inorganic salts that need to be overcome. Thus, aqueous micellar two-phase systems (AMTPSs), a particular type of ATPSs, seem to be an excellent alternative mainly because they only require water and small amounts of a surfactant to be formed, creating not only a benign extraction system, but also a cheaper one. These systems are temperature-dependent, which means that they only form two aqueous phases by the temperature manipulation. When the temperature reaches the critical value, known as the cloud point, two aqueous phases with high and low concentration of micelles are formed. Moreover, AMTPS have proven to be helpful in the purification of many biomolecules, such as proteins, antibodies, and antibiotics[5,6].

The aim of this work was to explore the possibilities of extremely efficient temperature control in the microflow systems for the development of an AMTPS within a microfluidic system, which has not yet been reported. Thereby, this work intends to demonstrate for the first time such accomplishment while being applied towards the purification of R-phycoerythrin, a phycobiliprotein from red seaweeds, used in analysis, diagnostics and medicine. Owing to the richness of red algae in natural chemicals, their exploitation is becoming of economic/industrial interest and is within the scope of a marine bio-based economy, showing a huge potential for various fine chemicals and materials production.

When the mixture of AMTPS components with the red algae extract was exposed to the rapid temperature change in the microflow system, the formation of AMTPS was achieved within less than 1 min (Figure 1a). Furthermore, R-phycoerythrin selective extraction was achieved in both macro and microscale (Figure 1 b). Nevertheless, in the case of using the microfluidic system, it took less than 20 min to have a complete phase separation, while 12 h were needed in the small batch system to achieve the same extraction performances.

Furthermore, various AMTPS with surface active ionic liquids could be screened in microflow system within very short times and with small material consumption, since these mixed systems have proven to boost the extraction performance.

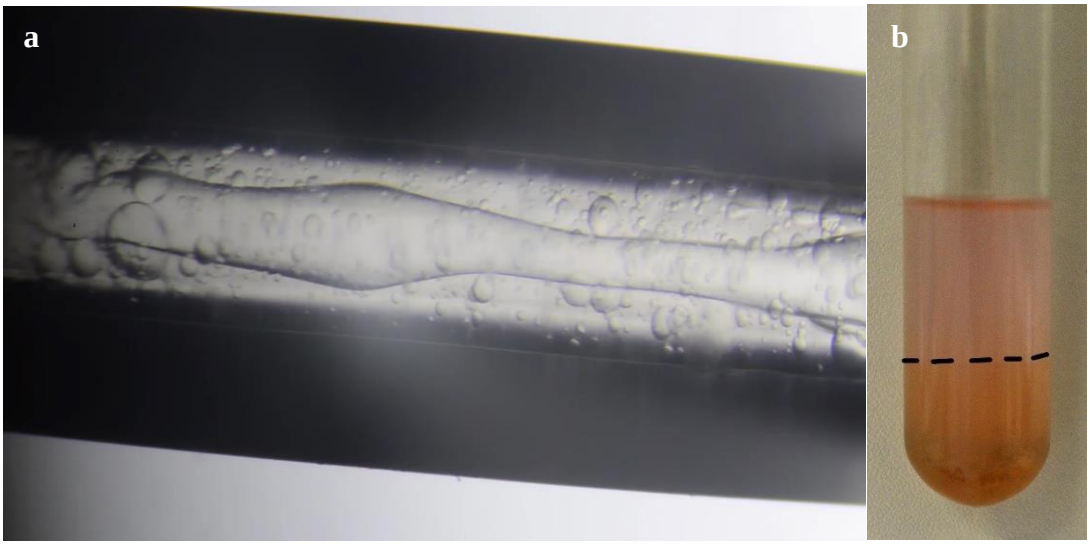

Figure 1: a) Phase separation after less than 1 min residence time within the microchannel; b) AMTP phases at the outlet of the microflow system with characteristic color of R- phycoerythrin in the top phase

Keywords: microfluidic device, aqueous micellar two-phase system, surfactants, proteins, macroalgae

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Bio-Lamina-Plates bioreactor for enhanced mass and heat transfer

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OSU's "bio-lamina-plate" (BLP) microscale-based bioreactor offers very high mass and heat transfer for two-phase gas-liquid reaction of methane gas to liquid biofuels and other high-value products. The bioconversion occurs within a thin (<500-μm) film of living methanotrophic bacteria entrapped in natural or synthetic hydrogels. The BLP reactor provides very high rates of mass transfer from flowing gas bubbles by reducing the characteristic distance for diffusion, from several millimeters between bubbles, to crossing the extremely thin (~10 μm) film of liquid separating the bubbles from the immobilized cell layer. On a biocatalyst mass basis, the oxidation of methane in the BLP microreactor substantially outperforms "traditional" packed bed and chemostat bioreactors. A variety of other biocatalytic reactions involving sparingly soluble gases, including oxygen or hydrocarbons, can also be carried out using this reactor. Thus, the BLP technology could enable efficient, on-demand synthesis of high-value products such as pharmaceuticals. Other potential applications include the oxidative biodegradation of solvents and other contaminants from wastewater or process streams. The OSU's demonstration scale BLP reactor is designed to facilitate scale-up and/or numbering up to reach industrial scale demand for bio-reaction processes. Techno-economic evaluation (CapEx & OpEx) produced entirely on microscale-based bio-reaction processes is available upon request.

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