Biotechnical Micro-Flow Processing at the EDGE – Lessons to be learnt for a Young Discipline

V. Hessel,* J. Tibhe, T. Noël, and Q. Wang

Micro Flow Chemistry and Process Technology, Department of Chemical Engineering and Chemistry, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, the Netherlands

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Review
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EDGE denotes "Explain, Demonstrate, Guide, Enable" and comes from a concept for youth leadership development training. Biotechnical and enzymatic micro-flow reactors are a young discipline. Here the enthusiasm level mirrors its technological growth and vice versa. Rather than being a linear function, enthusiasm first goes down after sudden realization of all bottlenecks and technological shortcomings which are to overcome in a Hercules task action. However, with increasing provision of technology and security in its performance, the enthusiasm level rises again. This "valley of death" needs to be crossed to bridge to a market. The enzymatic micro-flow reactors mirror the above depicted development of their counterparts – the chemical microreactors – which is already about 20 years old. This "Déjà vu" forms a feature story which encompasses a review about enzymatic microreactors and their synthetic applications which are shown in all their facets. This compilation is structured in chapters about reactor and enzyme support technology, transport intensification, chemical intensification, process-design intensification, and finally first steps into the bio-based economy.

Key words:

microreactors, enzymes, enzymatic microreactors, chemical intensification, process-design intensification

From Pioneering to Prognosis

Feature: From vision to illusion over disillusion to innovation

Innovations are fragile. What is called first vision easily turns to an illusion. Illusions have the moment of disorientation. That leads to disillusion. The fate of not overcoming that is clear. Frustration comes if high promises turn into dissatisfaction. If surviving, the status of an innovation has been reached.

The Frost & Sullivan research study entitled "European Lab-on-chip and Microfluidics Market" recommends that market participants need to address interoperability concerns that keep pharmaceutical companies from readily including lab-on-chip instruments in their existing workflows.[1] "Being a new technology that has to fit into an existing laboratory set up, it is essential that lab-on-chip equipment is compatible with the pre-existing robotics and automation lay-out of the lab," advises the analyst. "Companies that meet these standards will achieve greater success in attracting customers.

*Corresponding author: V. Hessel, email: v.hessel@tue.nl, tel. +31(0)402472973

Déjà vu – visions inspire researchers

Why mentioning this here? Writing about biotechnical micro-flow reactors is like a Déjà vu, something which comes again and again and again. At least for a senior scientist. Because it resembles what has been seen and experienced before. With their chemical counterparts – the 'microreactors' as these are shortly named. Some resistances have been surpassed here over 20-years development. Now, then same story starts again. Technology hype, dreams that blow away, hard work that finally turns into gold, demonstrated success. Like in the famous movie "Groundhog Day" which was released about 20 years ago and showing an endless repeated happening that occurs again each new day with exactly the same sequence – Déjà vu.[2]

Scientific psychology – Enthusiasm level

Such 'eternal law' is given for any scientific innovation. Market analysts typically like to refer to the 'Enthusiasm Level' and plot it versus time (Fig. 1). Then it first increases rapidly, topping and falling pretty soon down into a deep valley of frustration. Innovations which do not come out of here will dye and become illusions, fating away from

reality. Innovations which survive such danger through internal improvement and adaptation to reality rise again to enthusiasm like the stock exchange market after deep depression. A good description has also been given here from a completely different angle, by youth leadership development training.[3] In a first phase, explaining the new idea is so important. It is a phase of developing identity, termed "forming". The new technology is crystallized. Then demonstration is needed. The new technology turns out to be inferior to the well-researched long-lasting technology. This creates "storming" which easily can turn into frustration. Guidance is then necessary. Stop developments which lead to nowhere. Support developments which have the origin direction. This phase is termed "norming". Finally, the true unique selling points are recognized. The phase of enabling starts and the time is there for "performing".

In terms of "biotechnical micro-flow reactors" the four phases relate to (i) the formation of a scientific community, (ii) the design and provision of the microfluidic devices with enzymes incorporated, (iiii) the first finding of true applications, and (iv) the emerge of a market with small companies and rising business.

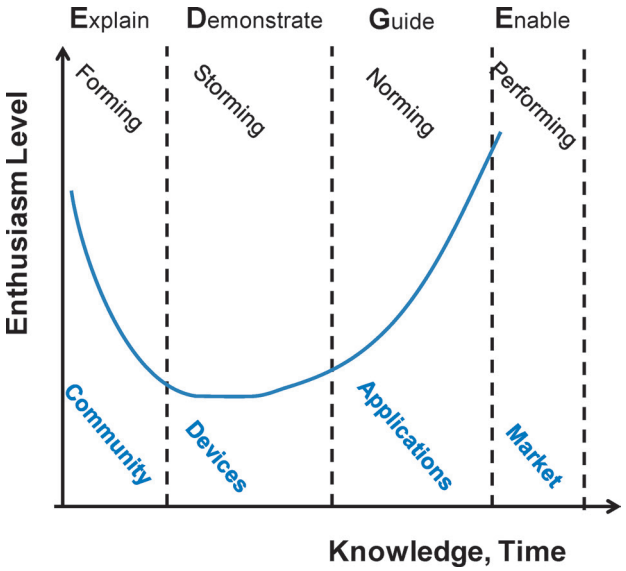


Fig. 1 – *EDGE-type switches of enthusiasm level with growth in knowledge which presents also a time axis. Here for about ten years. EDGE = Explain, Demonstrate, Guide, Enable. Redrawn after*[3] *and adapted to the content of this article.*

The first author has experienced such phases for the development of chemical microreactors.[4] It started 1993, yes, at that time the movie "Groundhog Day" was released. 20 years later, the Déjà vu is there with the biotechnical microreactors. For this reason, a review on this subject is given in the following. It is structured based on the technological enthusiasm phase assignment and will start for each of the chapters with a (personal) feature and then will give a scientific overview how detailed the development is.

Theoretical potential analysis – review compilations

From chemical to biochemical microreactors

Microreactors are widely used in various organic syntheses, where their characteristics of enabling decreased time constants for mass and heat transfer and thereby improving selectivity and yield are the most pronounced.[5] The biochemical synthesis in microreactors is of more recent date, and followed numerous analytical and diagnostic applications (such as Micro Total Analysis Systems (μ-TAS), enzyme linked immunosorbent assays (ELISAs) and enzyme-mediated protein fragmentation.[6] Efficient use of small amounts of reagents and catalysts (and expensive enzymes) in development studies is a clear motivation for the growing applications of microreactors in synthetic bio-organic chemistry.[7]

The development, optimization, and application of micro-flow enzymatic reactors are expected to open a new era in bio-based processing.[8] Enzymes are known as sustainable chemo- and stereoselective catalysts that can significantly increase reaction selectivity and yield. The utilization of such smart synthesis in micro-flow allows to broaden the microreactor applications towards biocatalysis and biotechnology. Vice versa, microreactor processing allows for a better control of specific reaction conditions, e.g. pH and temperature, which are very important for enzymatic activity.

The new field bio process engineering needs new types of reactors

In a review compilation, biotechnical micro-flow reactors are seen as a cornerstone in the emerging process intensification in biotechnology which has started around the turn of the century.[9] They are seen as counterpart to the successful trend in the chemical engineering area, where micro- and small-scale flow devices are used up to production scale. Some general information, stemming from the chemical microreactors is provided such as to discuss different designs, configurations and modes of operation of micro-flow reactors. Reference is also given to their use in downstream processing and the potential of integrating analytics. Current advantages and limitations in the use of micro-flow devices for process intensification are outlined and near-future developments are projected. The most valuable information, however, is a longer discus-

sion about several application studies of microstructured devices to bioprocesses. An (over several pages) extended table summarizes even more first-hand information about the reactors used, enzyme immobilization employed and a snapshot what the major outcome is.

Basic engineering features

The synthesis in enzymatic micro-flow reactors often needs longer residence time to achieve desired conversion.[7] The design of such microreactor should, therefore, be such to allow for efficient residence time and a proper conversion level. The engineering of the enzymatic micro-flow reactors just has started. By far the most contributions so far were given from researchers experienced in biocatalysis and thus chemistry. The following diagram shows operational windows determined for enzymatic segmented flow as described in[10] (and in this paper below in more detail) (Fig. 2). The Damköhler number (Da) and effectiveness factor (E) were taken as main engineering parameters and so determine productivity (P). A feasible operational area is proposed (shaded area in Fig. 2).

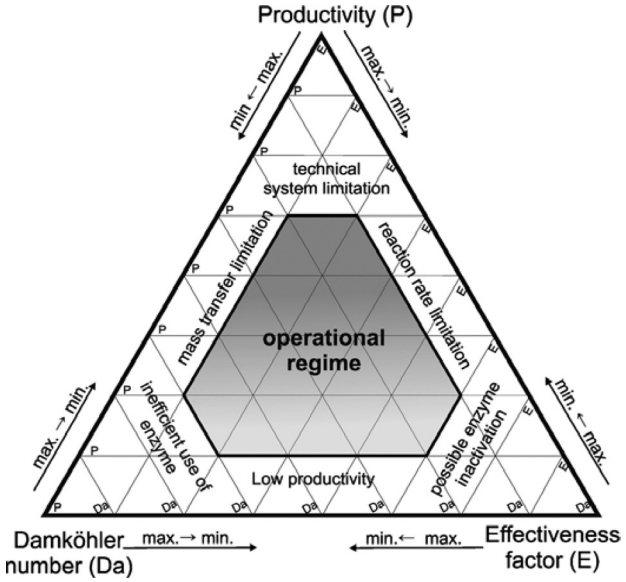


Fig. 2 – *Operational window diagram for segmented flow given in[7] as determined by the Damköhler number (Da) and effectiveness factor (E), yielding productivity (P). The shaded area represents a feasible operational area (by permission of Wiley-VCH[10]).*

Main further challenges for efficient use of enzymes are their short lifetime and mutual deactivation (in case of multi-step reactions). A development of an efficient miniaturized flow system can enable a long term use of the biocatalysts, while the reactor milli-dimensions will facilitate the hydrodynamics and the control of reaction conditions.[11,12] The advanced knowledge on immobilization techniques, close control of operating conditions and factors that will increase productivity are important items to allow for tailoring of enzymatic flow reactor design, and thereby to support process intensification needs. It is important to note that benefit from the miniaturization can be found in other steps than conversion (such as efficient mixing and separation).[7,9] Thus, the issues of process integration and a holistic process design approach have considerable importance.

Some observed advantages and challenges for using micro devices for conversion of biomolecules are shown in Fig. 3.[12]

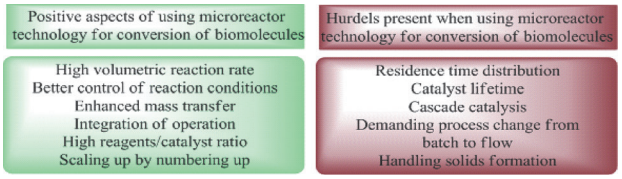


Fig. 3 – *Benefits and bottlenecks of using microreactor processing for biocatalysis and other biotechnological operations (by permission of deGruyter[12])*

Securing the innovation – Intellectual property rights

Patents on (chemical) microreactors

The patent development for chemical microreactors was for over ten years characterized by a strong linear increase in the number of patents filed.[13,14] Both applied institutes and companies took the lead here.[13] Initially, most patents came from Europe, here in particular from Germany, and from USA which had at that time the lead in the technological development.[14] Later Japan, China and other countries took over the latter and also filed a number of patents.[14] After about ten years the number of new patent applications settled to a constant level or even slightly decreased. Only a few devices were patented, probably because their design is most often not so generic and innovative over existing lab-chip and static mixer technology. In the first years of the chemical microreactors thus most patents were filed on complete laboratory bench-scale systems and numbered-up single reactors for production.[12] Concerning the latter, the DuPont patent in 1995 was regarded as a landmark in the public attention, yet probably never reached that importance as well for the true technological implementation. It appears that there are too many circumventory solutions for the equipment and there is not the "one and only" solution which beats in performance all others. Another point from today's perspective is that the first microreactor systems patented were (by far) too visionary, generic (more

on idea status, than being manufactured) and complex to satisfy the needs of the chemist's real world. 5–10 years later the second generation of manufacturers developed microreactor systems which are still in use in industrial practice and the developers probably have stronger patents for protection of their ideas. Lonza and Corning are profound examples. A third patent claim direction beyond devices and systems, relates to processes. Merck in Germany was the first chemical company to massively file micro-reactor based process patents. A broad diversity of medicinal chemistry patents were filed and granted, all of which based on the claim that to the surprise and satisfaction of the researchers the microreactor process performed so well and maybe better than with conventional technology. Today, such patent strategy probably is not applicable anymore. It is well known and commonly accepted that microreactors are suitable tools for most of the chemistries and in some cases even can be used in production. Thus, a micro-flow process-based patent probably has almost the same threshold in process innovation than has any other process patent (with conventional technology). Still there is probably enough room for such process patents, since microreactors allow to work under unusual conditions and even access novel process windows for which conventional technology cannot be used.

Forecast on patents on biotechnical micro-flow reactors

So far, only a few enzymes have been applied to microreactor-based process development, and only a few patents describe the construction of micro-flow enzymatic reactors, which is an indication that the field is still in its initial stage. A microreactor that carries a silica-based nanoporous material–enzyme composite, i.e. a microreactor using immobilized enzymes, and a sandwich-structured enzymatic membrane reactor patent[15] have been described in a patent review.[14] Therein is also reported about a US patent describing a microreactor system to perform a biocatalytic reaction with high yield, small enzyme/reactant ratio, and high rate (Fig. 4).[16] The drawbacks of using enzymes in solutions such as autolysis are overcome by filling the microchannels with functional beads. In this way, trypsin was introduced into the silicon microreactor.

Following the above mentioned industrial trends for developing systems and processes rather than new microreactor devices,[13] it is expected that in the near future new will happen also with biotechnical micro-flow devices. Particularly for enzymatic micro-flow reactors potential for various combinations of innovations exists – many novel supports for enzyme immobilization, novel engineered enzymes and microreactor devices are patentable as individual innovations and also synergistically. It appears that the system complexity of a biotechnological micro-flow benchtop plants and respective numbered-up devices for production is higher as given for their chemical counterparts. However, their mutual effects and benefits they bring to the entire process design have still not been addressed systematically. In the same way, good chances for patents in the field of biotechnical micro-flow processing are foreseen. Yet, the new processing windows and challenges are not so well developed and not as clear as in chemistry. Maybe the overall processing chances are smaller, since the most important development target is yet not fully solved, which is to achieve sufficient productivities in the small-scale micro-flow reactors. In turn, naturally any major innovation here or in another central process issue can make big impact at that time of development being in infancy.

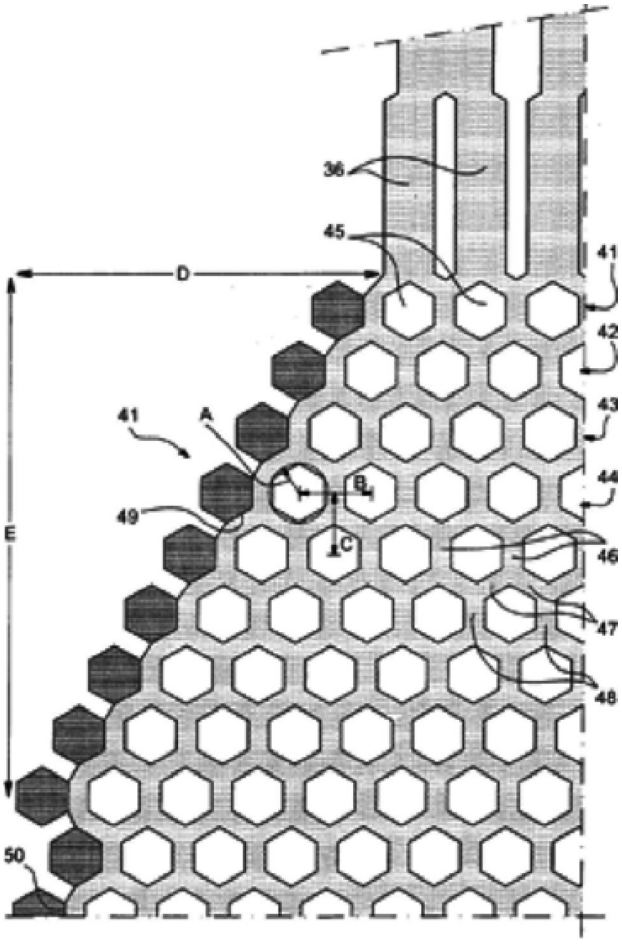


Fig. 4 – *Schematic of a patented enzymatic micro-flow reactor: 36) Channels 41–44, 46–48) reaction zones for fixing beads to immobilize the enzymes (not shown), 45) blocks, 50) wall. A: radius of a circle fitting a block; B, C: distance between the blocks; D, E: dimensions of reaction (by permission of Wiley-VCH[16]).*

Snapshot footprint on molecular diversity and performance achieved

In recent investigations, immobilized enzymes together with microfluidic devices have been applied in various hydrolysis and esterifications, C-C bond formation reactions, condensations and additions, oxidations and reductions, and polymerization reactions.[17] Some of these reactions associated with the conversion of biomolecules are collected in Table 2.

Technology fascination, technology infatuation, market uncertainness

Feature: From push to pull

Chemical microreactors initially were much influenced by the strong technological push coming from microtechnology and lab-chip technology.[18] The latter is persistent until today. Yet, what is good for one application – biochemistry and chemical analysis – does not have to be good for another application such as synthetic chemistry. It took nonetheless quite long time until that has been received as common sense in the microreactor community. Likewise for the proper technology orientation, it took even longer time until clarity was there which objectives to reach best with microreactors and for which applications conventional technology still will be superior.

The enzymatic microreactor technology is exactly in the same technology-market mismatch dilemma as chemical microreactors were about 15 years ago. In the following the state of current development will be shown in two chapters on "enzymatic lab chip applications" and first true "enzymatic micro-flow reactor concepts". Finally, it will be shown that there is first light at the horizon. A chapter about first true "enzymatic micro-flow reactor applications" will give an idea what origin objectives have been identified and what might be the markets in 5 to 10 years here.

An overview about some relevant researches with enzymatic micro-flow reactors giving some insight in the main achievements claimed and is a snapshot on the technology performance level which will be further detailed downwards (Table 1).[9,12]

Reactor design and systems assembly

Enzyme immobilization – technique optimization

A set of techniques were evaluated for the immobilization of enzymes in microchannels made of polycarbonate.[32] Physico-chemical immobilization on a layer of polyethyleneimine was found to be best suited and reproducible. In this way, alkaline phosphatase was entrapped into a tubular reactor. The known suitability of polycarbonate for industrial applications may offer chances for respective implementation of future enzymatic micro-flow reactor and its possible numbering-up.

Hierarchical porous monolithic rods

Monolithic silica rods of 4 mm diameter and 2–5 cm length have uniform 3D hierarchical pore structure of high porosity, which is demonstrated by achieving very high flow rates up to about 1200 mL h^{-1} at a pressure drop of 2.5 bar.[33] The macropores and mesopores were 35 μm and ca. 20 nm wide, respectively, and synthesized by sol–gel processing combined to pore templating and phase separation (Fig. 5). The enzyme invertase was immobilized on the silica rods and compared to mesoporous cellular foam-bound invertase for performance in the sucrose hydrolysis. Michaelis–Menten kinetics were applied and it was found that the hydrolysis of sucrose was more than 1000 times faster than given for invertase immobilized on mesoporous cellular foam. The invertase embedded in the mesopores of silica monoliths showed a remarkably larger affinity (lower K_M) to the substrate than the native enzyme. Notable results were also achieved concerning process stability and reliability. Storing the micro-flow

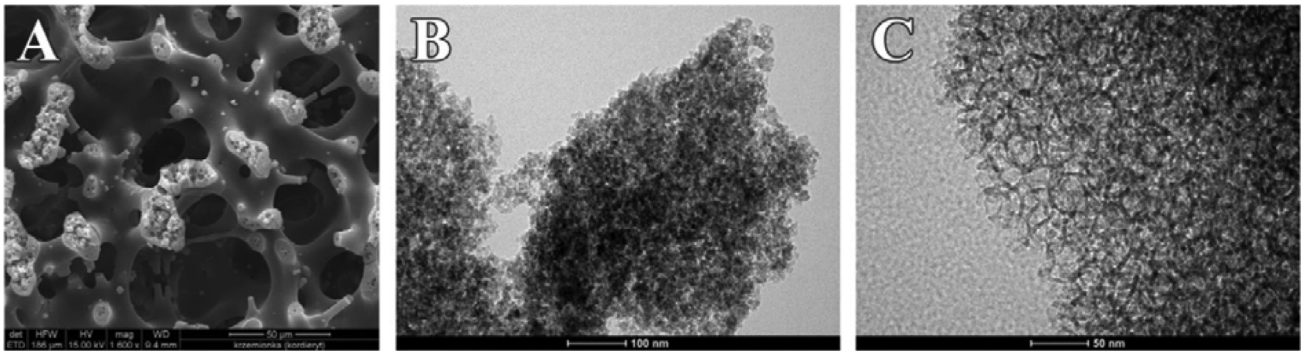


Fig. 5 – *SEM image of silica monolith surface morphology (A); TEM images of silica monoliths (B) and mesoporous cellular foam (C) structure (by permission of Elsevier[33])*

Table 1 – *Some relevant researches with enzymatic micro-flow reactors for some insight in the main achievements claimed. This serves as a snapshot on the technology performance level. More examples and the references of these researches can be found in.*[9,12]

Enzyme	Reactor type/ immobilization method or fluid system	Reaction type	Outcome
β-glycosidase[19]	Stainless steel microreactor/ covalent binding by surface activation of the wall	Hydrolysis of lactose	More than 70 % conversion yield, can be used for reaction optimization, screening and preparative synthesis
β-glycosidase (thermostable)[20]	Stainless steel microreactor/ covalent binding by surface activation of the wall	*β*-Glucosylglycerol synthesis using cellobiose and glycerol	Same conversion as in batch Fast reaction optimization
Fumarase[21]	Glass microchannels/ surface modification via silanization of inner wall using APTES and glutaraldehyde	Fumaric acid hydration to L-malic acid	25 % activity retention relative to that of free enzyme; Conversion yield up to 80 %
Lipase[22]	Borosilicate micro-capillary tubes/ thin film of lipase adsorbed on mesoporous silica coated on inner walls	Enantioselective transesterification of vinyl acetate	Long term operational stability, yield of 64 % with >99 % enantioselectivity
Lipase[23]	Silica microstructured optical fibers/ Covalent binding after silanization by APTES and glutaraldehyde used for cross-linkining	Conversion of *n*-butanol to butyl laurate using Lauric acid	Yield up to 99 %, long term operational and storage stability, can be used at production scale
Yeast cells[24]	Glass, PS, PTFE, PFA and FEP micro-channels/covalent binding on inner surface by silaninzation with APTES +cross-linking with glutaraldehyde	Surface activation of PS, FEP, PFA and PTFE	Round about 70 % cell coverage observed for all tested materials
Peptide-N-Glycosidase F and glutathione-S-transferase-fusion PNGase F[25]	Methacrylate based monolithic microreactor/ surface functionalization with glutathione via a succinimidyl-6-(iodoacetyl-amino)-hexanoate	Removal of glycans from glycoproteins	By the use of oriented and non-oriented immobilization, complete deglycosylation achieved in seconds or in minutes
Cholesterol oxidase[26]	Glass microchip reactor/ aqueous phase (enzyme): *n*-heptane phase (substrate)	Cholesterol oxidation to 4-cholestene-3-one	Around 70 % conversion, recovery of product in organic phase
Hydroxynitrile lyase[27]	Glass microchip reactor/ aqueous phase (enzyme + HCN): organic phase (aldehydes)	Enantioselective Cynohydrins synthesis from aldehydes	Conversion >90 % and enantioselectivity >99 %
Laccase[28]	Glass microchip reactor/ phosphate buffer (enzyme): phosphate buffer (L-DOPA)	Oxidation of L-DOPA	Up to 87 % conversion within a short residence time of 100 seconds
Lipase[29]	Glass microchip reactor/ aqueous phase (enzyme): *n*-hexane phase (substrate)	Isoamyl synthesis using isoamyl alcohol and acetic acid	Around 35 % conversion, recovery of the product in organic phase
Lipase[30]	Glass microchip reactor/ aqueous phase (enzyme): *n*-decane phase (substrate)	Esterification of propionic acid to butyl propionate using 1-butanol	The reaction equilibrium shifted by recovery of the product in organic phase
Lipase[31]	Glass microchip reactor/ IL ([bmpyr][dca]) phase (lipase, isoamyl alcohol): IL ([bmpyr][dca]) phase (acetic anhydride; *n*-heptane)	Isoamyl acetate synthesis using isoamyl alcohol and acetic anhydride	Compared to batch 3 times increase in the reaction rate better productivity

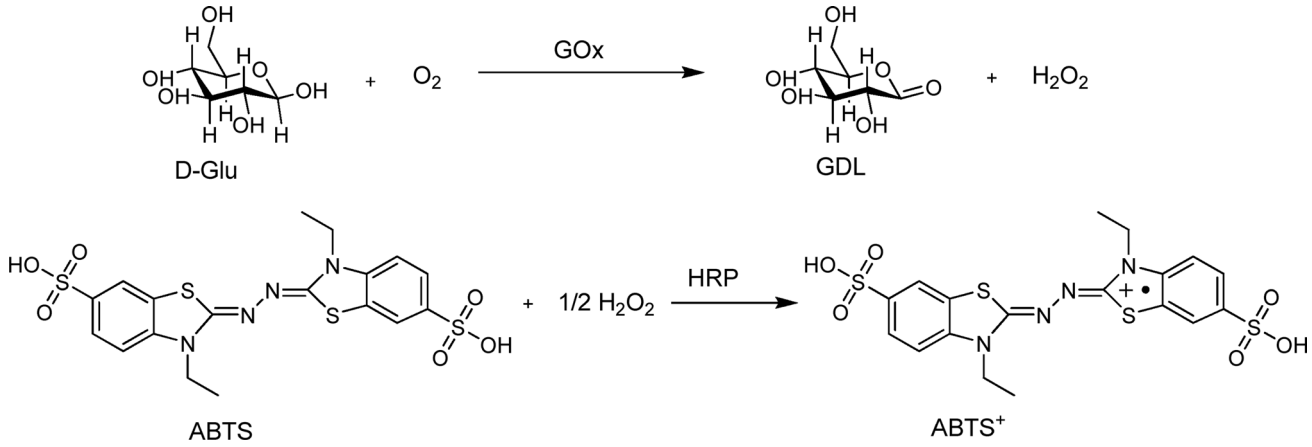
L-DOPA: 3,4-dihydroxy-L-phenylalanine; IL: ionic liquid; [bmpyr][dca]: 1-butyl-3-methylpyridinium dicyanamide

reactors at 4 °C kept the initial activity constant for at least 6 weeks. When used for continuous operation, no change in performance was observed for at least 2 weeks.

Supramolecular enzyme supports – polymer brushes

Microfluidic glass chips were functionalized with poly(2-hydroxyethyl methacrylate) polymer brushes as anchors for co-immobilization of the enzymes glucose-oxidase and horseradish peroxidase.[34] In this cascade reaction glucose oxidase converts D-glucose into δ-D-gluconolactone and hydrogen peroxide (**Scheme 1**).

Atomic force microscopy, FTIR spectroscopy, and field emission scanning microscopy were used for characterization of such supramolecular architecture. The enzyme-functionalized glass chips performed a bi-enzymatic cascade reaction to measure glucose in human blood samples with high selectivity and repro-



Scheme 1 – *Multienzymatic cascade reaction using glucose-oxidase and horseradish peroxidase*

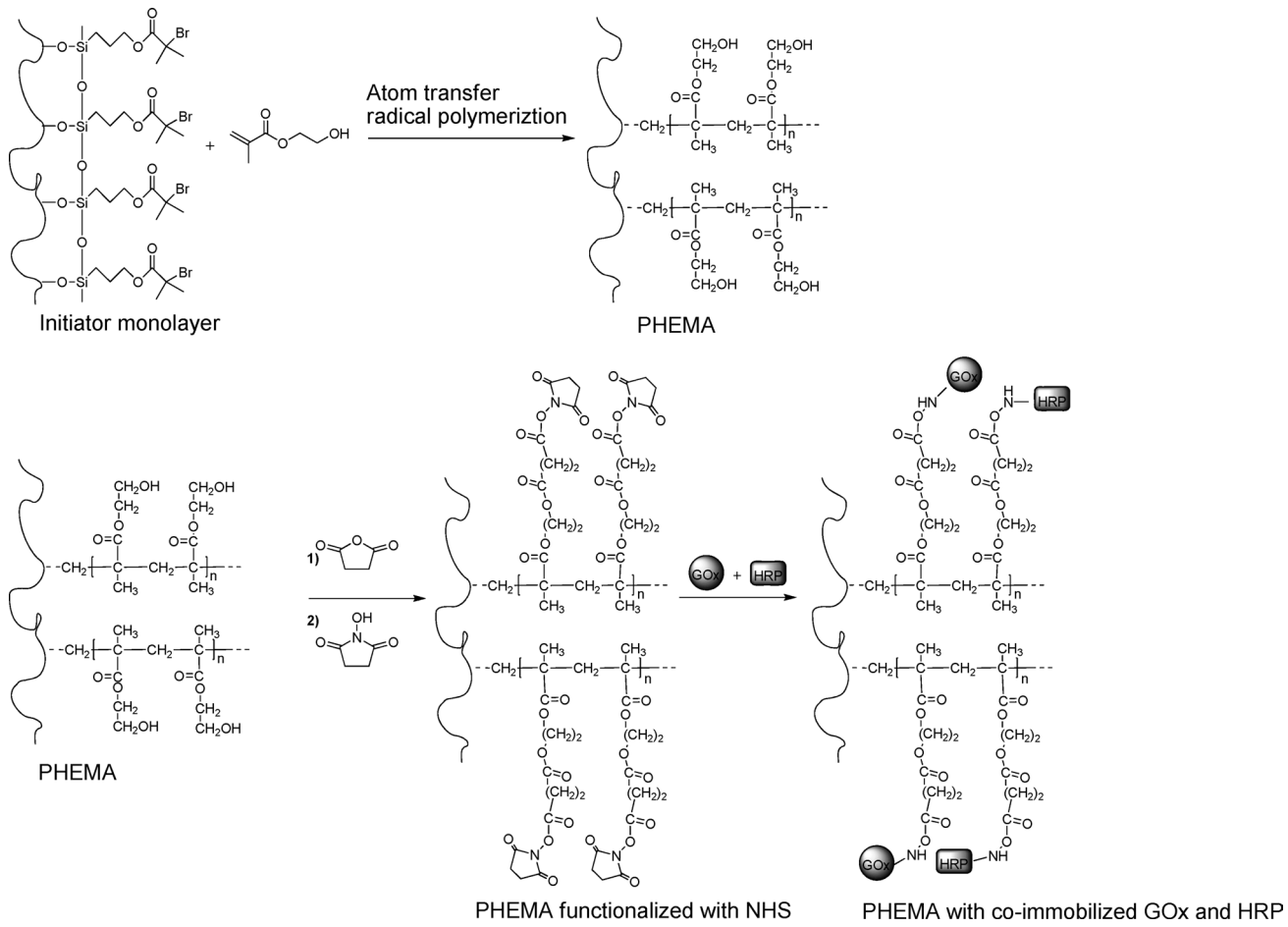


Fig. 6 – *Enzyme immobilization onto glass surface. From the first monolayer (a) via various chemical functionalizations (b) – (d) toward the co-immobilization of the two enzymes glucose-oxidase and horseradish peroxidase (by permission of Royal Society of Chemistry[34]).*

ducibility (Fig. 6). A fast analysis was reached in 20 s with a detection limit of 60 mM and the results agreed to analysis common to conventional hospital laboratory.

Foam made of silica nanowires (Nanosprings)

As smart enzyme support for threonine aldolase, the high-surface area and high-porosity silica material Nanosprings (Fig. 7) was compared to the commercial polymeric support material Eupergit CM.[35] The latter can be purchased as single beads to give a packed bed when filled in a mini tube. The first is available through chemical vapor deposition in sheet form, which simply can be cut further to any shape.[36] By stacking block-type shape (e.g. cylinder) can be realized, similar to monoliths. For Eu-

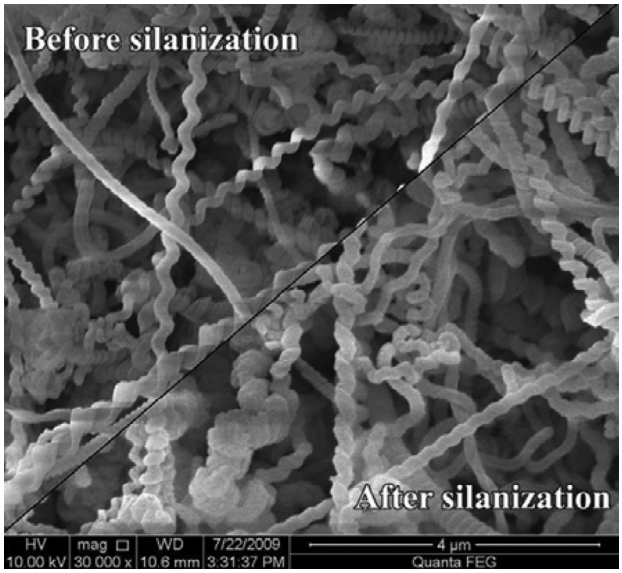


Fig. 7 – *Morphology of silicon dioxide nanosprings before (top) and after (bottom) vapor-phase silanization (by permission of Wiley[36])*

pergit CM, the share of chemically fixed enzymes and active enzymes were within the reported literature performance for enzyme immobilization. The values of immobilized enzymes on Nanosprings were somewhat lower. However, this is overcompensated by the larger specific surface area of Nanosprings and the availability of a new, second generation flow reactor concept, which involves a tight packing of stacked Nanosprings disks.

Interconnection of reactor plates – bonding

Both a temperature-robust (trypsin) and temperature-labile (pepsin) enzyme were patterned with little-to-no loss in enzyme activity.[37] Such functionalized PMMA microchips were sealed at 35 °C by a low-temperature solvent bonding system using methanol and water. High substrate/cover plate adhesion strengths were achieved up to 4,000 kN m^{-2} for unmodified PMMA substrates. In this way, pepsin was immobilized and encapsulated in a micro-flow reactor. Pepsin is a pH, temperature and solvent sensitive enzyme. Enzyme stability was dependent on the functionalization method. When immobilized water soluble carbodiimide considered only, a decrease of activity over a period of days was detected. In turn, immobilization with both water soluble carbodiimide and N-hydroxysuccinimide allowed to remain activity even after a month of use. A high protein sequence coverage was achieved and short column lengths (10 mm) and respectively short residence time (a few seconds) sufficed for operation. The schematic diagram of both immobilization methods has been shown in Fig. 8.

Community formation and forum for public-private partnership

Feature: No mirroring – no image

Although there has been microreactor research as early as in the late 80ies, this was centered in two research centers only and there were hardly any publications documented for about 4–5 years; at least not in chemical or chemical engineering literature.[4] The very few papers published, not more than 10 in total in peer-reviewed journals, targeted microfabrication literature and audience. The two institutions were the Kernforschungszentrum Karlsruhe KfK (now Karlsruhe Institute of Technology, south centrum, KIT) and the Pacific Northwest National Laboratory, PNNL). PNNL worked for aerospace and military applications mainly, both of which is quite prohibitive towards publishing. KIT had prime collaborations with a few companies interested in thermal engineering such as Messerschmidt-Boelkow-Blohm.[11]

Community as mouthpiece for technology promotion

Two moments then gave the push needed to let such individual, scattered researches become a strong, widely recognized momentum and to give it a new name, establishing it as a new field. One was

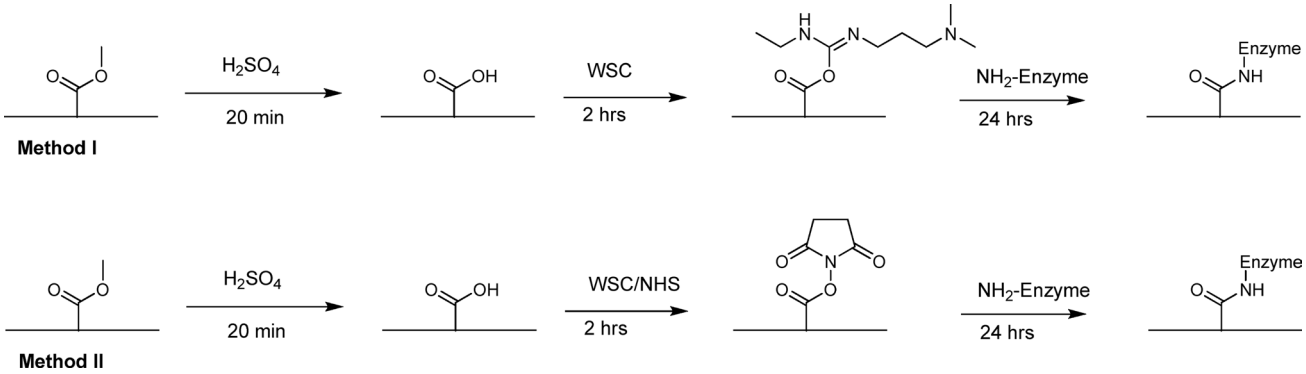


Fig. 8 – *A schematic representations of both methods of enzyme immobilization on the surface of PMMA*

the emerging of new research approaches which bundled together demanded for completion and a final counterpart. This was lab-chip technology, process intensification, and green chemistry; all of which started in the early 90ies. The second momentum was the formation of a conference series which served as platform for formation of a new scientific community and thereafter for communication and growth thereof.[38] An initial workshop on the microreactor topic in 1994 and, motivated by the success, the 1st International Conference on Microreaction Technology (IMRET) in 1995 landmarked the whole microreactor development almost 20 years thereafter. Thus, there has to be a "need" and an "enabling moment".

Biotechnology merges with miniaturization

Biotechnical micro-flow reactors needed the same to be recognized and to grow. The need comes here from the ever increasing importance of biocatalytic and biotechnical processing. The last push may come here from the biobased economy which will change all of chemistry's platform chemicals. These days, enzymes are increasingly penetrating the chemical industry as catalysts for numerous reactions. The global market of enzymes was estimated at around US$1.5 billion in 2005.[39a] A growth of 6.8 % annually is predicted to US$8.0 billion in 2015.[39b] Experts predict a doubling in the next ten years, casually said, every forth chemical product stems from a biotechnical route. Naturally the most innovative reactor and processing concepts must adapt a disproportional share of such innovation, meaning biotechnology should have a leading role in the overall microreactor development. Until 4 years or so ago, this was practically not given, however. Then, similar to their chemical counterparts, some scattered researches were reported enzymatic micro-flow reactors. Lastly, the momentum of having a communication platform needs to be added. This has been the international conference on Implementation of Microreactor Technology in Biotechnology (IMTB). Meanwhile IMTB has become the platform as I remember IMRET being in the first years – with about 100–150 researchers and some new researchers coming and others leaving – having a core concept, yet not firm in all its directions – being between the disciplines, yet with researchers not being truly interdisciplinary, being still rooted in the major discipline.

Miniaturization of biochemical analysis/sensing in microchips

Biochemical assays

L-Asparaginase was bound to monoliths and coatings which are part of enzymatic micro-flow chips. D,L-amino acids produced by the enzyme were chirally separated using microchip electrophoresis with a laser induced detector.[40] D,L-Aspartic acid (D,L-Asp) was used for a kinetic study for which a microchip electrophoresis–laser induced fluorescence method was set up. Further, a human serum containing L-asparagine (L-Asn) was hydrolyzed by the enzymatic chip. This was all done in the context of establishing a biochemical assay to eliminate unwanted side effects of the enzyme L-asparaginase when being applied as an anticancer drug to patients by injection. There are a number of these such as hepatic toxicity, allergic reaction, pancreatitis, central nervous system toxicity and decreased synthesis of blood clotting factors. A microchip electrophoresis–laser induced fluorescence method was set up for a kinetics study of the enzymatic reaction. Thus, a first step has been made towards an *in vitro* therapeutic method in an extracorporeal shunt system for acute lymphoblastic leukemia (ALL) treatment.

Proteomic sample processing

Miniaturization and automation in proteomic sample processing was the objective of researching proteolytic digestion in a digital enzymatic micro-flow reactor filled with hydrogels (agarose discs with immobilized trypsin or pepsin).[41] The processing was termed digital microfluidic (DMF) as it is based on moving and merging droplets (Fig. 9). Maximum digestion efficiency was achieved when using 31 g trypsin for 2-mm diameter discs. In an integrated method and using micro-flow droplet op-

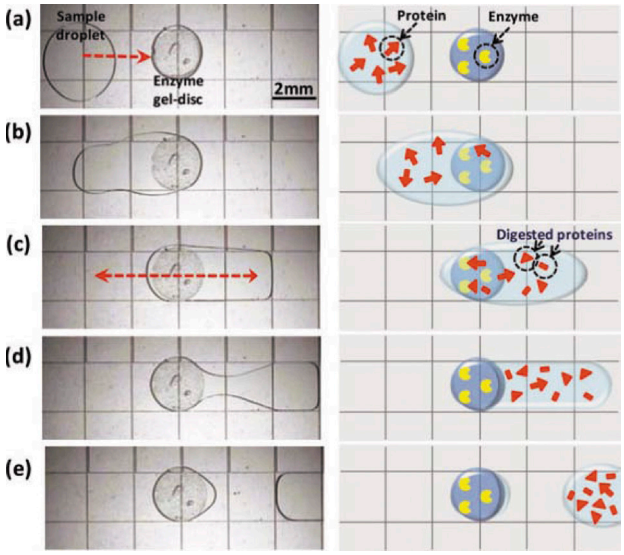


Fig. 9 – *Hydrogel proteolytic enzyme 'microreactors'. Movie images (left) and a schematic (right) monitor a typical digestion in a 2-mm-diameter gel disc on a DMF (digital microfluidic) device. A 2 μL droplet containing a proteomic sample is delivered to the gel (a and b), actively incubated (c), dispensed from the gel (d), and then isolated (e) (by permission of Wiley[41]).*

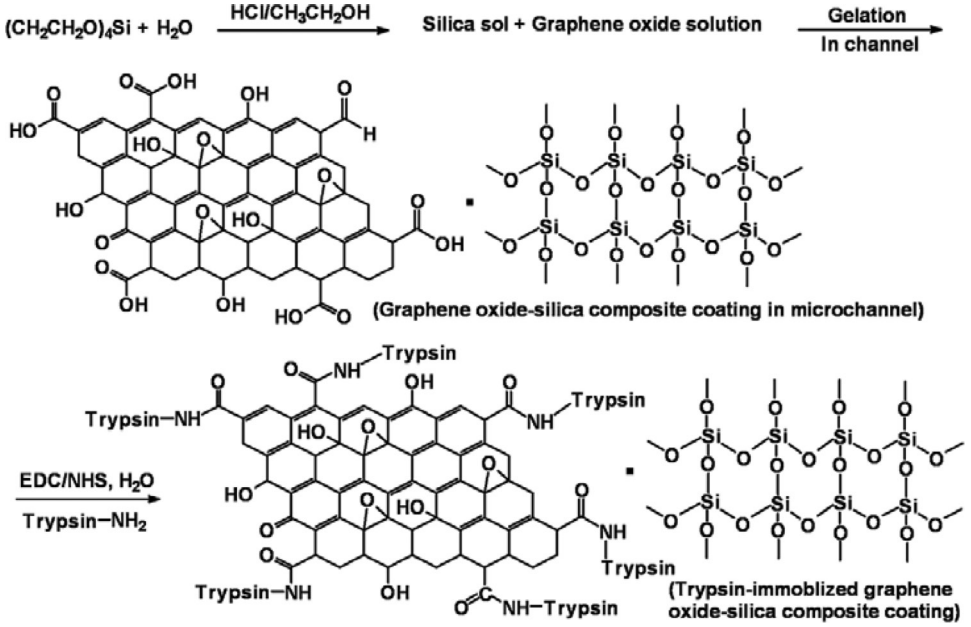


Fig. 10 – *Preparation of CO-silica composite coating for immobilizing trypsin (by permission of Elsevier[42])*

eration, proteomic samples were sequentially reduced, alkylated, and digested. The trypsin gel discs resulted in higher sequence coverage as compared to conventional homogenous processing. A parallel digestion system was demonstrated by the simultaneous digestion of a single sample on multiple gel discs carrying different enzymes.

Proteolysis and digestion

Trypsin was covalently immobilized on a graphene oxide-silica composite coated channel wall in a PMMA microchip for efficient proteolysis.[42] 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide were used to crosslink trypsin via its amino groups to the carboxyl groups of the entrapped GO sheets (Fig. 10). The digestion time of hemoglobin, cytochrome c, myoglobin, and ovalbumin was notably reduced to 5 s. The sequence coverages were 95 %, 76 %, 69 %, and 55 %, respectively. Moreover, the digestion of human serum demonstrated the suitability of using the functionalized chips for treating complex proteins.

Transfer intensification – merging with discipline and not loosing identity

Feature: Old wine is often the better one

Microreactors became strong when truly merging with reaction engineering and when the best reaction engineers took them over. Today, the best chemists take microreactors over and the teaching of chemists is complemented with flow chemistry lecturing.[43] Tomorrow the best biochemists will do. And bio process engineers will be truly needed. In the biotechnology the order of approaching has been upside down to the chemical counterparts. First, the biochemists, now the engineers are so much needed.

From multiple effects towards one-criteria focus

Microreactors traditionally were engineered by scientists from the fields of mechanical engineering and physics (rooting from the microelectronics main education streams) and later by scientists of the newly emerged field of microfluidics. Demonstration of effects was in the foreground.[4] A variety of creative designs came out; yet often not with the chem eng goggles of minimizing pressure drop, fouling, backmixing, ensuring scaleability, and simply heading for the most efficient design as predicted by common chem eng criteria and modern simulation tools.

As a result, very targeted and focused studies are found these days, e.g. developing a multi-injection microreactor to minimize hot-spot formation by smearing reaction heat release over a large area.[44] Using a concept known in conventional chemical engineering and with using the same tools and virtually the same models a powerful tool is developed. In turn, the early microreactor papers contained a cornucopia of research objectives. Naturally none fully developed. The direction just was not clear and approach was more playful. Publications on biotechnical micro-flow still have such feature and go for many foci. Yet the high specialism of the researchers and their biochemical background has already led to an enhanced level of result achievement

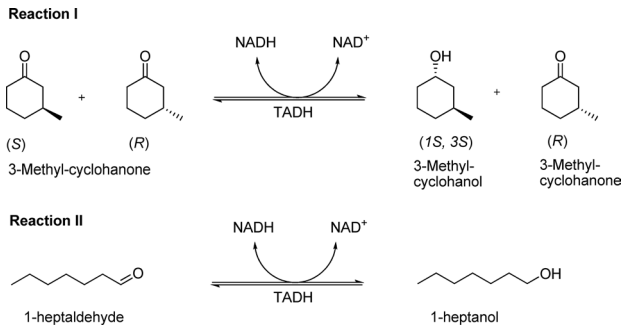
which made it possible to classify the researches in a manner introduced for the Novel Process Windows concept.

In the following the three stages in intensification will be discussed: (i) transfer, (ii) chemical and (iii) process-design based. The main focus of the research was taken for classification. Naturally this is not in each case fully rational. But the intention is here to give the reader insight in the multidimensionity of bioprocess intensification at microscale, rather than heading for perfect assignment.

Mass transfer and residence time intensification

Segmented flow

Only a very few uses of a segmented micro-flow capillary reactor are so far reported, albeit this has been proven to be a powerful processing concept for chemical microreactors.[10] To fill that gap a generic study was done for the enzyme-catalyzed conversion of 1-heptaldehyde to 1-heptanol in a liquid-liquid phase system (**Scheme 2**). Relevant parameters for segmented flow such as capillary diameter, flow velocity, phase ratio, and enzyme as well as substrate concentration were varied to show their impact on the enzymatic reaction. The sum of all this information led to the formulation of a novel operational window for an easy assessment of the various system constraints. Three coupled capillaries of different diameter were finally arranged in series (Fig. 11).



Scheme 2 – *TADH catalyzed reactions*

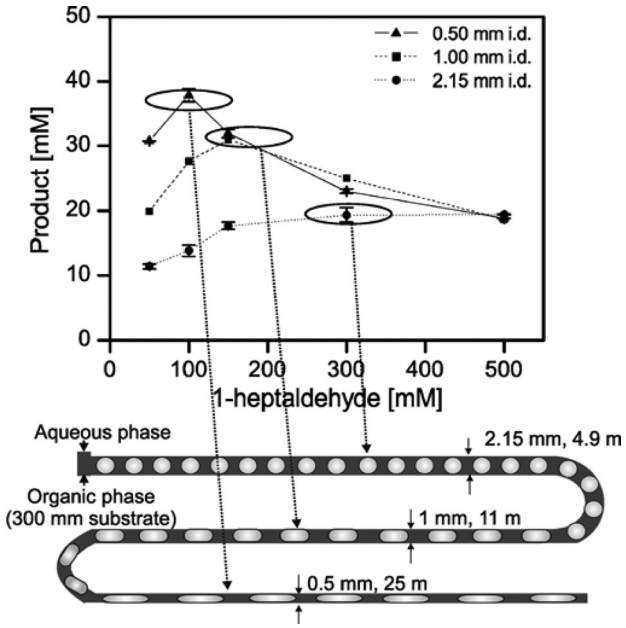


Fig. 11 – *Product generation at various reactant concentrations opposed to schematic of coupled capillaries (2.15 mm, 1 mm and 0.5 mm inner diameter) in series (by permission of Wiley-VCH[10])*

In another example of segmented flow a simple method for making macroporous silica-monoliths with well-regulated porosity was used for the lipases immobilization to make an active and stable enzymatic microreactor. The reaction of hydrolysis of 4-nitrophenyl butyrate in water–decane media was selected. For the immobilized *C. antarctica* lipase A, 96 % conversion was obtained due to the significant increase in the interfacial activation occurred by formation of favourable biphasic system. While only 23 % conversion for the free lipase was obtained. The stability of immobilized enzyme was also increased resulted in 64 % conversion at 80 °C and 70 % conversion when continuously run for 480 hours.[45]

Some more examples on multiphase biotransformations[46] and enzymatic microreactors using non-aqueous media[47] are reviewed.

Reaction rate enhancements

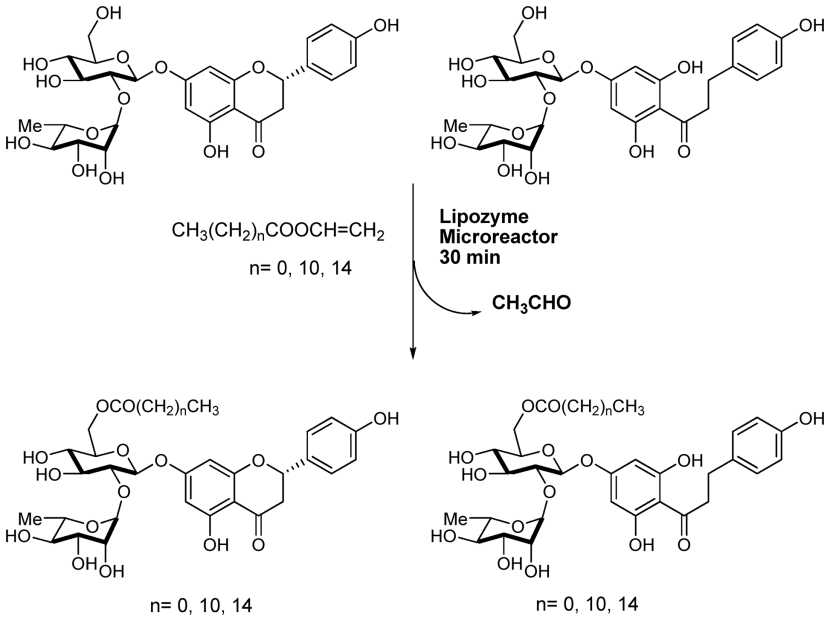
Catechol and L-DOPA oxidation/laccase

Laccases oxidise a wide range of substrates by radical mechanism accompanied by a reduction of oxygen to water in a four-electron transfer process.[48] The enzymatic oxidation of phenolic compounds, catechol and L-DOPA, using commercial laccase (from *Trametes versicolor*) was performed in a micro-flow microreactor. At a residence time of 72 s, a catechol conversion of 41 % and a L-DOPA conversion of 45 % were achieved, respectively. For catechol oxidation, the micro-flow based oxidation rates were 76 to 704 g dm^{-3} d^{-1} which is 18 – 167 fold higher as given for a macroreactor. Kinetic investigations showed that the maximum reaction rate achieved in micro-flow was two times higher than given for synthesis in a cuvette.

Selectivity

Regioselectivity

The regioselective acylation of the flavonoids naringin and naringin dihydrochalcone using Lipozyme TL IM (from *Thermomyces lanuginosus*) was conducted in a micro-flow microreactor[49] (**Scheme**



Scheme 3 – *Lipase-catalyzed regioselective acylation of naringin and naringin dihydrochalcone in microreactors*

3). The reaction conditions were mild (52 °C) and relatively short reaction times of 30 min were taken. High conversions up to 90 % and high regioselectivities in the range of 99–100 % were achieved.

Enantioselectivity

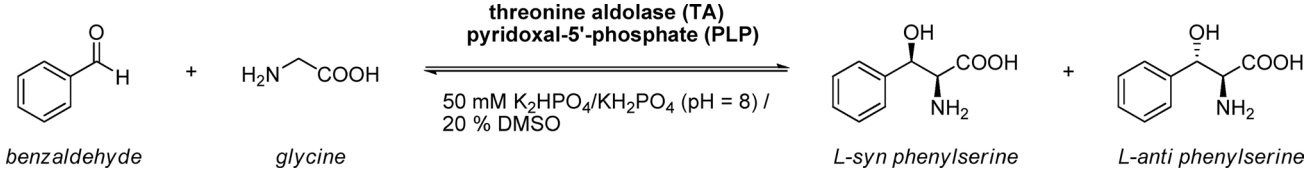
A micro-flow packed-bed microreactor with threonine aldolase (TA) from *Thermotoga maritima* immobilized on a Eupergit support conducted the synthesis of phenylserine from benzaldehyde and glycine[50] (**Scheme 4**). For immobilization two ways of different complexity were used, a so-called direct and an indirect method. The effects of temperature and residence time were studied in particular. The maximum yield of 35 % – due the equilibrium nature of the reaction and substrate inhibition – is realized both in the batch and flow process. In addition to using immobilized enzymes, a flow synthesis with free enzyme was realized. The product yield was slightly improved when operating under slug flow conditions. In all cases 20 % diastereomeric excess (de) and 99 % enantiomeric excess (ee) was observed. A stable run for 10 hours without enzyme deactivation was demonstrated at 70 °C and at 20 minute residence time. The productivity of the reactor was extrapolated to parallel run units and compared with the result in a previous publication.

From those and other experimental results together with assumptions, calculations on the (theoretical) productivity of enzymatic micro-flow reactors were made.[35] This included results for the glucose oxidase immobilization on Nanosprings. High enzyme loadings and adequate space-time yields were the objectives. These productivities were benchmarked to industrial production targets for a high-value pharmaceutical intermediate (a dichiral amino alcohol, using threonine aldolase) and for a large-volume fine-chemical (gluconic acid, using glucose oxidase). This shows that the present loading performance suffices (at best) for making high-value products, such as pharmaceuticals, but not for larger-volume, low-cost chemicals.

Productivity – scale out

Micro-flow packed bed reactor

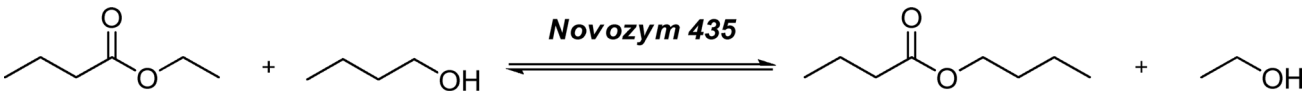
A packed micro-flow – bed reactor comprised acid phosphatase which was covalently immobilized on PMMA beads with an epoxy linker (Immobeads-150 or Sepabeads EC-EP).[51] After immobilization 70 % of the activity was retained and stability was given for many months. The enzyme



Scheme 4 – *Phenylserine synthesis using threonine aldolase in a micro-flow packed-bed microreactor*

Table 2 – *Different micro-flow reactors used in[35] and some key data on enzymes and support. This is used to extrapolate to (theoretical) reactor productivities. GOT: glucose oxidase; TA: threonine aldolase (by permission of Elsevier[35]).*

Reactor	Enzyme	Support & mass (mg)	Amount of immobilized enzyme (mg)	Amount of active enzyme (mg)[a]	Reactor productivity (g h^{-1})[b]
Single Porous Plate Flow Reactor (SPPFR)	GOT	Nanosprings 11.5	7.1	0.71	0.099
Stacked Multi-Porous-Plat Flow Reactor (SMPPFR)	GOT	Nanosprings 100	87	6.17	0.397
Single Porous Plate Flow Reactor (SPPFR)	TA	Nanosprings 17.5	3.51	0.45	0.212
Stacked Multi-Porous-Plat Flow Reactor (SMPPFR)	TA	Nanosprings 100	20.1	2.59	1.210
Micro Fixed Bed Reactor (MFBR)	TA	Eupergit beads 133	1.15	0.60	0.280
Membrane Microreactor[c]	TA	Membrane 750[d]	0.88	0.088[e]	0.041



Scheme 5 – *Transesterification reaction of ethyl butyrate and 1-butanol using Novozym 435*

catalyzed the transfer of a phosphate group from pyrophosphate to different molecules with primary alcohol groups. This included glucose-6-phosphate, N-acetyl-D-glucosamine-6-phosphate, allyl phosphate, dihydroxyacetone phosphate, glycerol-1-phosphate, and inosine-5'-monophosphate. Operation on a gram scale was achieved.

Via kinetic analysis and higher enzyme loading towards conversion-rate intensification

A packed micro-flow – bed reactor was compared to the batch slurry reactor for the transesterification of ethyl butyrate with 1-butanol to give butyl butyrate catalyzed by *Candida antarctica* Lipase B (CALB) in Novozym 435[52] (**Scheme 5**).

Novozym 435 was stable up to 80 °C in the micro-flow packed bed reactor. Stable 12 h operation at 70 °C was achieved, without enzyme deactivation. External and intra-particle transport limitations notably impact the observed reaction rate. The same packing can be reused for several days without activity loss.

A complete kinetic description allows to make use of transfer intensification in the micro-flow reactor stemming from higher specific enzyme loading, shorter diffusion pathway, with low reactant molar ratio (ethyl butyrate:1-butanol) and use of high concentration. This results in higher overall conversion and higher reaction rate per unit of catalyst volume. Total conversion was achieved in approximately 4 min residence time giving 0.04 mol L^{-1} of product, while in batch more than 30 min were needed for maximum conversion of 87 % (Fig. 12). An outline on enzymatic microreactor production performance is given.

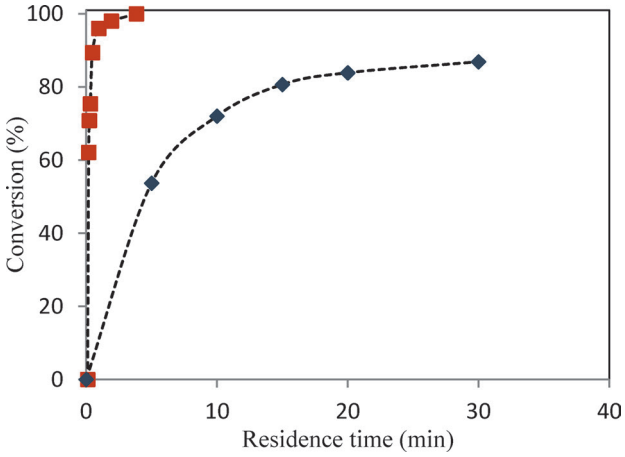
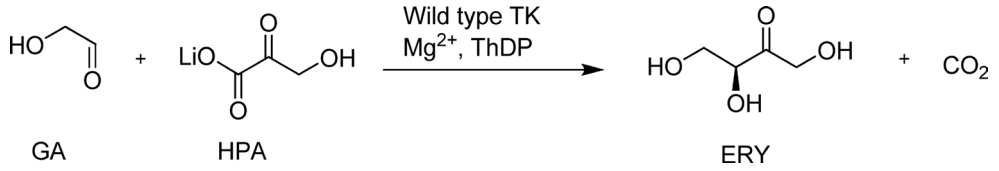


Fig. 12 – *Conversion for batch and packed-bed micro-flow reactor at 60 °C. (■) Microreactor; 0.04 M EB, 0.4 M B; (♦) Batch reactor: 0.04 M EB, 0.2 M B in t-BuOH as a solvent. EB: ethyl butyrate, B: 1-butanol (by permission of American Chemical Society[52])*

Fed batch in flow – multi-input reactor

While chemical microreactors typically perform best at high concentrations or even in solvent-free operation, biocatalytic pathways suffer



Scheme 6 – *Reaction of lithium hydroxypyruvate (HPA) and glycolaldehyde (GA) to l-erythrulose (ERY) using transketolase*

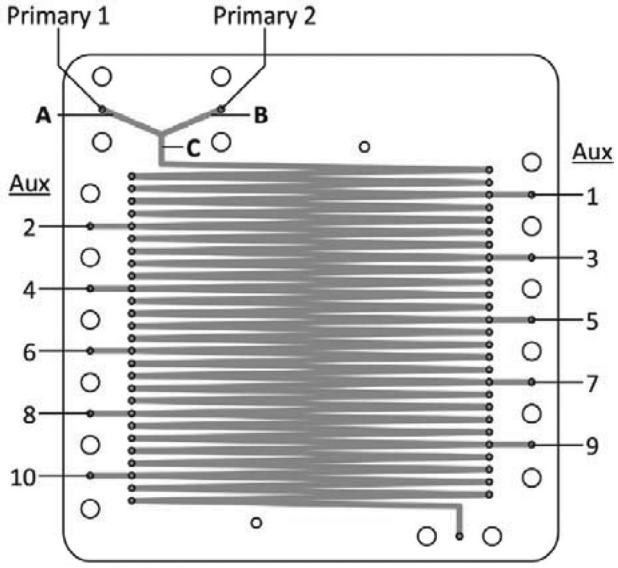


Fig. 13 – *Multi-input microfluidic reactor for multiple substrate feeding (by permission of Elsevier[53])*

from adverse effects of high substrate concentration on the biocatalyst, including inhibition and denaturation.[53] Thus, to achieve high productivity remains an open question. A solution to this is provided by fed-batch reactors and thus it is advised to search for micro-flow analogues. To realize this, a multi-input microfluidic reactor was developed which is capable of substrate feeding at multiple points (Fig. 13).

The transketolase-catalysed reaction of lithium hydroxypyruvate and glycolaldehyde to l-erythrulose was investigated (**Scheme 6**). A 4.5-fold increase in output concentration and a 5-fold increase in throughput was achieved compared with a single input reactor.

Chemistry intensification – push enzymes to their limits

Out of the niche and become accepted

There was a time when chemical microreactors were considered to be niche applications.[4] Only a few chemical reactions seemed "to keep speed" with the fast-tuneable chemical microreactors. Meanwhile it is known how to speed chemical reactions; actually by virtue of utilizing the transport intensification enabled by the microreactors. That was game changing and is a main result of the chemists entering the microreactor world in the shape of the flow chemistry development. While the catalytic action of enzymes seem to be suited only for slow reactions, first examples give some hope that at least "niches" for sufficient productivity exist. Assuming similarity to the chemical microreactor development and finding of similar (or other new) intensification means, there might be a larger use of the enzymatic micro-flow reactor for future's production needs. This development is regarded to be much more relevant than to further optimize the transport intensification means.

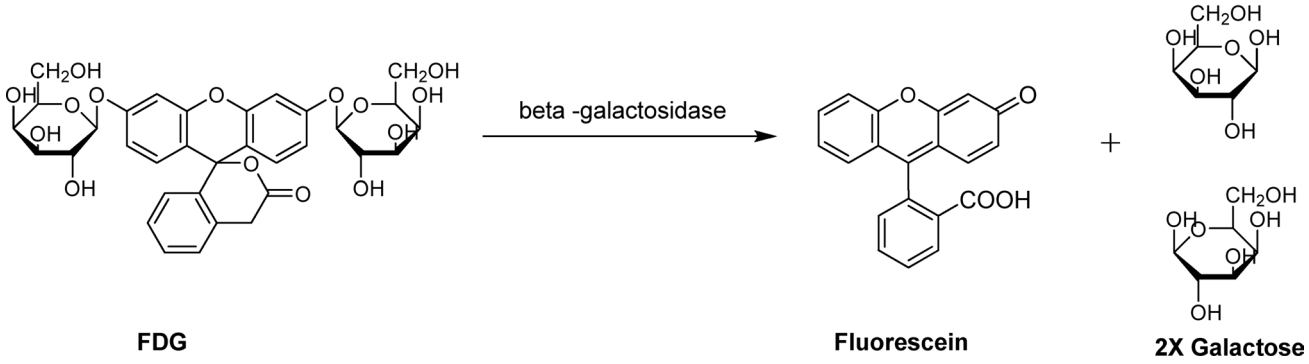
Bioflow chemistry – optimization of process conditions

Concentration – enzyme and coenzyme

The enzymatic oxidation of hexanol to hexanal (as green note fragrance) using NAD^+ dependent commercial alcohol dehydrogenase (from *S. cerevisiae*) was performed in tubular micro-flow microreactors with internal volumes of 6 and 13 μL.[54] Another tubular micro-flow microreactor with a volume of 2 μL internally comprised micromixers, as one had to deal with mass transfer in immiscible phases. The impact of enzyme and coenzyme inlet concentrations and flow ratios on the conversion of hexanol and the volumetric productivity of hexanal was analyzed. Best result was achieved for c (hexanol) = 5.5 mmol L^{-1}, c (NAD^+) = 0.55 mmol L^{-1}, and c (NADH) = 0.092 g L^{-1} which outperformed the batch processing. 12 % conversion of hexanol was given for the 6 μL microreactor after 72 s, whereas the macroscale reactor had only 5 % conversion after 180 s.

Preconditioning – of enzyme and substrate ratio

A preconditioning study utilized mixing enzyme and substrate in predefined ratio before reaction and enabled so a millisecond temporal analysis of enzymatic reactions.[55] Crucial element was a micromixer generating four lamination layers through a narrow channel, thereby reducing the diffusion lengths to a few micrometers and providing mixing times in the millisecond range. In this way, the enzymatic hydrolysis using β-galactosidase was performed using enzyme/substrate ratio from 1:1 up to 3:1. Kinetic data collection was achieved in a very short time in the order of minutes. The fast and easy



Scheme 7 – *Hydrolysis of FDG using β-galactosidase to produce fluorescein and two galactose molecules*

handling of the mixing device makes it a very powerful and convenient instrument for millisecond temporal analysis of bioreactions. The enzymatic activity determination of β-galactosidase was done by optical measurements using fluorescein di-(β-D-galactopyranoside) (FDG) as a substrate which gets converted to fluorescein and galactose by sequential hydrolysis (**Scheme 7**).

Process-design intensification – push enzymes to their limits

Essay: Two is more than one, synergy of integration

One 'relict' from miniaturization remains as big advantage for chemical and biotechological micro-flow reactors, even if these cannot give transfer or chemical intensification. Their smallness allows for compact process designs. The big commercial success of microelectronics stems from the system integration in the microprocessor as central element of every computer. Same for microchemistry. The compact process integration achievable can go beyond conventional process integration and is often accompanied by process simplification.[56,57] Integration can be hierarchical from device to system to full-plant level. As a net result, synergy is achieved, e.g. when coupling of reaction to extraction or endothermic reactions to exothermic ones.[57]

The closer the developments go to industrial level, the more attention will be given towards the process-design improvement with the specific possibility the compactness allows. Some of which is given in the following.

Coupling of micro-flow enzymatic reactions to micro-flow separation

Micro-flow extraction – hydroxylation of progesterone/ Rhizopus nigricans

The hydroxylation of progesterone in 11α position by *Rhizopus nigricans* was performed in a bench scale reactor, while the extraction was carried out in a microchannel system with a Y-shaped inlet and a multiple bended channel.[58] This is one of the key steps in the production of corticosteroid drugs and hormones. The integration of reaction with flow extraction of progesterone and 11α-hydroxyprogesterone from water into ethyl acetate has been suggested.[59] This allows increase in productivity as compared to the batch process both because the reactant is readily recovered for reuse and the product is easily purified. For increasing the capacity of the system, a numbering-up approach by using parallel microreactors is proposed. It is predicted that a continuous filtration unit is then needed upfront.[59]

Micro-flow extraction – Esterification to isoamyl acetate/lipase

The esterification of isoamyl alcohol and acetic acid to give isoamyl acetate using Lipase B (from *Candida antarctica*) was done in a microchannel reactor, for which all details were given above.[29] Isoamyl acetate is one of the most employed flavors in food industries. An increase in temperature from 25 to 45 °C resulted in higher reaction yields. The residence time needed for the flow reaction was even below 1 min. Reaction rates were orders of magnitude higher than for batch esterification were obtained at similar reactant concentrations. A further benefit stems from the direct coupling of the flow reaction to flow extraction. The enzyme is dissolved in aqueous phase and continuously separated from the organic phase (*n*-hexane) and the product. Lipase mediated synthesis of isoamyl acetate was carried out in a two-phase solvent system consisting of a water miscible ionic liquid and *n*-heptane within ψ-shaped microfluidic device.[31] Complex flow patterns arise due to the amphiphilic nature of the enzyme causing droplet formation aside the induced slugs of the segmented flow. The latter is then surrounded by an increasing corona of droplets (Fig. 14).

A microreactor consisting of a glass microfluidic chip comprising an X-junction was also used for this reaction in the above mentioned solvent

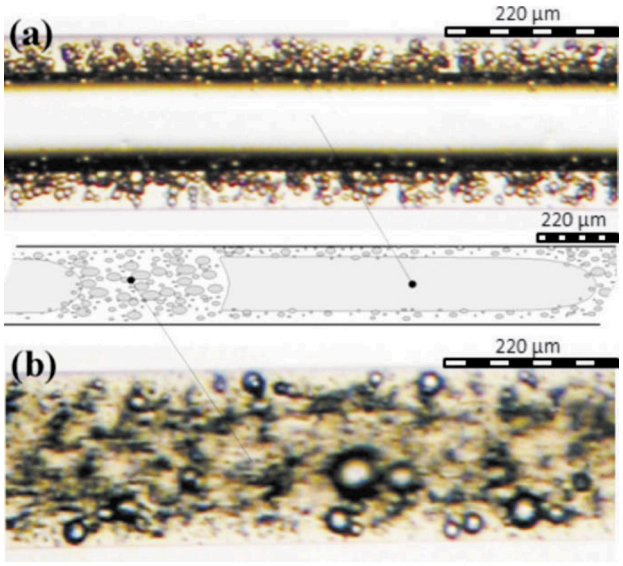


Fig. 14 – *(a) Long slug of n-heptane with concave tail surrounded with small droplets of n-heptane and (b) fine dispersion between the two neighboring n-heptane slugs (by permission of Royal Society of Chemistry[31])*

system. This allowed to generate high interfacial area for continuous product removal, and further recovery of the enzyme in solvent when coupled with a phase separator.[60]

Micro-flow extraction – Esterification to butyl butyrate/lipase

A packed-bed micro-flow reactor using immobilized lipase B was coupled with a miniaturized separator in continuous manner for the synthesis of butyl butyrate using ionic liquid as a solvent. 100 % conversion was achieved within only 5 min at 25 °C and 0.5 M equimolar concentration of both the reactants. The coupled separator resulted in 90 % of product isolation efficiency (allowing also to reuse the ionic liquid).[61]

Coupling of enzymatic micro-flow reactions to online-analytics

Photoionization mass spectrometry – Esterification to ethyl oleate/lipase

Lipid transformation was achieved in a lipase immobilized on a silica monolith providing a micro-flow network.[62] The monolith was placed into a 320 mm internal diameter fused silica capillary and such micro-flow reactor could be used up to flow rates of 60 mL h^{-1} (Fig. 15). *Candida antarctica* lipase was covalently bound onto the silica monolith using glutaraldehyde as the cross linking reagent. Triolein was quantitatively transformed into ethyl oleate at room temperature. No loss of activity was found for 15 runs. The micro-flow lipid transformations were coupled online with atmospheric pressure photoionization mass spectrometry. Feasibility could be demonstrated, yet a further reduction of dead times of the prototype system is needed to enable the potential of such innovative analytical monitoring.

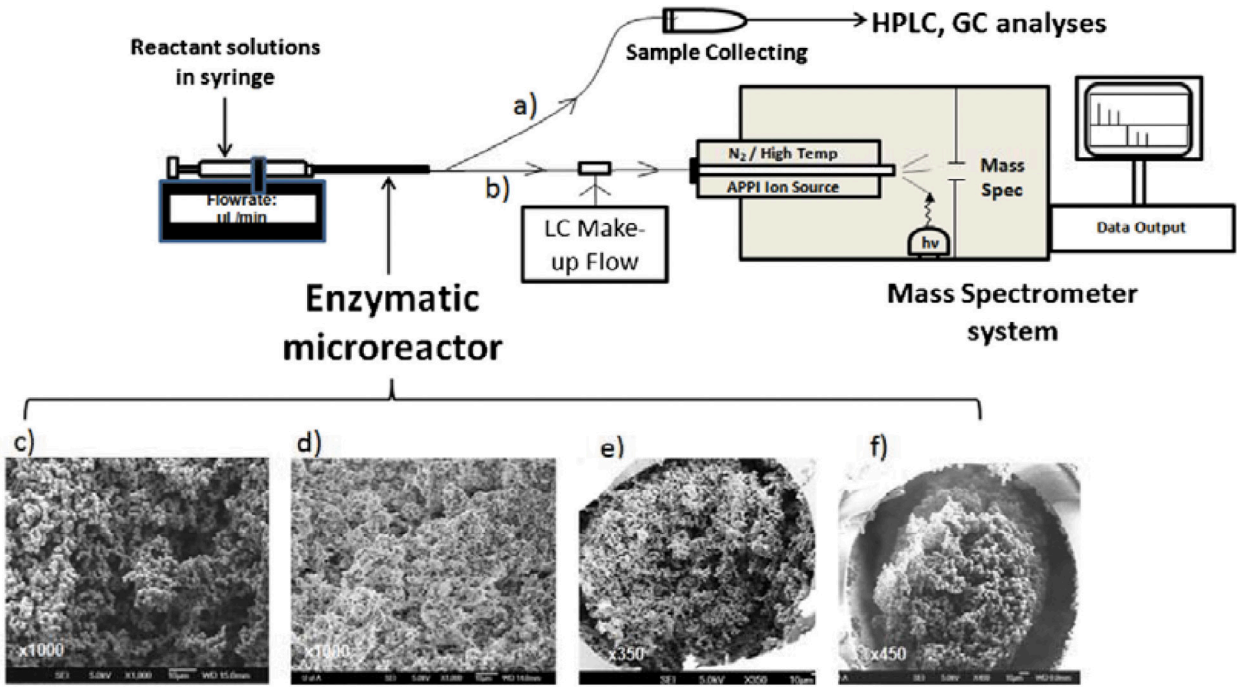
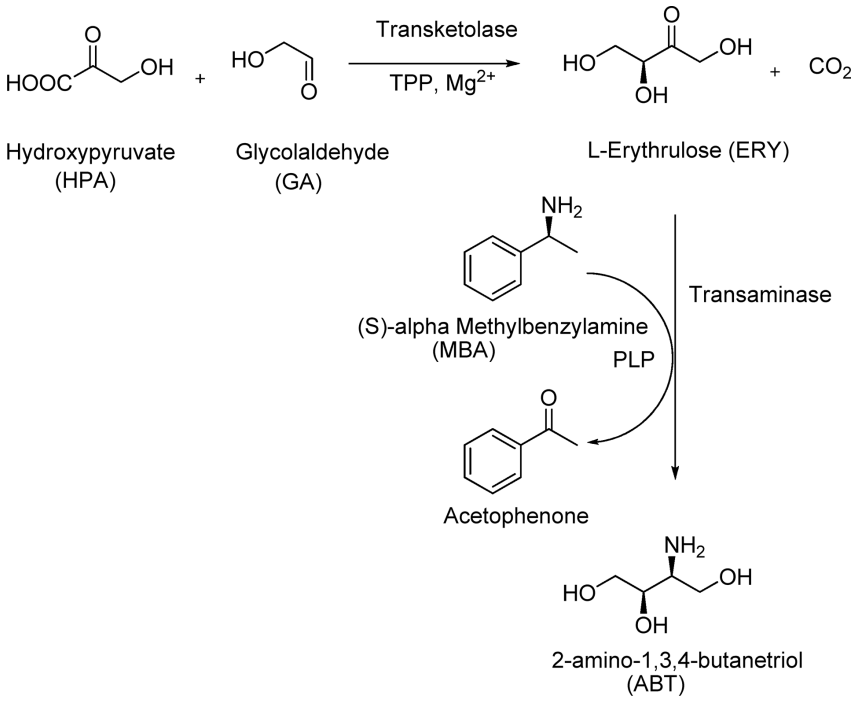


Fig. 15 – *Enzymatic micro-flow reactor based on monoliths: (a) offline reaction (b) online coupled with the mass spectrometry system; SEM image of a cross-section of: (c) a silica monolithic capillary (1000×), (d) an enzymatic monolithic microreactor (1000×); (e) an NaOH-treated silica monolithic capillary (350×); (f) a non-treated silica monolithic capillary (450×) (by permission of Elsevier[62])*



Scheme 8 – *Two step pathway for the synthesis of ABT*

Multi-step enzymatic synthesis

De novo metabolic engineering offers chances for multi-step enzymatic synthesis to give in one step complex molecules.[63] The pharmaceutical building block and diastereoisomer 2-amino-1,3,4-butanetriol is synthesized from simple achiral substrates using two enzymes, transketolase (TK) and transaminase (TAm). His6-tagged TK and TAm were immobilized onto Ni-NTA agarose beads which were packed into a tube to give each an enzymatic micro-flow reactor. The two latter microreactors were cascaded in series. In a first step l-erythrulose was synthesized from lithium-hydroxypyruvate and glycolaldehyde and thereafter converted to 2-amino-1,3,4-butanetriol using (S)-methylbenzylamine as amine donor (**Scheme 8**). 83 % conversion was reached in 20 min at 60 mM concentration. A kinetic investigation yielded the reaction constant.

New Economy – Synthesis of biomass platform molecules

Essay: New Windows of Opportunity – Back to the future

The use of microreactors for valorization of biomolecules is gaining increasing attention. Some examples of biomass "platform molecules" are sugars (glucose and xylose), polyols (sorbitol, glycerol), furans (hydroxymethylfurfural and its derivatives), acids (lactic acid, levulinic acid) and alcohols (ethanol).[14] Platform molecules are selected based on their availability and potential to produce corresponding intermediates and final products. However, the production of platform chemicals often proceeds in a multi-step process with low overall selectivities and yields. Therefore the amount of final product is low, costs are high and their further applications are limited.[64] To overcome these limits, not only novel synthesis routes are required but – virtually hand in hand – also novel process technologies that will improve the efficiency of the conversion processes.[65] A few examples of microreactor technology based valorization of biomolecules are shown below.

Start with sugars – like in nature

5-Hydroxymethylfurfural

Biotechnical micro-flow inventions need to follow the track of their chemical counterparts have done already.[66] A new continuous production method of 5-hydroxymethylfurfural (HMF) from glucose and fructose in a high-pressure and high-temperature aqueous medium has been developed. Within 10 s of reaction time a high selectivity and high yield in HMF synthesis were achieved. By using the high-pressure and high-temperature microreactor, heating and cooling within 0.01 s was achieved. As a result, production of HMF with a selectivity exceeding 80 % and yield higher than 70 % became possible.

L-Malic acid synthesis

Whole-cell biocatalysis was performed in flow in a commercially available plastic tube with permeabilized *Saccharomyces cerevisiae* cells.[67] The modification was carried out by using 3-aminopropyltriethoxysilane and glutaraldehyde. Such low price tagged, disposable and easy to use whole-cell microdevices have their promises, e.g. might lead to rapid optimization of the treatment time for permeabilization, pH of the medium and kinetic data gathering. The synthesis of L-malic acid by hydration of fumaric acid was tested. The optimum time for permeabilization and pH of the medium are 6 min and pH-7, respectively. The volumetric and biocatalytic productivities of the microreactor were determined, and were found to be 616 mM day^{-1} and 11.8 mmol $g^{-1}_{\text{ww of cells}}$ day^{-1}, respectively.

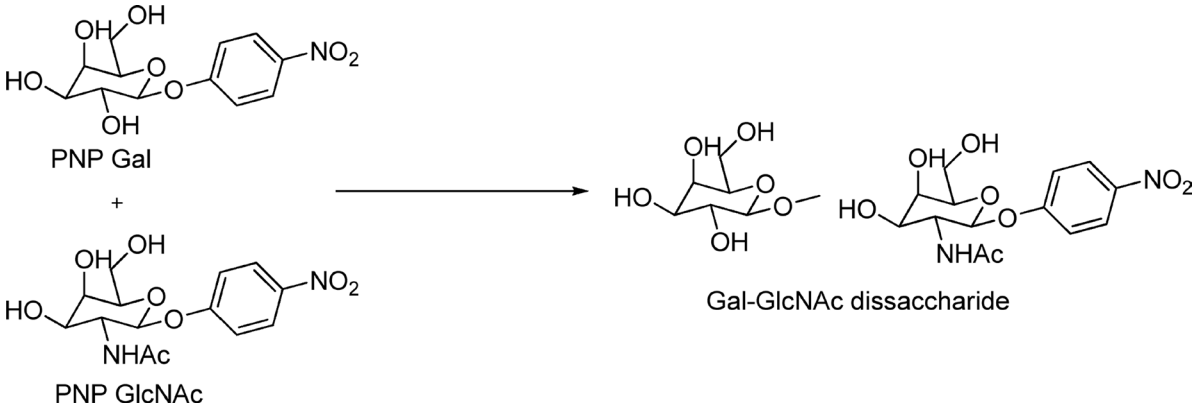
Oligosaccharides

As one of the first developments in the field of biotechnical micro-flow technology for biomass platform synthesis, a silicon microreactor was used for the enzymatic oligosaccharide synthesis.[68–70] The authors reported for the first time the synthesis of higher oligosaccharides in a micro-device with microchannels 200 μm wide, 200 μm deep, and 40 cm long. The reaction between *p*-nitrophenyl-*β*-D-glucopyranoside (PNPGal) and nitrophenyl-2-acetamide-2-deoxy-*β*-D-glucopyranoside (PNPGlcNAc) was performed using *β*-galactosidase (from *Escherichia coli*) (**Scheme 9**).

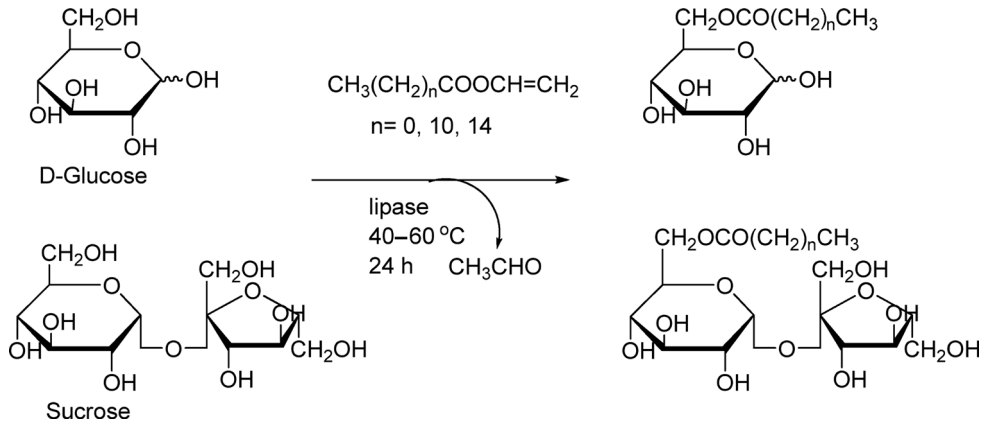
The obtained disaccharides with nitrophenyl group represent an important class of glycoconjugates. The enhancement of the reaction rate compared to the micro test tube is explained by the efficient diffusive mixing obtained within the microchannel. The microreactor synthesis enabled also rapid reaction optimization and faster development of important products such as carbohydrate based vaccines, while batch optimization was assigned much more time consuming and costly.

Sugar 6-monoesters

Sugar 6-monoesters were synthesized from sugar and vinyl carboxylate in a micro-flow reactor using Lipozyme TL IM (from *Thermomyces lanuginosus*)[71] (**Scheme 10**). The processing was done under mild reaction conditions, short reaction times (30 min), high yields and high regioselectivities.



Scheme 9 – *Transglycosylation between p-nitrophenyl-β-D-glucopyranoside and nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside using β-galactosidase*



Scheme 10 – *General approach to give sugar 6-monoesters from sugar-vinyl carboxylate by transesterification*

Lactulose synthesis

Lactulose, non-digestible galactooligosaccharide, 4-*O*-*β*-D-galactopyranosyl-D-fructose were synthesized in a microreactor with immobilized beta-galactosidase.[72] The oxygen plasma-manufactured glass microreactor comprised a 13-mm-long trench structure with 12 channels of 390 μm width and 150 μm depth. The lactulose synthesis was conducted for 48 h at a flow rate of 2.5 μL min^{-1} and using a concentration of 1.29 g L^{-1}. Increase of the flow rate can influence lactulose synthesis, because of the shorter reaction time for the lactose hydrolysis and the transgalactosylation reaction of galactose.

Polymers beyond oil

Polycaprolactone

The synthesis of polycaprolactone was done using 100 mg of Novozym 435 beads (400 +/– 50 μm) filled into a microchannels 2 mm wide, 1 mm deep and 260 mm long.[73] The microchannels were mounted on a 10 mm thick aluminum block which served as heating sink. Polycaprolactone is an important biodegradable polymer used in the manufacture of specialty polyurethanes and in biomedical applications. The enzymatic process represents a considerable sustainability advantage over the commonly used organic tin-based catalyst in the ring opening polymerization reaction of caprolacton. The tin catalyst is highly toxic and difficult to dispose. Yet, there are also drawbacks of the enzymatic process issues which relates to catalyst stability, leaching and deactivation. The conversion rate in the microchannel reactor is higher. 90 % conversion is obtained within a residence time of 5 min, while the batch system gives 70 % conversion in 30 min. This is mainly due to the much higher catalyst concentration per unit volume of the millireactor compared to the batch reactor. The micro-flow processing provides a small molecular weight distribution which is assigned to good control of process conditions achievable in flow, in particular concerning temperature and reaction time. This microreactor platform can be easily extended to be used for efficient enzymatic synthesis of various biopolymers.

Conclusions

Enzymatic micro-flow reactors will follow the enormous prospects that enzymes and biotechnology generally have. In the just recently formed, yet strongly emerging bio process engineering they are likely to play a key role. Compared to the EDGE classification given above, the whole development is still in the "storming" phase. It is much technology-driven and the market pull is hardly feasible. The devices of today may not be the devices of tomorrow as it has been observed for their "older" counterparts – the chemical microreactors. These certainly have reached the "norming" level and partly the "performing" level which relates to applications and market readiness, respectively. Decisive will be the uptake of the new technology by industry and the emerge of first commercial providers. There will be external stimulus as well, as given by the increasing capabilities of engineering enzymes and by the emerging bioeconomy. Thus, with having passed the "forming" phase, for which this CABEQ Special Issue of the IMTB Conference is a powerful document, an opening of the technology to impacts of other disciplines is needed. Here the bio process engineering is urgently needed. While the reactor engineering is often relatively simple, e.g. with functionalized monoliths being filled in tubes, the engineering on a process and production level will give a new momentum and also a smart process control may be required.

Outlook – What's next?

Keep speed in innovating

As mentioned in the Conclusions a large innovation push is needed for the next years in development of enzymatic micro-flow reactors. It is needed to merge to the flow chemistry community and openness for engineering solutions is needed. Cost arguments so far have hardly been given with very few exceptions.[14,35,52] Benchmarking to industrial technology is seldom and the same holds for real-life applications. Speed in innovation needs to be maintained and the innovations needs to weighted and selected by process-intensification and finally cost criteria.

New grounds – controlled drug release and tissue engineering

The multidisciplinary opening does not demand only for more innovations, but also new market directions which simply are open for the new technology. Controlled drug release and tissue engineering are new windows of opportunity; yet certainly it is uncertain if this will be a main future direction. In one such example, surface-adhered hydrogels equipped with enzymatic cargo served as enzymatic microreactors with vision for application in controlled drug release and tissue engineering (Fig. 16).[74] Polyvinyl alcohol (PVA) chains with narrow polydispersities (1.1−1.2) and molecular weights of 5, 10, and 28 kDa were synthesized via controlled radical polymerization (RAFT). The substrate-me-

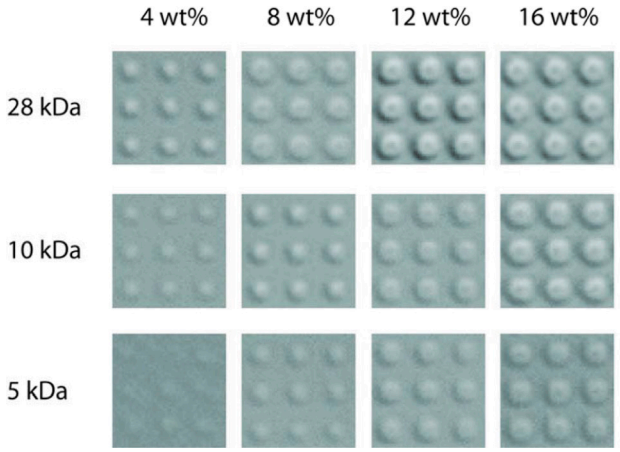


Fig. 16 – *Differential interference contrast micrographs of surface-adhered microstructured PVA hydrogels prepared using polymers with varied molecular weights and made from solutions with a polymer content (by permission of American Chemical Society[74])*

diated in-situ conversion of an externally added prodrug into a final product was monitored as a function of the polymer molecular weight and concentration of solution taken for assembly of the hydrogels. The PVA molecular weights have to be suitably high to give mechanically robust hydrogel matrices, yet need to be suitably low to allow gradual degradation of the hydrogels since biodegradability is an issue for the applications under consideration.

From enzymes to whole cells in flow – fermentations/cell cultivations

Schäpper *et al*. have reviewed the advantages of the microbioreactors over conventionally lab technology used in the field of fermentations/cell cultivations such as use of shake flasks and microtiter plates. The latter have limitations for reasons of most of the times only end-point measurement and non-ideal control on cultivation conditions. The use of microbioreactors can lead to more supple and well-regulated operation at bench scale in the mentioned field. A midterm outline on the development of microbioreactors is provided, coming to the main conclusion it will be ready to be used in the fermentation industries on such timescale.[75]

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