Invited Paper

Microbioreactors for Cell Cultures: Analysis, Modeling, Control, Applications and Beyond

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Abstract: *Microbioreactors offer many advantages over macro-scale reactors for the screening and cultivation of microbial cultures. However, some weaknesses of microbioreactors are yet to be overcome. Optimum mixing under laminar flow, miniaturized on-line measurements and control, integration of upstream and downstream microdevices with the microbioreactor on a single chip, and adequate mathematical models to optimize the performance are some major issues that need to be resolved. Nevertheless, recent research indicates rapidly growing understanding of these problems and it has presented some promising solutions that have led to useful commercial applications. While specialized reviews have covered specific aspects of microbioprocesses exhaustively, the present article reviews microbioreactors per se in a holistic way, analyzing their functions both in a stand-alone setting and in conjunction with other microdevices. We also consider technological developments arising out of the standard microbioreactor and its possible future ramifications.*

Keywords: *Microbioreactors, Cell cultures, Performance analysis, Modeling and control, Integrated microprocesses, Applications, Future developments.*

Introduction

Microbial cells are cultivated in different kinds and sizes of vessels, under varied conditions and for a variety of purposes. The vessels (or reactors) used for cell cultures may vary from as large as a few hundred liters in industrial production through tens of liters for pilot scale bioreactors to about a liter or less for bench-scale reactors. Reactors of these sizes are topically called *macro-scale* bioreactors to distinguish them from the more recent *micro-scale* bioreactors (or microbioreactors). Microbioreactors differ from large reactors not just in their sizes but in more significant features such as fluid mixing, heat and mass transfer, control methods, fabrication technologies and performance. These aspects have been discussed in different degrees of detail in recent reviews [1-4]. Each of these has analyzed certain aspects in depth, and possibly to maintain the focus of the review and a reasonable length, touched upon other features in a more superficial manner. The present chapter attempts both to introduce microbioreactors to readers not familiar with them and collates all significant information from the reviews as well as some original sources to present a comprehensive account of the subject.

Both macroscale bioreactors and microbioreactors try to create conditions that mimic *in vitro* as closely and reproducibly as possible the *in vivo* microenvironment of the cells that

are being grown. Replication of the cellular environment is not easy because the performances of living cells in their native habitats involve "a complex set of physical, chemical and biological conditions that surround the cells" and enable (them) to function efficiently [3]. The larger the reaction vessel, the more difficult it is to mimic ideal conditions faithfully and over practically useful stretches of time. This makes it difficult to cultivate microbial cells efficiently in large bioreactors, more so for sensitive cells such as hybridomas and mammalian cells. Large bioreactors have other drawbacks also. They are (i) bulky and hence it is difficult to maintain a homogeneous state in the entire reactor volume, (ii) labor intensive and expensive to operate and maintain because of the repeated requirements of cleaning, assembling and sterilizing, (iii) costly and impractical for high-throughput screening because many batches have to be run in parallel, and (iv) not suitable where significant heat and/or mean transport are involved [3, 4].

Micro-scale bioreactors overcome these limitations for many reasons. Their small sizes (typically 50 µl to 0.1 ml [5-7]) result in large surface-to-volume ratios, thereby enabling efficient transport of heat and mass between the cells and their microenvironment. Another significant advantage of smallness is that many reactors can be operated simultaneously under different conditions at reasonable costs. This makes microbioreactors eminently suitable for high-throughput screening studies, whereby the performances of a large number of cultures can be determined simultaneously. High-through screening has been exploited to great advantage by the pharmaceutical industry in the design and selection of new drug molecules or the modification of existing molecules. Other benefits that accrue from the sizes of microbioreactors include low power consumption, less space requirements, small quantities of reagents and cells per batch and portability.

The nature of fluid mixing is an important feature that distinguishes microbioreactors from their larger counterparts. To illustrate this, we note that a rectangular deep channel microreactor used by Sotowa et al. [8] in an industrial setup had a channel width of 0.1 mm, depth of 400 mm and an aspect ratio of 4000. Owing to the small cross-section area and the large aspect ratio, flow through the channels in such reactors is in the laminar range and largely diffusion-controlled [9, 10]. The absent of turbulent mixing allows microbioreactors to be applicable for shear sensitive cells but it also implies that axial gradients may make it difficult to obtain a spatially and temporally uniform output. This weakness is alleviated by introducing controlled mixing that still maintains laminarity. Methods to active this are described in Section 4.

It might seem paradoxical that despite their small sizes, microbioreactors are considered to be good candidates for high-throughput operations. The solution to this apparent paradox lies in the so-called "numbering-up" approach, i.e. many microreactors are stacked in a bundle such that their combined output is large enough to produce an economically viable process. Numbering-up is a useful concept for production-scale reactors but it is not suitable for large-scale screening because individual microbioreactors may require different operating conditions. Even when the same product in desired from all the microbioreactors, maintaining nearly identical environments for all of them requires careful distribution and control of the external fluid environment. Some methods to achieve this are outlined in Section 4.

A complete biotechnology process requires one or more bioreactors to receive processed feed streams and generate outlet streams that require further processing to obtain either the biomass or a desired product in the required purity. This interlinking of upstream and downstream units with the bioreactor is easier and less expensive with microbioreactors than with larger vessels. Early versions of microbioreactors coupled them to macroscale processing units such as PCR amplification devices, capillary electrophoresis, cell cytometers, ELISA apparatus and mass spectrometers [11, 12]. However, recent developments in fabrication technologies have enabled micro-scale upstream and downstream units to be positioned on a single chip with the microbioreactor itself. This level of miniaturization allows all processing

units and their layout to be so designed that smooth synchronous operation is readily achieved. This was difficult when microreactors were coupled to micro-scale equipment because their time constants and measurement and control methods were often widely different.

Like any new equipment or experimental method, microbioreactors too have weaknesses and difficulties. Although laminar flow protects the cells from damage due to shear forces, it also creates nonuniformity, introduces diffusion resistances and allows free cells to settle down and thus become nonprofitable for product formation [13]. From a more direct practical perspective, microbioreactors are susceptible to leakages at the junctions between interconnecting fluid streams, have limitations on accurate on-line monitoring and control of a large number of reactors operating simultaneously ("numbering-up"), evaporation losses, which can be significant even when small quantities of liquid are lost as vapor, and possible dilution of reactor contents upon addition of acid or base to maintain a particular pH [2, 4, 14]. Nevertheless, new developments arising out of both laboratory research and industrial uses are helping to overcome many of these limitations, thereby promoting increasing preference for microbioreactors for processes which have conventionally been carried out in large bioreactors.

Physics of microfluidics

All microfluidic devices exploit the physics of fluid flow through microcapillary channels. However, most reviews [1-4] do not discuss the underlying physics that determines how successful a particular application will be. Here we will review this aspect briefly, leaving some details undiscussed, either because they are too complicated and not relevant to the practical understanding of microbioreactors or because they are discussed in specialized articles [15, 17].

A basic understanding of the physical phenomena associated with microfluidic flow may be obtained through certain dimensionless numbers that characterize these flows (Table 1).

Table 1. Main dimensionless numbers used to characterize fluid flow through microtubes [17]

Dimensionless number	Symbol	Definition	Physical significance
Reynolds	Re	$\frac{\rho u_0 L_0}{\eta}$	$\frac{\text{inertial forces}}{\text{viscous forces}}$
Peclet	Pe	$\frac{u_0 L_0}{D}$	$\frac{\text{convection forces}}{\text{diffusion forces}}$
Capillary	Ca	$\frac{\eta u_0}{\lambda}$	$\frac{\text{viscous forces}}{\text{interfacial forces}}$
Weissenberg	Wi	$\tau_p \gamma$	$\frac{\text{polymer relaxation time}}{\text{shear rate time}}$
Deborah	De	$\frac{\tau_p}{\tau_{flow}}$	$\frac{\text{polymer relaxation time}}{\text{flow time}}$
Elasticity	El	$\frac{\tau_p \eta}{\rho h^2}$	$\frac{\text{elastic effects}}{\text{inertial effects}}$
Grashof	Gr	$\frac{\rho u_b L_0}{\eta}$	Re for buoyant flow
Rayleigh	Ra	$\frac{u_b L_0}{D}$	Pe for buoyant flow
Knudsen	Kn	$\frac{\beta}{L_0}$	$\frac{\text{slip length}}{\text{macroscopic length}}$

The Reynolds number, Re, is fundamental to both macroreactors and microreactors, but for different reasons. It expresses the ratio of inertial forces to viscous forces:

$$\mathrm{Re} = \frac{\text{inertial forces}}{\text{viscous forces}} = \frac{\rho u_0 L_0}{\eta} \quad (1)$$

Whereas Re can differ widely for large bioreactors, it is small enough in microbioreactors so that inertial effects may be neglected. Typically, for aqueous media Re ranges for *ca.*10^{-6} to *ca.*10, indicating that the fluids move in strongly laminar linear Stokes flows.

Since flow in a microbioreactor is driven by viscous forces, it is strongly diffusion-controlled. Different kinds of mixing devices have therefore been designed to enhance diffusion rates and improve the mixing of two or more fluids. These are discussed in Section 4. The basic physics of diffusion-mediated mixing is illustrated by the simple T-mixer (Fig. 1 [18]).

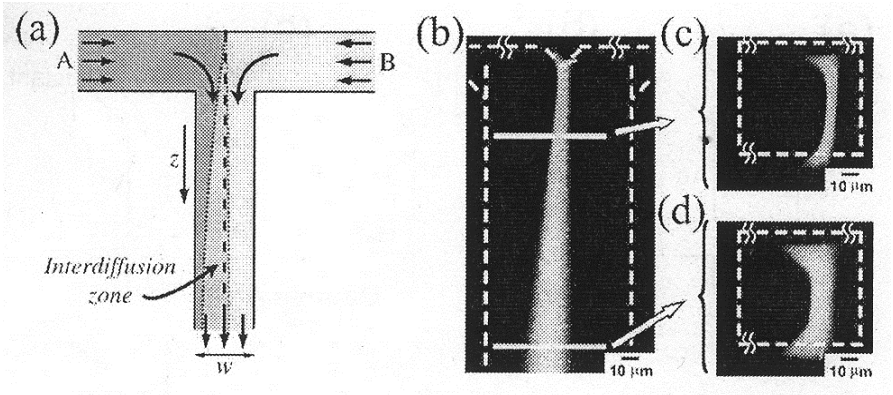

Fig. 1 (a) Schematic diagram of a microfluidic T-sensor; reprinted from Kamholz et al. [242] with permission from American Chemical Society, Washington DC ©1999;
(b) Pictorial depiction of the no-slip nature of the top and bottom walls that affects the flow profile. Reprinted from Ismagilov et al. [243] with the permission of American Institute of Physics, College Park, MD ©2000.

Three regimes of operation are possible, according to the value of the Peclet number, Pe:

(i) Diffusion-dominated mixing

This occurs when the flow rate u_0 is small, and hence

$$\mathrm{Pe} = \frac{\text{convection}}{\text{diffusion}} = \frac{\mu_0 h}{D} << 1$$

(ii) Taylor dispersion-mediated mixing

At very large flow rates (Pe >> 1), Taylor dispersion becomes the controlling factor in axial mixing. The mixing time, τ_{TD}, is then

$$\tau_{TD} \approx \mathrm{Pe}^{-2}\,\tau_R \quad (3)$$

where τ_R is the tracer diffusion time constant. While Pe should be much greater than unity, it is limited to $2\pi R/h$, where R is the radius of the mixer.

(iii) Convection-controlled mixing

For Pe >> $2\pi R/h$, i.e extremely rapid flow, both diffusion and Taylor dispersion are sufficiently fast and mixing is then controlled by convection currents. The convection time constant is:

$$\tau_{con} \approx \mathrm{Pe}^{-2/3}\left(\frac{\tau_R}{h}\right)^{2/3}\tau_D \approx \mathrm{Pe}^{-2/3}\left(\frac{h}{\pi R}\right)^{4/3}\tau_R \quad (4)$$

Sometimes a much greater degree of mixing is required than is possible by conventional stirring, as with viscous liquids. C*haotic advection* [19] is one method of achieving this; the staggered herringbone mixer [20, 21] works on this principle (Fig. 2). Chaotic flow systems too may have ordered regions similar to the dead pockets in large stirred bioreactors. Since the fluid in these regions is poorly mixed, they should be minimized. The forgoing discussion applies to miscible fluids. Often however, two- or three-phase systems are used. Then other factors, notably surface tension, govern droplet formation and performance. Primarily there are two competing stresses: surface tension tends to reduce interfacial area, and viscous stresses try to extend and drag the interface downstream [17]. Balancing these two stresses determines the resulting radius of a droplet:

$$R \approx h/\mathrm{Ca} \tag{5}$$

where Ca = $\eta u_0 / \gamma$ is the Capillary number, η is the viscosity of the two-phase mixture and γ is the interfacial tension.

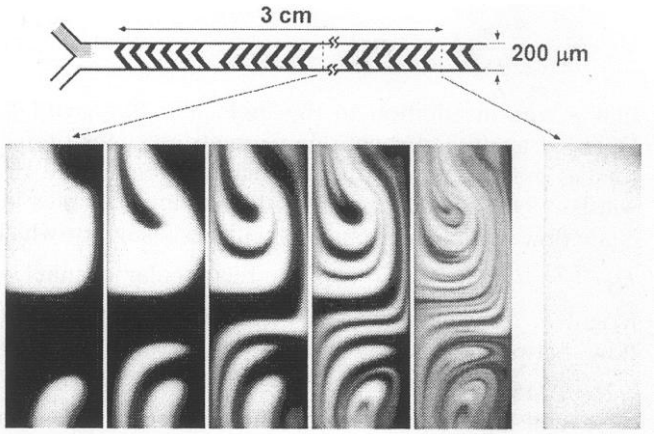

Fig. 2 Continuous flow staggered herringbone mixer (upper figure) and photographs of tracer distribution at successive times that show diffusive mixing.
Reprinted from Stroock et al. [115] with permission from American Association for the Advancement of Science, Washington DC ©2002.

Since the flow is linear and deterministic in a microchannel, it is possible to generate monodisperse droplets more easily in microbioreactor than in a large macro-bioreactor, where there is usually a distribution of drop sizes owing to breakage and coalescence [22].

For viscous liquids, e.g. polymer solutions, elasticity becomes a significant factor in addition to inertia, diffusion and surface tension. The flow of such liquids through microtubes is characterized by two other dimensionless numbers. The Weissenberg number relates the (polymer) relaxation line to the flow deformation time:

$$\mathrm{Wi} = \tau_P e^{-1} \text{ or } \tau_P \gamma^{-1} \tag{6}$$

where e is the extension rate and γ the shear rate.

The Deborah number

$$\mathrm{De} = \tau_P / \tau_{flow} \tag{7}$$

expresses the relative rates of relaxation and flow. Here $\tau_{flow} = L_0/u_0$ for linear flow and $\tau_{flow} \approx \omega^{-1}$ for oscillatory flow, where ω is frequency of oscillation.

Apart from phase differences and differences in surface tension, elasticity, etc., two fluids may also differ in their densities. Then gravitational forces need to be considered. For stable flows in such situations, viscous forces should balance buoyancy forces; this results in a buoyancy velocity scale

$$u_0 \approx \alpha_c \rho_0 \Delta c g h^2 / \eta \quad (8)$$

where α_c is coefficient of thermal expansion, ρ_o is reference density, c is solute concentration differential, h is characteristic dimension, g is gravitational force, η is viscosity of the mixture.

Given u_0, the Rayleigh number expresses the ratio of convective flow to diffusive flow:

$$\mathrm{Ra} = u_0 h/D \quad (9)$$

where D is the diffusion coefficient. When Ra << 1, diffusion dominates over convection; alternately, for Ra >> 1 the flow is fast and a gravitational current of each fluid penetrates the other [17]. For very fast flow, inertial forces become significant compared to viscous forces, and their ratio is measured by the Grashof member:

$$\mathrm{Gr} \approx \rho_0 u_0 h/\eta \quad (10)$$

Segments of the foregoing theory form the basis of many important biological applications of microfluidics, even though this is often not explicitly recognized. Microfluidic protein crystallization is based on free-interface diffusion, which requires both Ra and Gr to be much smaller than unity. In one approach to a polymerase chain reaction (PCR) to replicate and amplify DNA, buoyancy flows at large Ra values are employed to perform PCR at a constant temperature without an external pump [23].

As with large bioreactors, microbioreactors too may require the inflow of gases, notably air or oxygen, to promote metabolic reactions that are aerobic. Then we have gas-liquid flow through microcapillary tubes, and inter-phase mass transfer may become a rate-controlling factor. The Knudsen number quantifies this phenomenon, and it is defined as:

$$\mathrm{Kn} = \lambda_f / L \quad (11)$$

where λ_f is the mean free path length of the gas molecules and L is a characteristic length scale of the microtubes. For Kn <<1 the gas behaves as a non-slip fluid, for Kn ~ 1 it behaves as a continuum but slips at the boundaries, and Kn >> 1 the continuum approximation breaks down [24]. Fluid slip at a boundary generates interesting possibilities which will be discussed later.

Types of microbioreactors

Microbioreactors may be classified in a manner similar to macro-scale bioreactors but, as explained above, for any particular type of reactor a microtubular array functions very differently from a single macrobioreactor or an array of such reactors.

Shake flasks and microcell plates

Most biotechnological studies begin with experiments in shaken flasks since these are cheap, easy to operate and hence can be run in a pool of several flasks operated simultaneously. In a sense, shaken flasks, and more recently micro-cell arrays, have been the work-horse of process development to the extent of accounting for over 90% of all cell culture experiments [25]. Shaken flakes are also popular because of their versatility, being usable for bacteria [26], fungi [27], yeast [28] and mammalian cells [29].

Offshoots of the traditional array of shaking flasks are the microcell plates. These are more compact units that typically contain 48 or 96 cells, each of which is a miniature shaken bioreactor. Since a microcell plate is more compact and better automated than an equivalent set of flasks, it is possible to align a number of plates together so that hundreds of small-scale experiments can be performed simultaneously. Microcell arrays have therefore been successful for the high-throughput screening of cultures [30], for microbial fermentation optimization [31], demonstrations of proof of concept [32] and also for automated whole bioprocess sequences [33]. Micheletti and colleagues [34] extended these studies to evaluate scale- up possibilities

from microliter-scale culture experiments. Promising results were obtained for two systems: (i) an automated micro-scale process (1000 µl) for the aerobic fermentation of *Escherichia coli* JM107:pQR706 overexpressing transketolase, and (ii) antibody production in suspension cultures (800 µL) of VPM8 hybridoma cells.

Microcell plates overcome one major limitation of shaken flasks, the absence of sufficient automation; both suffer from low aeration rates since both rely on surface aeration and not on bubbling of gas through the culture both. However, relatively better oxygen transfer rates to the culture are possible in microcells, this being directly proportional to the shaking amplitude and frequency and inversely proportional to the fill volume [35]. The advantages of microcell plates over shaken flasks has led to the development of large arrays of microcells, going up to 1536 and 3456 wells per plate for industrial use [36].

While their small sizes make microcell arrays attractive for conducting a large number of parallel exploratory experiments, this is also a limitation because evaporation of even small amounts of liquid can significantly reduce the volume of material in a well. One solution is to place breathable membranes on the plates [37] but this also reduces the oxygen transfer rates. An alternative is the use of spin tubes, but microliter scale spin tubes are not yet well developed, thus limiting their current use for high-throughput experiments. Present sizes are of 1 mL to 5 mL; even these relatively large tubes have been useful for slow-growing mammalian cells [38].

Miniature stirred bioreactors

Just as laboratory-scale bioreactors are the next logical step from shaken flasks, microbioreactors follow the studies with microwell plates. However, unlike normal bioreactors which are usually of 1-10 L size microscale devices have volumes in the µL to mL range. They are usually made from Perspex, Pyrex, polymethylmethacrylate (PMMA) or stainless steel [39, 40]. Sometimes more than one kind of material may used, such a as PMMA for the main body and stainless steel for the joints and connections.

This reactor generates better mixing and oxygen transfer from gas to liquid than is available with shaken flasks and microcells. Both features can also be controlled more effectively, thus allowing more accurate studies of microbial behavior under different process conditions. These microbioreactors are usually of the stirred type although bubble column reactors (described next) are also used for certain applications. Stirred reactors have been used for a wide variety of organisms and bioprocesses, such as *Saccharomyces cervisiae* for ethanol production [41] cultivation of the microalgae [42], and the traditional cultivation of *E. coli* [43]. In the last application, Lee et al. [43] demonstrated long-term cultivation of *E. coli* up to 500 h in an integrated system that combined rigid materials with polydimethylsiloxane (PDMS) membranes to minimize evaporation losses and reliable flow control without concentration drift. Acetate formation remained within tolerable limits, in spite of such long operations, a significant advantage are larger bioreactors.

Despite avoiding high agitation rates, mass transfer rates in stirred microbioreactors are superior to those in both shaken flasks and large bioreactors. Typical k_La values reported are between 360 h^{-1} [44] and 1650 h^{-1} [39], for *E. coli* cultivations, depending on the flow rate, stirring speed and method of measurement. The latter device, designed in association with an industrial partner (HPP Labortechnik AG, Oberschleissheim, Germany), is an integrated unit than can handle up to 48 cell-cultivations simultaneously, thereby accelerating process development studies significantly. The industrial value of miniature stirred bioreactors is further underscored by a second application, in which Flowrometrix Corporation (Stow, Massachusetts, USA) developed a multi-vessel unit that permits *in situ* on-line monitoring of up to 12 parallel cultivations. Its long-term usability (for over 70 h) has been demonstrated for a mammalian cell culture process [45], thus encouraging improvement of the design to a 24-well platform [46].

Other companies have concentrated on the development of platforms with large microbioreactors to promote studies that mimic industrial systems more closely and enable process parameters to be varied more accurately. These devices include Dasgip AG's (Villach, Germany) 16- vessel unit with each reactor having a working volume of 200-275 mL [47] and another from Infors AG (Bottmingen, Switzerland) that comprises 4 to 16 chambers, each of 100 mL working volume and integrated with dissolved oxygen (DO) and pH sensors.

Miniature bubble column reactors and novel designs

In some situations, such as microbial systems in which the cells are sensitive to shear or systems that involve viscous solutions or suspensions, stirring may not be a good method to provide adequate mixing and/or gas transfer to the broth. Then bubbling of air or an inert gas is preferred. In such miniature bubble column reactors, the gas is sparged upward from the bottom through a fine mesh or a sintered porous plate so as to insure uniformly distributed small bubbles. It may be recalled here from Section 2 that the linear laminar flow and small diameters of bubble column microbioreactors make it easier to generate and maintain a uniform distribution of bubbles than in large columns.

A prototype 12-microwell bubble column microbioreactor with a working volume of 2 mL has been described by Doig et al. [48]. k_La values comparable to those in equivalent stirred reactors were achieved. The lack of agitation implies that oxygen transfer is easier to model than in stirred microbioreactors and there are fewer design parameters, thus enabling easier scale-up/scale-down [49]. Earlier investigators designed and tested somewhat larger microreactors with gas sparging. Many of these devices have been described by Hessle et al. [50]. However, they were one or two orders of magnitude larger than that of Doig et al. [48], thus limiting the extent of parallel processing possible.

Some novel bubble column designs have emerged in recent years. One such is the falling biofilm reactor employed by Villena and Butierres-Correa [51] to study cellulose production by *Aspergillus niger*. They reported that cellulose activities and volumetric productivities with biofilms grown in shake flasks were 70% higher than by freely suspended mycelium, and this advantage increased to three fold (i.e. 300%) when the biofilms were grown in microbioreactors. Two other unconventional types of bubble column microreactors have been discussed by Jensen [52], who has also described a similar integration of a membrane reactor that allows simultaneous chemical reaction and product separation. To enable the rapid determination of reaction kinetics, McMullen and Jensen [53] recently fabricated an automated spiral microreactor of 120 μL volume and coupled it to an HPLC. The spiral design makes the microreactors usable for reactions with a solid phase, thus enabling kinetic studies with porous pellets and slurries.

The types of microreactors described here certainly do not exhaust all available configurations but they do cover most of the major types. Indeed, new configurations emerge frequently as new technologies develop and new applications are perceived.

Mixing in microbioreactors

Similar methods to promote mixing apply to microreactors for both biological and nonbiological processes. Thus the methods reviewed here are common to both. This overview is intentionally brief because excellent detailed reviews of mixing alone in microreactors have appeared recently [54-57].

Unlike large bioreactors, even of the stirred kind; flow in microchannels is usually laminar, Re being usually in the range 0.01 to 10 [3, 13]. Hence turbulent mixing is absent and the main driving forces are diffusion [18], chaotic advection [19, 20] and Taylor dispersion. Depending on whether or not the cells can endure shear forces, either molecular diffusion or chaotic advection is employed, Taylor dispersion being an extension of the former. Aref [19]

highlighted one important difference between the words "mixing" and "stirring", which are sometimes loosely interchanged. Stirring refers to the act of creating mixing, the latter being the phenomenon itself. Pasirayi et al. [3] pointed out two basic approaches to achieve micromixing. In *active mixing* energy is added to a system and external forces, not necessary physical, are employed to stir and mix. Electrokinetic, ultrasonic, magnetic, thermal, petistaltic and piezoelectric mixers are all of this kind [58-61]. This method may not be suitable for delicate shear-sensitive cells or tissues. In such situations *passive mixing* in preferred; the principle is to use channel geometry and the energy provided by the flow to stir, stretch and fold the liquid elements to as to increase the interfacial area over which molecular diffusion occurs. Several ingenious designs have been reported for microchannels and fluid contacting models to promote passive micromixing. Some significant representative examples are considered here.

Active mixing

Table 2 summarizes the main methods of active mixing. Some of them are novel and some ideas are implementations of similar methods employed successfully for large bioreactors.

Table 2. Comparative data of the recent performances of some active micromixers [57]

Category	Mixing technique	Mixing time (ms)	Mixing length (μm)	Mixing index
Acoustic/ Ultrasonic	Acoustically driven side-wall trapped microbubbles	120	650	0.025
	Acoustic streaming induced by surface acoustic wave	600	10 000	0.9
Dielectrophoretic	Chaotic advection based on linked twisted map	--	1000	0.85
Electrokinetic time-pulsed	Chaotic electric fields	100	Width*5	0.95
	Periodic electroosmotic flow	--	200	0.88
Electro-hydrodynamic force	Staggered herringbone	--	825	0.2
	Staggered herringbone	--	2300	0.5
Thermal actuation	Thermal	--	600	--
Magneto-hydrodynamic flow	High operating frequency	1100	500	0.977
Electrokinetic instability	Low Reynolds number	--	1200	0.98
	Low Reynolds number	--	1500	0.98

The use of high frequency is conceptually analogous to the use of ultrasonication to break the cells in large bioreactors so as to recover certain intra-cellular products Liu et al. [60] used a mixer comprising a piezoelectric disk attached to a microreaction chamber. The chamber was so designed that air bubbles of a specified size were trapped in the solution, and their circulation promoted mixing. The time to mix fully the contents of a 22 μL microreactor was reduced from several hours (for pure diffusion based mixing) to tens of seconds.

Experiments with *E. coli* K12 suspended in blood showed that this kind of "acoustic mainstreaming" [60] was also the basis of Ahmed et al.'s [62] micromixer, which trapped air bubbles of a prescribed size within pre-designed grooves on the sidewalls of the microchannel. When acoustically driven, liquid/air interfaces of the trapped bubbles oscillated, thereby creating strong pressure and velocity fluctuations, resulting in fast and homogenized micromixing. For the mixing of deionized water and ink, the mixing time was just about 120 ms. Similar low mixing times have also been reported by Long et al. [63] for a different system, thereby demonstrating the versatility and effectiveness of this method.

It may be recalled that chaotic advection is one of the most effective methods of inducing micromixing [19-21]. Two studies illustrate the use of electric fields to generate chaotic advection. Deval et al. [64] presented a dielectrophoretic mixer that "induces chaotic trajectories of embedded particles through a combination in space and time of electrical actuation and local channel geometry variation". More recently, Campisi and coworkers [65] designed a soft-lithographed micromixer that generated chaotic advection as an implementation of a linked twisted map. The two liquids to be mixed were electrokinetically driven by generating rolls through AC electro-osmosis. While mixing times have not been reported, the instrument was effective over wide ranges of frequency (10-100 kHz) and voltage (15-20 V).

Similar to the dielectrophoretic apparatus described above, pulsed electric currents have also been employed to promote chaotic micromixing. The driving force here is the zeta potential distribution, which may be exploited to create complex flow patterns that are conductive to intimate micromixing. Wu and Li [66], for example; explored the effect of induced-charge electrokinetic flow in a rectangular microchannel with embedded conducting hurdles. Numerical simulations showed flow circulations generated by an induced non-uniform zeta potential distribution. This was experimentally validated using PDMS microchannels with embedded Pt hurdles. Chen and Cho [67] induced chaotic advection via four electrodes mounted on the lower and upper surfaces of the microreactor. Chaotic oscillating electric potentials were designed through simulations, which induced complex flow circulations that created mixing efficiencies of up to 95% of perfect mixing. Lim et al. [68] improved mixing in T-type micromixers by introducing a constriction in the microchannel under periodic electro-osmotic flow. The degree of mixing was maximized by optimizing the amplitude and frequency of the AC electric field and the length of the constriction. This observation and that of Wu and Li [66] are compatible with Kang et al.'s [69] numerical evidence that for every system there exists an optimum period of electric modulation to generate the best time-varying zeta potential.

Analogous to the time-varying electrical pulses used by dielectrophoretic methods is the introduction of kinetic instabilities by electrical means. In Shin et al.'s [70] work, a time-periodic electric field was applied to excite instability in a micro-channel. They found that instability was most pronounced when the period of the applied field was close to half of the period of the kinetic instability, and consequently the degree of micromixing was the highest. Chun and coworkers [71] also generated a periodic electric field through a pair of positively charged polyelectrolyte gel electrodes (pPGEs) connected to an external AC signal source. Periodic reversal of the direction of charge flow created alternating depletion-enrichment regions, thereby generating instability and mixing. The method efficiently lysed human red blood cells with little damage of the white blood cells.

Electro-hydrodynamic and magneto-hydrodynamic methods to promote micromixing work on principles similar to those just described. In Zhang et al.'s [72] simulated study, electrical pulses aided the periodic oscillations of fluid in a microchannel that were caused usually by grooves running along the length of the channel. The electric field thus supplemented the mixing created by the groove-generated helical flow. Significant improvements were observed with a T-mixer. Du et al. [73] applied the same principle to a staggered herringbone mixer (SHM), and computed the length of microchannel required for complete mixing, L_m, for different concentrations of two fluids being mixed. The computed values of L_m agreed well with experimental values, thus underlining the usefulness of a simple parameter such as L_m to characterize hydrodynamic mixing in a SHM. Magneto-hydrodynamic methods rely on the motions of magnetic particles to enhance further the mixing created by channel design itself. The motions of the particles may themselves be generated by electric feeds, thus illustrating the analogy with electro-hydrodynamics. For instance, Gleeson and West [74] employed AC current to churn the magnetic particles in such a way that two fluids that initially flow in separate semi-circular halves of a microreactor are forced to mix with each other.

Through similar experiments, Wang and associates [75] observed that mixing efficiency was maximized at an optimum AC frequency, which depended on the size of the magnetic particles and the diameter of the microchannels. These observations were further corroborated by Wen et al. [76] in their studies of the micromixing between magnetic nanoparticles suspended in water (i.e. ferro-fluids) and Rhodamin B. By optimizing the electrical frequency, mixing efficiencies up to 95% could be obtained within 2.0 s and a distance of 3.0 mm from the inlet.

A non-electrical non-hydrodynamic method that also achieves significant chaotic advection creates pressure perturbations within the fluid streams to be mixed by pulsing the velocities of the streams [77]. In a typical device (Fig. 3) the mixer has one main channel and a number of side channels. The streams flowing in through the side channels are pulsed; as a result, pressure fluctuations induced in the main channel create stretching and folding of the fluids, which in turn generate chaotic advection.

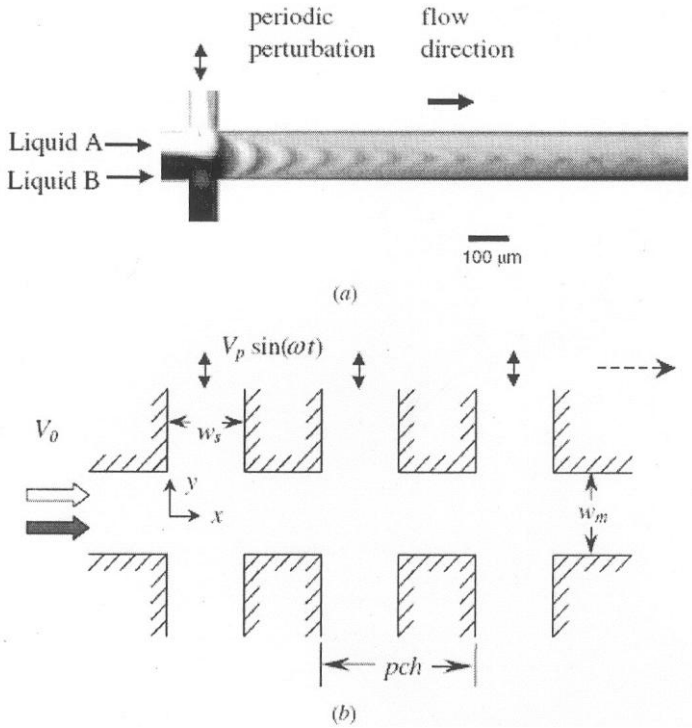

Fig. 3 Model of a chaotic micromixer with multiple side channels:
(a) Experimental observation of fluid mixing in a device with one pair of side channels;
(b) Schematic of a mixer with multiple side channels.
Redrawn from Niu and Lee [77] with the permission of IOP Publishing,
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Since most microreactor applications are in the diffusion controlled regime [1-4], it may not be surprising that the control of heat input/output can help micromixing by controlling the diffusional movements of molecules. However, thermally-controlled mixing has just recently been recognized as a practically workable tool. Two recent studies have demonstrated this. Pradere and associates [78] used infrared thermography and computer-processing of the temperature frames to estimate online the longitudinal temperature distribution in microreactor for an acid-base neutralization reaction. The Peclet number Pe was used to quantify molecular diffusion. The online temperature profiles were related to Pe so as to enable the heat removal rate to be controlled so as to maximize diffusive mixing. Xu et al. [79] induced the thermal mixing of two miscible fluids in a T-shaped microchannel. In their analysis the microchannel was conceptually divided into two zones: the T-junction and the mixing channel. Thermal diffusion was dominant in the T-junction whereas convective heat transfer was also significant

in the mixing channel. By correlating these with the fluid flow, the degree of mixing could be enhanced by regulating heat inflow.

As microreactors move rapidly from laboratory curiosities to real commercial applications, there has been a tendency to return to simple principles of mixing so as to minimize the cost. Piezoelectric micromixers are of this kind. One simple model was devised by Massimo et al. [80]. Their micromixer generated unidirectional rotation of a liquid inside a hole of a metallic cube. This was achieved by bonding four piezoelectric elements on the four sides and applying AC voltages. The equipment is simple, cheap, efficient, portable and disposable. Martinez-Lopez et al. [81] focused on a strictly biological application – an integrated injection system for the perfusion of Chinese hamster ovary (CHO) cells into microbioreactors. This was done by a piezoelectric micropump characterized by the voltage, frequency, viscosity and microfluidic design. Mixing of the CHO cells was optimized by optimizing three parameters. Kong and and Devasia's [82] interest was somewhat different. Their attention was on the distorting vibrations caused by a piezoactuator when used to improve biometric-cilia-based mixing in microreactors. Their iterative approach reduced the mixing time substantially without significant distortions, thereby establishing a new method to generate different wave forms for such actuators. Although these are all recent investigations, piezoelectric mixing was employed tacitly in 2002 by Liu et al. [60], whose work has been cited in the context of acoustic streaming.

The use of microbeads is another example of basic principles being exploited for practically viable applications. As in Liu et al.'s [60] study, magnetic microbeads have been used in magneto-hydrodynamic mixing experiments [74-76]. Ukinta et al. [83], however, used polystyrene (non-magnetic) microbeads in a vertical microreactor stack with multi-functional fluid filters for immunoassays. ELISA results indicated very effective mixing performance, which depended on a combination of Coanda effect [84] and Taylor dispersion [16, 17]. Srinivasan and coworkers [85] used paramagnetic (and not ferromagnetic) microbeads to bind streptavidin molecules and fluorescent microbeads for biotin-coupling. The two types of beads conjugated on being injected into a microtube. Fluorescence emission spectra indicated that bioconjugation efficiency, which is linked to micromixing efficiency, peaked at specific flow rates, similar to the optimal frequencies for best mixing in electrokinetic methods [66-69].

Passive mixing

Since passive mixing derives its energy requirement from the fluids themselves, without any external energy input, the mode of contacting of the fluids being mixed is a critical feature of this method. As for active mixing, a number of contacting methods have been proposed and more methods are emerging continually. The most important methods are summarized in Table 3.

Repeated splitting and recombining of inlet streams is a common method to enhance passive micromixing. The two processes may be carried out either inside a common microtube, including the microbioreactor itself, or by allowing two fluids supplied separately to come into contact in a suitably designed mixing device. The former method was adopted by Howell et al. [113] and by Comesasca et al. [114]. The former group fabricated a rectangular micromixer with grooves in the top and bottom faces. Chevons pointing in opposite directions created a pair of vortices adjacent to each other. Stripes along the width of the channel generated a pair of vertically stacked vortices. The design could generate advection patterns not possible with grooves in the bottom face only. Comesasca et al. [114] also induced chaotic advection by means of grooves on the reactor surfaces, but located the grooves on the basis of results from the Weierstrass fractal function. Their micromixer achieved substantially better mixing than a similar herringbone mixer [115].

Even though it is the cheaper option, internal splitting and recombination of fluids does not enable easy control of the process. So some investigators have preferred to combine two or

more fluid streams by contacting them at suitably designed junctions. Some common contacting schemes have been described by Doku et al. [116], but they relate to publications before 2005. Later improvements include rotation-cum-splitting [117], in which the fluids go through a 90° rotation of a flow cross-section followed by a split into several channels. Each daughter channel is rotated further by 90° and then recombined, and this process is repeated until the desired degree of mixing is obtained. Buchegger and coworkers [118, 119] also exploited laminarity of flow to promote micromixing. Their design used wedge shaped inlet channels to create four lamination layers, thereby reducing the diffusion lengths to just a few micrometers. Since diffusion is the main driving force for mixing in microchannels [16-18], such a reduction is likely to enhance micromixing considerably. This was demonstrated by an application to the enzymatic hydrolysis of *β*-galactosidase, where it was possible to follow the reaction at millisecond time intervals.

Table 3. Comparative data of the recent performances of some passive micromixers [57]

Category	Mixing technique	Mixing time (ms)	Mixing length (μm)	Mixing index
Lamination	Wedge shaped inlets	1	1	0.9
	90° rotation	--	--	0.95
Zig-zag channels	Elliptic-shape barriers	--	10 000	0.96
3D serpentine structure	Folding structure	489	--	0.01
	Creeping structure	--	--	0.015
	Stacked shim structure	--	--	--
	Multiple splitting, stretching, and recombining flows	--	--	--
	Unbalanced driving force	--	815	0.91
Embedded barriers	SMX	--	--	--
	Multidirectional vortices	--	4255	0.72
Twisted channels	Split-and-recombine	730	96 000	1
Surface chemistry	Obstacle shape	--	1000	0.98
	T-/Y-mixer	--	1000	0.95

Two basic methods to induce intimate micromixing are (a) by suitable contacting of the fluids and (b) by appropriate designs of the microchannels. The second method has encouraged many creative designs, with more evolving continually. A simple design is to have zig-zag channel (Fig. 4) through which the liquids have to change directions frequently, thereby forcing them to mix. Lee et al.'s [120] micromixer had channels running parallel and across the flow at 90° to each other. They reported complete mixing within 200 μm as compared to 3000 μm for conventional straight line channels. Yang et al.'s [121] circulation-disturbance micromixer had slanted grooves on the bottom and a zig-zag barrier at the top. This produced transverse motion perpendicular to the flow direction. Compared with a slanted groove mixer without the zig-zag structure, mixing improved by 132% to 208% at Re = 10.

A zig-zag shape is, of course, not the only possible deviation from simple right-angled contact, and this idea has led to many innovative channel designs. Many of these channels may be categorized broadly as having a serpentine structure. This classification nevertheless allows considerable variations in the topology of the layout of the channels. For instance, Kim et al. [122] used an F-shaped arrangement in which each stream flows in through one arm of the F and the two mix in the vertical long segment; this design featured splitting-recombination and chaotic advections. Earlier, Liu et al. [123] had shown that a C-shaped serpentine microchannel with repeating units generated 1.6 to 16.0 times better mixing than

conventional square wave and straight channels. This device was later optimized by Kang et al. [124] to produce almost complete global chaotic mixing in the Stokes flow regime that extended beyond the ranges of Re studied by Liu et al. [123].

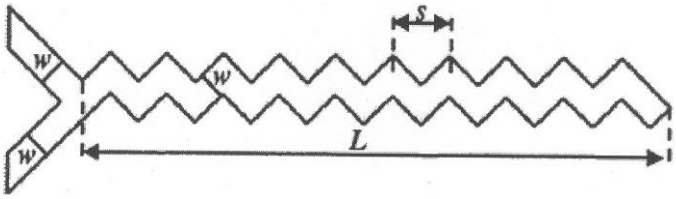

Fig. 4 A microfluidic mixer integrating a Y-junction with a linear zig-zag microchannel.
Redrawn from Mengaud et al. [101] with permission
from American Chemical Society, Washington DC ©2002.

Given the endless variations possible, it is difficult to provide an exhaustive overview of all serpentine micromixers. Two variations of the serpentine structure are shown in Fig. 5 and the upper portion of Fig. 6.

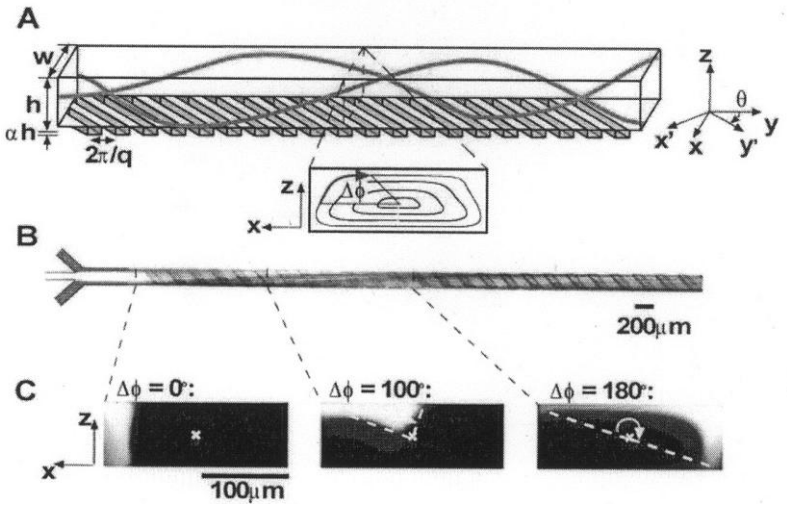

Fig. 5 (A) Schematic of a microchannel with ridges; (B) Optical photograph
showing two other streams flowing on either side of a main stream;
(C) Fluorescent confocal photographs of vertical cross-sections of the microchannel.
Redrawn from Stroock et al. [115] with permission from American Association
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Process needs and ingenuity can, of course, other possibilities and this is illustrated by two novel configurations. Neerinex and coworkers [125] improved the traditional splitting and recombination method through a circularly shaped parallel multiple mixer that used fan-shaped channels to guide the flow into the splitting channels, where it is reversed and re-collected in a second fan-shaped channel. Repetition of this twelve-way splitting design produces 24 layers in the first mixing unit, 228 layers in the second unit, and so on, thereby significantly enhancing mixing efficiency. Moon and Migler [126] were interested specifically in the micromixing of two molten polymers. For this they developed a planar micromixer that "drives streams of molten polymer through mixing chambers". Different complex flow patterns could be generated by suitably stacking the chambers. This design was not only suitable for viscous liquids but could also operate with sample sites much smaller than for traditional micromixers (< 100 mg).

Twisted channels are an alternative to serpentine structures, and some investigators have preferred this either because of their effectives for particular applications or/and because of ease of fabrication. The latter reason motivated Cha et al. [127] to develop a new

polydimethylsiloxne (PDMS) bonding technique to fabricate a "chessboard" micromixer, which, unlike most conventional mixers, had a precisely aligned complex 3-dimensional microchannel array. This improvement enhanced micromixing significantly, nearly complete mixing being achieved within 1400 μm. Hardt et al. [128] also employed twisted channels for the split-and-recombine method; their topology accounted for hydro-focusing and complex flow dynamics, and the main benefit was the slow increase of the mixing distance with the Peclet number. Splitting and recombination of fluids was also the basic principle of Ansari et al.'s [128] passive micromixer. It comprised two sub-channels of unequal widths which repeatedly undergo splitting and recombination; the result was unbalanced collisions between two fluid streams and the generation of Dean vortices. Four variations of twisted channels were explored by Jen et al. [103], one based on the conventional T-mixer configuration and the other three featuring inclined, oblique and wave-like channels (Fig. 7). It may be recalled that the repetition of a particular structure to enhance mixing was also the rationaleof Liu et al.'s [123] serpentine micromixer, and vortex generation as a tool was also employed by Yang and associates [121] in their zig-zag channels. The exploitation of two or more methods together thus seems to enhance micromixing more than any one method alone, as demonstrated also by Hashimoto and Whitesides [131], who exploited flow-focusing in a unit containing multiple rectangular mixing sections. By varying the operating conditions, it was possible to generate a monodisperse or bidisperse or tridisperse distribution of gas bubbles in a two-phase mixer.

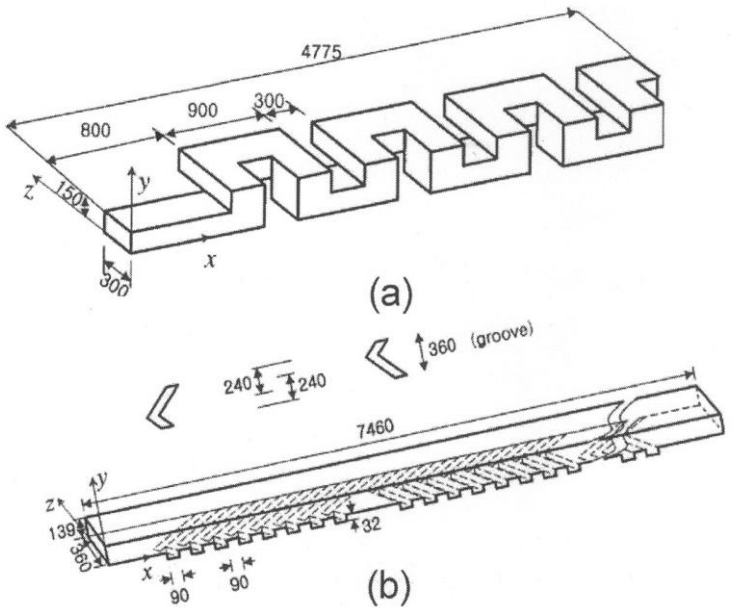

Fig. 6 Geometry of (a) three-dimensional serpentine and (b) staggered herringbone mixers. Redrawn from Liu et al. [102] with the permission of Elsevier B.V., Netherlands ©2004.

Apart from channel geometry, it is also possible to promote micromixing by introducing obstructions in the flow paths. The idea is of course borrowed from the use of baffles in large bioreactors. An example of a successful commercial design is that of Kim et al. [132, 133], which uses embedded barriers along the upper surface of the microchannel and helical flow structures along the lower surface to induce pressure-driven chaotic flow (Fig. 8); recall that the principle is similar to that of Niu and Lee's [77] zig-zag channel. An alternate approach is that of Singh et al. [134], who optimized three design parameters of SMX static mixers: the width of the channel, $N(X)$, the number of parallel cross bars per element, $N(p)$, and the angle, Θ, between opposite cross-bars. They derived the optimum design rule $N(p)= (2/3)N(X) - 1$ for $N(X)= 3, 6, 9, 12, \ldots$. Surprisingly the rule does not contain Θ. Tsai and Wu [135] combined

two modes of mixing enhancement: curved microchannels to induce vortices and internal radial baffles to compel the fluids to mix. Like that of Liu et al. [123], this mixer too had C-shaped channels and was fabricated out of PDMS to obtain an exact configuration [22]. Excellent mixing was reported over a wide range of Re. Two-method enhancement is also the theme of Sotowa et al.'s [136] microreactor, which had baffles embedded in deep microchannels. A deep microchannel has a much larger aspect ratio than a standard channel. While the width of Sotowa et al.'s channels was 100-1000 μm, the same as in standard channels, the depth was at least a few millimeters (Fig. 9). Its effectiveness was demonstrated for the enzymatic hydrolysis of o-nitrophenylgalactopyronaside by β-galactosidase for throughputs up to 10 000 tons per year by stacking several microreactors in parallel [137].

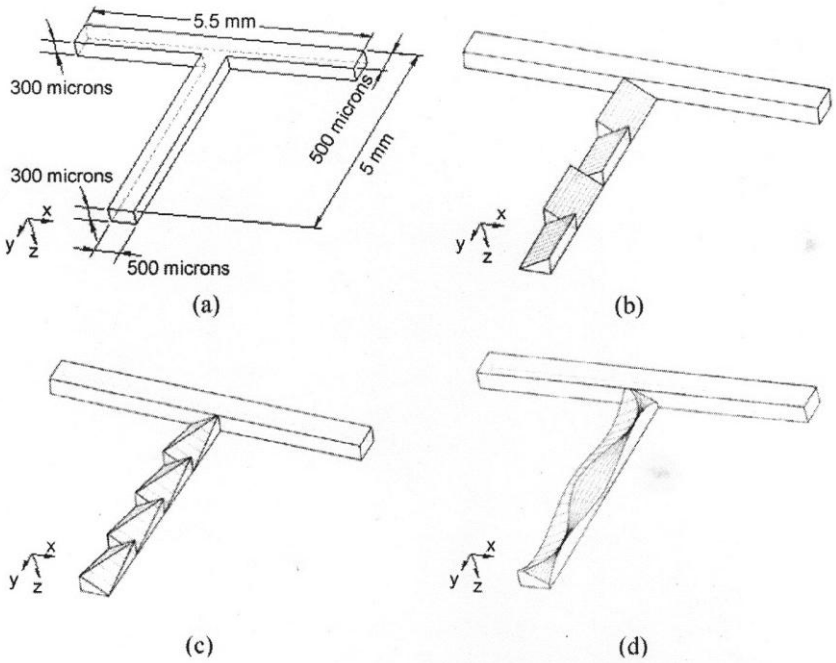

Fig. 7 Schematic diagrams of (a) a T-mixer; (b) an inclined mixer; (c) an oblique mixer and (d) a wavelike mixer. Redrawn from Jen et al. [103] with the permission of Royal Society of Chemistry, London ©2003.

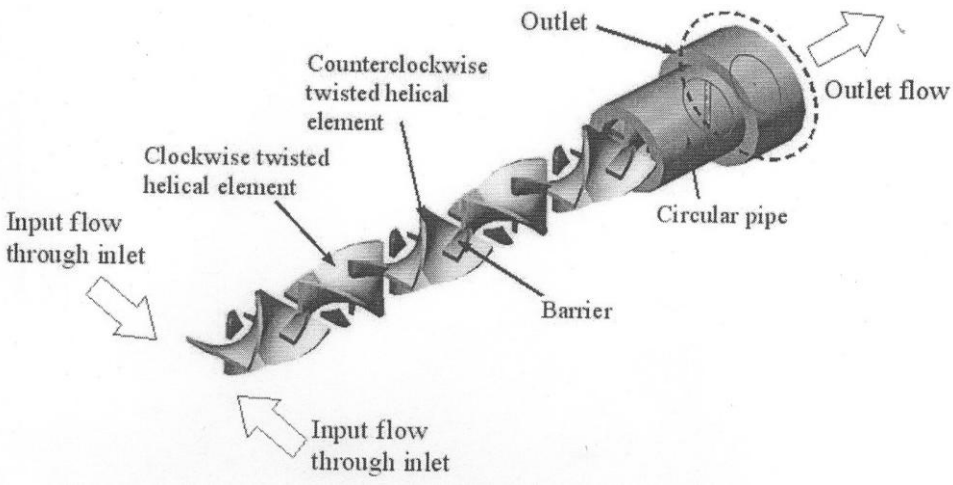

Fig. 8 Schematic diagram of a Kenics micromixer with embedded barriers. Redrawn from Kim et al. [104] with the permission of IOP Publishing, Philadelphia, PA ©2004.

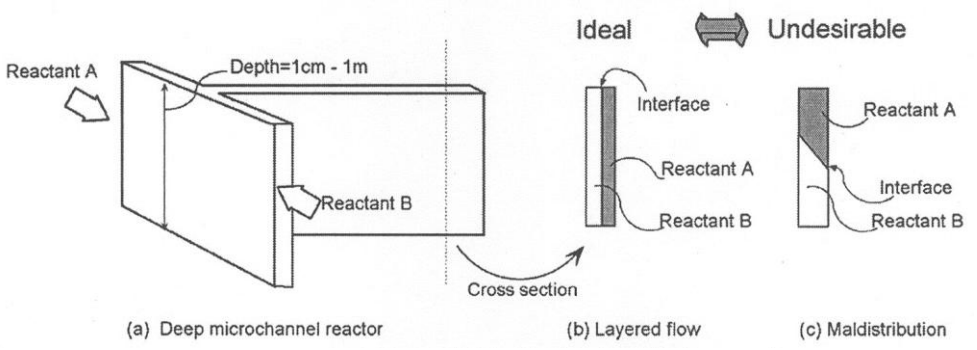

Fig. 9 (a) Concept of a deep microchannel reactor; (b) expected flow pattern in such a reactor. Redrawn with permission from Sotowa et al. [133] with permission from Elsevier B.V., Netherlands ©2007.

While the micromixers discussed so far have been predominantly of the active or the passive type, one interesting design was proposed by Chen and Cho [138] that combined both kinds of mixing. The microchannel had a wavy-wall section to disrupt the flow, and active mixing was induced by applying periodic velocity perturbations to the inflow. Mixing was favored by increasing the amplitude and the length of the wavy surface, as might be expected, but interestingly there was an optimum range of velocity perturbations corresponding to a Strouhal number between 0.33 and 0.67. The relevance of an optimum velocity perturbation range was not clear. Nevertheless, this study suggests that combination of active and passive mixing is both feasible and beneficial.

4.3. Evaluation of micromixing efficiency

Given the large and rapidly increasing, number of different micromixing strategies, it is important to have some quantitative measures of the mixing efficiency of a given device or a particular method of mixing. The most widely used method is based on the Villermaux-Dushman reaction [139]:

$H_2BO_3^- + H^+ \leftrightarrow H_3BO_3$ (Instantaneous reaction)

$5I^- + 10_3^- + 6H^+ \leftrightarrow 3I_2 + 3H_2O$ (Fast reaction)

$I_2 + I^- \leftrightarrow I_3^-$

To study mixing efficiency two solutions, one a buffer solution of $H_2BO_3^-$, I^- and 10_3^-, and the other being diluted sulfuric acid, are fed through the microchannel. For ideal (or complete) mixing, the acid gets instantaneously distributed homogeneously in the buffer and is thus completely neutralized by the borate ions to form boric acid, H_3BO_3. As a result, no free acid is available for the second reaction to take place and hence no free iodine is formed. However, for less-than-complete mixing, pockets of unreacted acid are available for the second reaction to generate iodine. Thus the concentration of iodine in the outlet is a measure of the degree of mixing, the two being inversely proportional to each other. Note also the presence of the third reaction, which consumes iodine to produce iodate (I_3^-) ions. Thus, in practice it is these ions that are detected through absorbance in a UV-VIS spectrophotometer.

On the basis of these measurements Guichardon and Falk [139] proposed a segregation index, X_s, defined as:

$$X_s = Y/Y_{st} \quad (12)$$

where Y is the ratio of acid moles consumed by the second reaction and Y_{st} is the value of Y for complete segregation i.e. no mixing. Therefore $X_s = 0$ indicates perfect mixing and $X_s = 1$ corresponds to complete segregation.

Two recent reviews have compared different mixers through this reaction. In the first review [140] the authors compared twelve micromixers, some commercial and some generic, based on different mixing principles. The comparison was done through the mixing times and the energy efficiencies. Experimental determination of the mixing time, t_{mix}, is described by the authors and how it may be related to the segregation index X_s. t_{mix} may then be related to the energy dissipation, ε, in a microchannel for laminar flow as:

$$t_{mix} = \frac{1}{\sqrt{2}}\left(\frac{\nu}{\varepsilon}\right)\ln(1.52\mathrm{Pe}) \tag{13}$$

where ν is the kinematic viscosity of the fluid mixture. Likewise, the energy efficiency of mixing is defined as [141]:

$$\eta = \frac{\dot{\gamma}}{\gamma_{\max}} = \frac{\dot{\gamma}}{\sqrt{\varepsilon/(2\nu)}} \tag{14}$$

where $\dot{\gamma}$ represents the shear rate that is effectively used for micromixing and $\dot{\gamma}_{\max}$ is the total shear rate used for the flow.

From the preceding two equations, it is easily derived that

$$t_{mix} = \frac{d}{8\bar{u}\eta}\ln(1.52\eta\mathrm{Pe}) \tag{15}$$

where d is the diameter of the microchannel and $\bar{u}$ is the average fluid velocity.

Falk and Commenge's [140] analysis showed that: (a) mixing times much smaller than 1 ms are realistically possible, and (b) under most practical conditions the mixing time is inversely proportional to the energy consumed for mixing.

In the second review, Kashid et al. [142] compared five generic micromixer configurations through the same Villermaux-Dushman reaction [138]. They reported more definitive conclusions than did Falk and Commenge [140]: a microchannel with structured internal surface (the caterpillar design) was better than the T-square, T-trapezoidal, Y-rectangular and concentric designs. The caterpillar design has been elaborated elsewhere [143].

To evaluate their deep microchannel with indents and baffles, Sotowa et al. [136] expressed the mixing performance by the ratio of the area of the outlet surface where the mass fraction of one fluid was greater than 95% of the mixture to the total surface area of the outlet. By this definition the mixing efficiency is 0.50 when there is no mixing (i.e. complete segregation) and it reduces as mixing improves. They observed that increasing the flow rate favored mixing.

Yet another index was proposed by Jain and Nandakumar [144]. The main thrust of their work was that even though micromixing is often improved at the expense of reduced flow rate [145-147], and there is a tradeoff between mixing and transport [148], these effects have not been considered in previous studies. They argued that most evaluations of micromixers have been based either on deviations from perfect mixing or the length required to achieve perfect mixing. Jain and Nandakumar [144] proposed a new micromixing index that accounted for flow rate changes through the residence time. Like many earlier studies [141-147, 149], their index too evaluates mixing performance relative to perfect mixing:

$$\eta = 1 - \frac{\sqrt{1/N\sum_1^N (C_s - C_s^*)^2}}{\sqrt{1/N\sum_1^N (C_s^0 - C_s^*)^2}} \tag{16}$$

In this equation, N is the number of points in the cross-section used for estimation of η, C_s represents the normalized concentration at the point of interest, C_s^* is the concentration for perfect mixing and C_s^0 for complete segregation. With these definitions it follows that $C_s^0 = 0$ or 1, $C_s^* = 0.5$, and $0 \leq \eta \leq 1$.

Jain and Nandakumar [144] pointed out that η as defined above does not account for flow rate (or residence time) variations. To do so, they introduced a comparative mixing index (CMI) defined as:

$$\alpha_{A,B} = \frac{\eta_A}{\eta_B} \tag{17}$$

$\alpha_{A,B}$ essentially evaluates a mixing design A relative to a different mixer B. For "standard" values, the T-mixer is often used as a reference design, in which case $\eta_B = \eta_T$, where the subscript T denotes a T-mixer.

Given the proliferation of mixing strategies and performance criteria, it is sobering to note that the intrinsic value of mixing time or mixing index *per se* is less relevant in real terms than the resulting improvements in the rates and selectivity of the chemical reactions that follow from the mixing of two or more fluids [140]. However, few studies have addressed this important facet of micro mixing, hence that of Aoki et al. [150] is a pioneering exception.

Monitoring, modeling and control

Making microbioreactors truly effective requires that they should be equipped with practically feasible automatic process monitoring and control systems. Key parameters that usually need to be controlled for on-line optimization are the optical density (OD), DO concentration, temperature, pH and the flow rates of the principal reactants [2, 3, 86].

Each of these variables may be monitored by more than one method and often the choice of method is guided by a balance between accuracy, speed, cost and amenability to automation. In view of detailed reviews elsewhere [1-4], each of the main methods is briefly presented below.

Optical (cell) density

The OD of the contents of a microbioreactor is the most commonly used indicator of cell concentration. Two basic categories of OD measurement are used: optical and electrochemical. Optical methods include fluorescence absorbance, refractive index, colorimetry, spectroscopy, chemiluminescence and bioluminescence. Absorbance of light is the simplest method, and it is based on the Beer-Lambert law [87]. The wave length for optical probes is normally chosen within the range of visible light (400-700 nm); for example, *Saccharomyces cerevisiae* cultivations use 600 nm.

Although it is simple, the absorbance method has limitations such as the risk of misalignment of the fibers and interference by gas bubbles. An alternate method that is widely employed is fluorescence; it can measure both cell concentration and cell viability. In one version, cells are tagged with fluorophores such as calcein, propidium or ethidium bromide. Focusing light of a particular wave length (or color) initiates certain intracellular reactions in viable cells, as a result of which these cells emit fluorescent light of a different color. For example, cells stained with calcein or propidium emit green fluorescence when excited by blue light. Another common fluorescence method used green fluorescent protein (GFP) to track specific genetic events [88, 89]. An analogous method is the use quantum dots of nanomaterials to follow cell viability [86].

Bioluminescence and chemiluminescence are experimental manifestations of controlled light emission by fluorescent methods. Zanzotto et al. [90] described an interesting application

in which both techniques have been used together in an integrated microbioreactor. A combination of methods was also used recently by Bower et al. [91] to monitor cell growth of *E. coli* by OD measurements and the synthesis of a plasmid DNA vaccine vector by GFP fluorescence.

Methods other than OD and fluorescence are also used but less frequently. Impedance spectroscopy is an example. The principle is to apply an AC electrical field to the cell culture and measure the conductivity as a function of frequency. Krommenhoek et al. [92] integrated an impedance sensor on to a multisensor chip and applied it to monitor a number of variables simultaneously in a bioreactor. However, the lowest concentration of biomass that could be measured was 1 g/L, which makes impedance spectroscopy less attractive than fluorescence.

Dissolved oxygen

As for the cell mass concentration, DO concentration too is measured by optical sensors with the use of fluorescence sensor spots [5, 6, 93]. These so-called optodes work on the principle of quenching of fluorescence by oxygen. The possibility of making optodes in small sizes at a low cost, their nonreactivity and ease of integration with other units on a multi-functional chip makes them suitable for simple disposable microreactors. Thus optodes have become popular monitoring devices in fermentations where more than one variable has to be monitored simultaneously. Some illustrative applications are described later.

An alternative to optical methods are the use of electrochemical sensors such as the ultra-microelectrode array (UMEA), which is based on the electrochemical reduction of oxygen [92]. However, this method is limited by the surface area of the electrode, which implies that detection of small concentrations requires large electrodes, thereby reducing the practical utility of such devices. So there has been a shift toward cell-based electrochemical sensors. Different sensors function on the basis of different signals generated by cellular reactions, e.g. electrode potential (potentiometry), oxygen reduction or electrochemically inactive molecules (amperometry) or electrochemically active molecules (conductimetry). Recent innovations based on detection of changes in cell membrane potential and potentiometric changes induced by light have been particularly useful in neurotransmitters, heart muscle cells, pancreatic beta cells and embryonic stem cells. Recent developments in cell-based electrochemical sensors for biological applications have been reviewed by Ding et al. [94] and medical applications in high-risk diseases have been discussed by various authors [95, 96].

pH

As explained in Section 5.1 for cell density measurement, optical detectors or optodes are preferred over ISFET chips because of the former's low cost, noninvasive nature and ease of integration, thereby making them specially suitable for single use disposable microbioreactors. This also adds to the benefit of not requiring such optodes to have long life-cycles.

However, ISFET pH sensors have advantages in covering a wider range of pH (2 to 12 versus 4 to 9 for optodes [99]), a linear response (unlike the nonlinear outputs of optodes), greater sensitivity than pH sensor spots and a wider temperature range of operation (-45 °C to 120 °C as against 0 °C to 50 °C). They have some drawbacks also. Apart from being more costly, they have greater measurement drift and are sensitive to ambient light [97-99].

Thus, pH sensors based on optical detection and ISFET have both advantages and weaknesses. Nevertheless, recent developments have succeeded in reducing the weaknesses, thus enabling both types of biosensors to be practically useful in a variety of applications, many of which have been highlighted elsewhere [2-4].

Integrated biosensors

Given the sizes of microchips, it is expected that linking a number of different miniature sensors to an operating microbioreactor to follow all the relevant variables might be practically difficult and costly. Recognizing this, a number of investigators have designed and implemented integrated biosensors which incorporate different individual sensors on a single chip along with a microbioreactor.

In two related studies, Krommenhoek et al. [92, 99] have described a multi-variable electrochemical sensor for the simultaneous online measurements of viable biomass concentration by impedance spectroscopy, DO by an amperometric ultra-microelectrode array, pH using an ISFET sensor and temperature variables by a Pt thin-film resistor. This multi-functional chip was designed for a 96-well microreactor array and tested with *S. cerevisiae* fermentations in microbioreactors as well as large industrial scale units. The versatility of such a microchip was further underlined by Boccazzi et al. [100], who performed differential gene expression analyses of *S. cervisiae* grown in galactose and glucose media in 150 μL bioreactors. A similar integrated microsensor had been reported earlier by Szita et al. [40], who employed optodes to monitor cell mass concentration, DO and pH in *E. coli* cultivations.

More recent studies have expanded integrated microchips in more innovative and useful ways. Funke et al. [105] combined a fiber optic online monitoring device for microwell plates with microfluidic control of cultivations of *E. coli* in disposable single-use integrated units. Bower et al. [106] also studied *E. coli* fermentations but their organism had a temperature inducible plasmid pVAX1 that synthesized a DNA vaccine vector. This recombinant microfermentation required accurate and sterile control of DO, temperature and pH, along with online monitoring of cell growth, glycerol and acetate concentrations. Unlike the studies reported above, which employed stirred microbioreactors, Fonseca et al.'s [107] work demonstrated the usability of integrated biosensors for immobilized cells. A similar departure from conventional technology has also been reported by Balagadde and associates [108]. Their microfluidic bioreactor focused on long term "unnatural" behavior of *E. coli* under time-varying conditions over long durations of hundreds of hours. Two features of their work stand out: (a) the ability to monitor the performance up to single-cell resolution and (b) long duration feedback control through quorum sensing.

Modeling and control

Since the measuring devices are usually integrated with the microbioreactor on one chip, the control of these reactors is understandably linked with the measurements themselves. This may be contrasted with large bioreactors, where the sensors and the controllers may be separate instruments that are inter-connected and programmed according to the process requirements [109, 110].

Initial studies of chip-based microreactor control focused on just one or two variables, usually pH or temperature or DO. For instance, Buchenauer's project [111] was intended to control standard microtiter plates. Zhang et al. [112] went further and controlled the cell density, pH and DO concentration. Multi-variable control was also practised by Maharbiz et al. [98]. Their system of eight 250 microbioreactors was designed to control the inflow of gas to the reactors so as to maintain the pH and temperature at optimum levels. pH control seems to be at the core of most control studies with microbioreactors because Buchenauer et al.'s [113] work also focused on this. Apart for controlling the pH, all these authors also used *E. coli* as a model system. A refreshing departure was that of Alam et al. [114], whose interest was in temperature control of a *S. cerevisiae* fermentation. They showed that with a Pt100 sensor and resistance wires embedded in the walls of a microreactor made out of polymethylmethacrylate and polydimethylsiloxane, simple on-off control could maintain the temperature within ±0.1 °C of the set point value.

The success and the importance of multi-variable integrated control strategies in research laboratories are highlighted by the rapid growth of commercial microbioreactor arrays implementing these schemes. The Automation Partnership (York Way, UK) offers the ambr™ microscale reactor [115] for cell line development; this is a disposable microbioreactor with integrated pH and DO sensors and controllers, an integrated impeller and a sparge tube for gas delivery. Applikon's micro-Matrix™ consists of 24 miniature bioreactors with measurement and control for pH, temperature, dissolved oxygen, liquid feeds and gas delivery [120]. Choi et al. [117] have referred to their use of another 24-well array from Pall Corporation. This system provides continuous monitoring and control of the same variables as the micro-Matrix™ and it has been effective in culturing stem cells for T-cell therapies of patients suffering from metastatic melanoma, lymphocytic leukemia, HIV and damaged pancreas or liver or spinal cord.

While these investigations and applications demonstrate the viability and importance of integrated online monitoring and control of microbioreactors through simple methods, they also reveal two weaknesses: (a) all have been experimental implementations without a strong modeling foundation, and (b) all have used on-off controllers, so the effectiveness of other control strategies have not been explored for microbioreactors, even though different modeling methodologies are well established for large bioreactors.

Given the complex behavior of cellular processes in bioreactors in general and microbioreactors in particular, it is not surprising that experimental understanding is still evolving and there are few rigorous models. The phenomenological complexities have led to a growing preference for computational fluid dynamic (CFD) simulations of microbioreactors. Even here, CFD simulations have addressed specific aspects of performance improvement and not a full scale depiction of reactor dynamics. Thus, Bailey et al. [151] used a CFD + ACE + multiphysics simulation package to study design features that will increase the destruction of undesirable products, and thereby improve selectivity, in enzymatic microbioreactors. Their simulations were validated for the destruction of urea catalyzed by urease. Li et al. [152] were interested in improving mixing efficiency by pressure-based recycle flow; experimental validation through the distribution of a colored dye in a 30 μL microbioreactor substantiated their simulations.

Since mixing is a central feature determining the performance of a microreactor, it is not surprising that many CFD studies have addressed this aspect of microreactors. Recent studies include those by Li et al. [153] and Chen et al. [154]. The former group applied varying pressure to a microchannel, as they had done earlier [152], to induce oscillating flow. This improved mixing by both diffusion and convection, thus supporting their earlier work and demonstrating the effectiveness of this method. Chen et al. [154] improved mixing and reaction of a split-and-reconfigure microreactor by modifying the generic configuration. By shrinking the structure of the splitting and reorientation regions in alternate directions, they induced rotation of the fluid inside the microchannel. This in turn generated internal multi-lamellation and chaos, which enhanced micromixing. They validated their method for the hybridization of two complementarily labeled oligonucleotides.

Compared to enzymatic reactors, those employing whole cells are more useful and also more difficult to analyze. However, both CFD and population balance methods, coupled with kinetic equations, have been useful in predicting and optimizing the performances of microbioreactors with living cells. For well-known reasons, the cultivation of *S. cerevisiae* has been a popular model system for such studies. Fernandez et al. [155] developed a multi-scale model comprising population balances, reaction kinetics and a flow model to predict point-to-point variation of the concentrations of the substrate (glucose), product (ethanol) and biomass, as well as cell size distributions, in a suspended culture. Schapper et al. [156] on the other hand focused on immobilized *S. cerevisiae* in a rectangular microbioreactor. Their strain also differed

from that of Fernandez et al. [155] in harboring a plasmid pGAC9 imported from *Aspergillus awamori* that expressed glucoamylase extra-cellularly. To have a realistic model they considered that at any given time some cells may be attached to the support and some may be freely suspended; their relative concentrations may vary with time. Unlike other investigators, Schapper et al. [156] showed through a deterministic set of equations that a nonuniform distribution of cells along the microcapillary results in greater glucoamylase activity than the conventional uniform distribution.

Like Schapper et al., Kaul et al. [157] also analyzed the microreactor optimization problem from a different perspective. They argued that there is two-way dynamic flow of material and information between cells and their microenvironment. So their model coupled an agent-based modeling platform with a transport phenomena computational modeling frame work. The validity of this approach was tested by comparing simulated cell population distributions and product synthesis with experimental results for bacterial chemotaxis.

The references cited above indicate there are two basic approaches to the modeling of both microbioreactors and microbioreactors. Deterministic models either ignore or use lumped approximations of fluctuations in cellular processes so as to derive mathematical descriptions that a simple, reasonably informative and can be implemented easily. Simplification however limits the applicability of such models for complex biological processes, especially in situations where subtle variations can have significant impact on reactor behavior, as in intra-cellular genetic processes [158] or when a bioprocess in under the influence of external noise in addition that within the cells [159]. In such situations stochastic models provide more faithful and useful descriptions of the observed behavior [156]; they also provide greater insight into cellular processes, thus enabling operational adjustments that are not evident from lumped deterministic models. Julien and Whitford [156] classified bioreactor models into three types: qualitative, mathematical and statistical. Each of these may be sub-classified into other types. This hierarchy is portrayed succinctly in Fig. 10. This classification, however, does not exhaust all kinds of models possible. For instance, fuzzy logic and neural networks are just two kinds of non-parametric artificial intelligence models, but there are others also (e.g. genetic algorithms and expert systems).

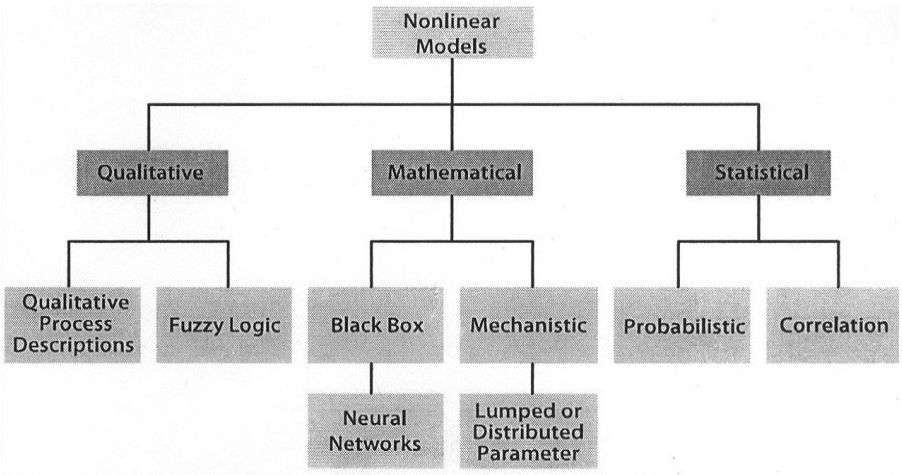

Fig. 10 Classification of some major types of nonlinear models used for bioreactors. Redrawn from Julien and Whitford [161] with permission from Bioprocess International, Westborough, USA (2007).

Integration of microbioreactors with other devices

To be practically useful, microbioreactors should be able to function in conjunction with other miniaturized devices such as detection systems, separation units and biological assays, all integrated on a single microchip. Moreover, for a cost-effective bioprocess, many such integrated devices should be linked functionally on a single multi-unit platform. The complexity of the problem should be evident by now, and it has led to new rules to design and develop such "microfluidic large-scale integrations (mLSI)". The main ideas behind major mLSI methods and some of their applications have been discussed elegantly by Mellin and Quake [162]. A central thesis of mLSI is *multiplexing*, which refers to the performing of a large number of tasks with a small number of steps or units.

The simplest multiplexer is, of course, of the binary type, which controls two tasks per device, e.g. distribution of a fluid between two microchannels. The scope of a binary multiplexer is however, limited and this recognition has led to the combinatorial multiplexer [163], which allows $N!/(N/2)!^2$ tasks to be performed simultaneously with N control lines to illustrate, with $N = 16$, the combinatorial method addresses 12 870 tasks as compared to 256 by the binary method.

The huge benefits from combinatorial multiplexers have been exploited in a matrix containing $20\times20 = 400$ reaction chambers, each functioning as a PCR unit [164]. Only $2\times20 + 1 = 41$ pipetting steps were required, compared with $3\times20^2 = 1200$ steps by conventional pipetting. The efficacy of these devices was demonstrated by the amplification of a 294-bp segment of human β-actin cDNA fragment in a matrix of 3200 reaction chambers with 98% success.

Methods other than multiplexing to widen the scope and versatility of chip-based microbioreactors include (a) the incorporation of affinity columns and (b) parallel processing by a number of miniature devices. The latter method is analogous to the "numbering up" of microchannels to increase throughput [136, 137], and its usefulness is exemplified by a microchip containing parallel process lines for cell isolation, cell lysis, mRNA purification, cDNA synthesis and cDNA purification [165].

DNA and PCR based applications in fact form a major fraction of integrated microchip applications to cellar processes. Recent designs of PCR based microfluidic devices include Liu et al.'s [166] rotary chip for rapid PCR cycling, West et al.'s [167] annular flow PCR microreactor with three temperature zones, and Yuen et al.'s [168] microchip which combined sample preparation with PCR.

Integrating PCR and capillary electrophoresis (CE) on one microchip is in fact a major area of PCR-based microfluidic devices. Legally et al. [169] designed a glass microdevice that performed eight PCR and CE analyses on a single chip. Khandurina et al. [170] demonstrated on-chip PCR and CE in a conventional cross microchannel chip by attaching a pair of Peltier type thermoelectric heating/cooling elements over the reaction vessel and separating the products by traditional on-chip CE. Other authors have demonstrated the feasibility of integrating a micro-PCR made out of one material with a CE unit fabricated out of a different material, e.g. silicon-glass [171] and PDMS-glass [172].

The focus of the present review is however on the integration microbioreactors with other micro-devices. Affinity columns referred to above form one specialized category of such devices when they are used for reaction chromatography [173]. More conventional microbioreactors too may be linked to upstream or downstream units on a single chip. Losey et al. [174] have described such a device for two-phase mixing of fluids in the context of heterogeneous catalytic reactions. Their device had separate gas and liquid ports, and the two-phase mixture flowed into one of 10 microchannels containing porous, solid high-aspect ratio posts with impregnated catalyst. Brivio et al. [175] presented a continuous flow glass/silicon

channel-based microunit integrated with a laser desorption ionization time-of-flight mass spectrometer on the same chip.

Increasing understanding of micro-integration and improvements in manufacturing technologies has enabled the design and fabrication of more intricate devices. Lee et al. [176], for example, developed an integrated array of microbioreactors, each of 100 μL working volume, comprising a peristaltic oxygenating mixer and microfluidic injector. This unit could provide high oxygen transfer rates without introducing gas bubbles and had closed loop control over DO and pH. The effectiveness of the system was demonstrated by promoting eight simultaneous *E. coli* fermentations. Buchenauer et al.'s [177] unit was of a similar kind. An array of microbioreactors based on the format of 48-well microtiter plates was monitored and controlled online by different integrated sensors for pH and biomass concentrations. The functioning of this device too was validated for *E. coli* fermentations.

The integrated microbioreactor described by Pohar et al. [178] is a noteworthy departure from those described above. It comprised a packed bed microtube coupled to a miniaturized separator to perform biocatalytic reactions in an ionic liquid medium. For butyl butyrate synthesis from butanol and vinyl butyrate, catalyzed by immobilized lipase B from *Candida antartica*, 100% conversion of 0.5 M equimolar concentrations of both reactants was achieved within 5 min at 25 °C, followed by 90% separation efficiency for butyl butyrate.

It may be recalled that the ability to carry out a large member of operations through a small number of steps, which is the heart of successful integrated microdevices, has been facilitated by (combinatorial) multiplexing. The idea and the architecture for a multiplexor were inspired by the miniaturization of computer circuits and microanalytical systems. In traditional control systems, one valve is used in each flow channel to switch the flow on or off. However, in this arrangement the number of control valves becomes prohibitively large for complex integrated microdevices. Analogous to electronic integrated circuits, Thorsen et al. [179] designed a fluidic multiplexor which was "a combinatorial array of binary valve patterns that exponentially increased the processing power of a network". Such multiplexors enable control of F fluid channels with just $C = 2\log_2 F$ control channels. For example, for $F = 1024$, only $C = 20$ control channels are sufficient, thereby reducing the control effort substantially.

Since both integrated micro-chips and model-based control for microbioreactors are still evolving, it is perhaps not surprising that not many control strategies have been explored. Those that have been implemented are more heuristic than rigorously optimized on the basis of cellular dynamics and fluid dynamics. Nevertheless, heuristic control methods with justifiable logic are useful, as commercial applications have shown, and Schapper et al. [2] presented a flow-sheet (Fig. 11) that illustrates the logic involved in many such control schemes.

Applications

Recent reviews by Pasirayi et al. [3] and Yeo et al. [180] have surveyed some major areas for applications of microfluidic devices. However, while the former covers cell culturing in general and the latter is more focused on applications, both reviews cover a broad range of applications, most of which describe protein manipulation and analysis, DNA hybridization, drug delivery, biosensors, and diagnostic devices. Relatively less attention has been devoted to microbioreactors *per se;* however, reactors are at the core of most microbiological processes of practical interest, as evident form the number of patents [181] and commercial products [110]. For this reason and because this review is concerned primarily with microbioreactors, applications using these devices are discussed here in greater detail.

Owing to their small sizes, many microbioreactors can be operated in parallel in a simple microarray, thereby allowing a large number of reactions to be run simultaneously. This feature has enabled the use of microbioreactors in place of large bioreactors for generating high throughputs at moderate costs. Many such applications have already been discussed earlier in

this review [30, 39, 40, 44, 46] and some authors [4, 33, 47] have provided succinct overviews. Hence discussion of this area of applications again will be repetitive.

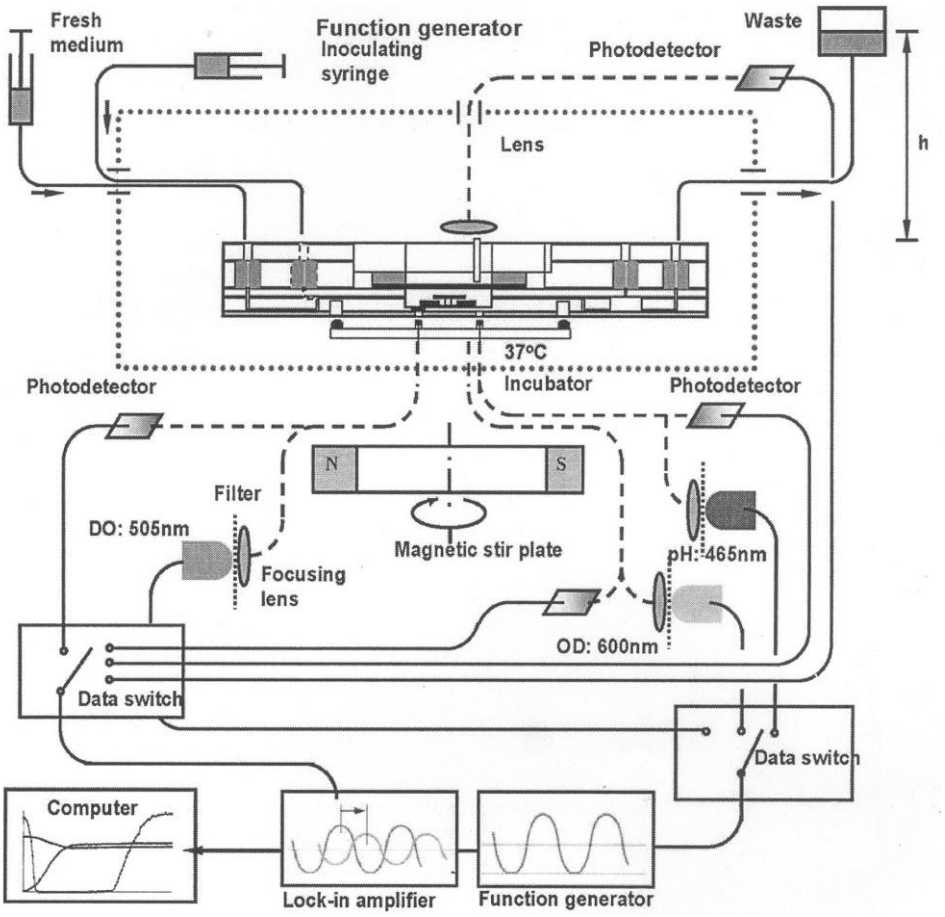

Fig. 11 Flow diagram of a microbioreactor set-up complete with actuation, fluid connections (solid lines) and optical fibers (dashed lines) for on-line sensing of OD, DO and pH. Redrawn from Schapper et al. [2] with permission from Springer-Verlag, N. Y. ©2009.

Although diagnostic devices may not strictly qualify as microbioreactors, many of them are based on biochemical reactions and therefore constitute one important area of applications. Since these kits should be manufactured and used in large numbers under rugged conditions, they should be cheap, easy to use, robust to variations in environmental conditions and preferably disposable after one use. From this perspective, Weigl et al. [182] have discussed many interesting diagnostic devices such as ABO blood typing cards, diffusion-based detectors and separators for blood processing to obtain pure white blood cells, and non-instrumented nucleic acid amplification kits that overcome some of the limitations of standard PCR-based methods. Another novel device was presented by Zhao et al. [183]. Theirs was a paper-based colorimetric probe using gold nanoparticles to perform rapid inexpensive bioassays. Being paper-based, the assay kits were disposable and environment friendly. Zhao et al. [183] suggested possible uses in blood testing for disease diagnosis, pathogen detection and quality monitoring of food and water.

More "genuine" bioreactor applications have been reported by Reis et al. [184] and Edlich et al. [185]. The former group operated a 4.5 mL (internal volume) microbioreactor with oscillatory flow mixing for fermentations by a flocculent *S. cervisiae* strain. With an inlet glucose concentration of 20 g/L, the microbioreactor achieved experimentally an increase of 83% in biomass concentration and a reduction of 94% in air requirement when compared

with a large scale stirred bioreactor; however, the monitoring instruments (for pH, DO, glucose consumption, biomass concentration and ethanol production) were externally linked and integrated with the microbioreactor on the same chip.

Edich et al. [185] also studied fermentations by *S. cerevisiae*. Their microreactor was much smaller than that of Reis et al. [184] and had DO and OD sensors integrated with the 8 μL reactors. In addition, several metabolite concentrations were followed through off-line measurements in a modified Verduyn medium described by the authors. Edlich et al. also observed greater outputs of biomass, ethanol and some key metabolites, and significantly lower utilization of glucose and air than in a macroscale bioreactor operating under comparable conditions. They also observed strong growth of cells along the microbioreactor walls, resembling a biofilm, which is not normal in large bioreactors. An obvious reason for this is that the flow through a microbioreactor is laminar and hence there is much less shear than in large vessels.

Two frontier areas of application underline the real worth of microbioreactors. One area is the cultivation of stem cells for the treatment of complex and potentially fatal diseases such as leukemia, nervous disorders and myocardial regeneration [186]. The therapeutic value of these cells arises from their ability to differentiate into other types of cells, each of which has a specific utility. There are two kinds of stem cells – pluripotent embryonic cells and adult stem cells [187].

Microbioreactors are suitable for stem cell cultivation because their laminar flows exhibit conductive physiological requirements such as constant solution-phase microenvironments, benign conditions due to fast removal of heat, large surface-to-volume ratios and high throughputs [188]. Such benefits and the therapeutic effects of stem cells without common disadvantages such as the negative ancillary effects of prolonged treatment by strong drugs have motivated increasing research into the controlled production of stem cells.

Chung and associates [189] reported a microfluidic platform with a concentration gradient generator to study the effect of growth factor concentration on the differentiation and proliferation of human neural stem cells. Kim et al. [190] developed a microfluidic device for culturing human embryonic stem cells under different biological conditions; best growth was observed at an optimum flow rate which was neither too low nor too high. At low flow rates the cells do not get sufficient quantities of nutrients for their metabolic requirements, while high flow rates cause shear damage to the cells. A somewhat different type of microbioreactor was used by Park et al. [191] to generate of various cytokines; the device was run continuously for eight days without any contamination or loss of concentration gradient or lowering of stem cell production. Long term cultivations have also been reported by Korin et al. [192] for human embryonic stem cells and by Wang et al. [193] for mouse mesenchymal stem cells. Korin et al. have also published another study [194] in which they examined the effectiveness of human foreskin fibroblasts (HFF) cells as feeder cells for human embryonic cell cultures in a microchannel perfusion bioreactor. A sobering but valuable result was that it was difficult to grow embryonic stem cells over a long period (> 7 days) under conditions that were suitable for the progenitor HFF cells because of the extreme sensitivity of the former to small variations in flow and culture conditions.

Despite their high sensitivities to small perturbations, stem cell cultivations in microfluidic reactors have produced many useful types of cells for disease treatment more effectively and without the ill effects of long-term drug therapy.

Tissue engineering is the second high value area of applications of microbioreactors. This inter-disciplinary area blends the principles of biology and engineering to develop functional tissues in the laboratory, starting from precursor cells such as stem cells [195]. Microreactors are more suitable than macroscale bioreactors to generate 3D tissue constructs because the former lend themselves more readily to the integration of microvalves,

microactuators, micromixers and microcroseparators, thereby allowing for low mass transfer limitations and accurate synchronous sterile operation (see Section 6).

Most of the currently used tissue engineering techniques grow the tissue cells on macrostructured porous scaffolds. This approach has been successful for the generation of simple constructs relying on the intrinsic natural ability of cells and tissue to self-generate [196]. However, this natural ability has limitations and does not enable the growth of complex thick tissues. This difficulty is being overcome by new designs for scaffolds and other micro-devices that allow fine control over cellular positioning, organization and interactions. For example, micropatterning integrates micro- and nanofabrication techniques with materials science and surface engineering to produce devices that allow deep exploration of embedded cells [197]. Such devices have smooth integration of microbioreactors with other microdevices that facilitates the formation of chondrocytes, a key component of tissue synthesis. Chondrocytes are mature cells present in cartilage, and they perform a number of vital functions in a tissue. The progenitors of these cells arise in the bone marrow in the form of stem cells. When stem cells differentiate into cartilage cells, they start as chondroblasts; these chondroblasts secrete chondrin, the main constituent of cartilage. Once a chondroblast becomes totally engulfed by chondrin, it is a mature chondrocyte [198].

Thus, the formation of chondrocytes is a fundamental requirement of tissue synthesis in the laboratory, and much attention has been devoted to this process. Barbucci and colleagues [199] showed that micropatterned hyaluronic acid surfaces induced higher adhesion, migration and alignment of knee articular cartilage than was possible with homogeneous surfaces. Cells cultured on to a microarray of micropatterned surfaces also maintain their morphology and their ability to retain important phenotypic features of the chondocytes [200]. Networks of microfluidic reactors also produce highly uniform flows that mimic physiological patterns, thus reproducing biological fidelity [201]. This similarity has been utilized beneficially (a) to create a functional liver organ that survived up to 2 weeks [202] and (b) to fabricate a multiwell with a micropatterned architecture of collagen that reproduces the phenotypic behavior of primary rat hepatocytes [203].

Microbioreactors used in tissue engineering offer a number of benefits: (a) they enable high density cultivation of cells and (b) they can sometimes be used as extracorporeal devices, notably to liver and kindly diseases. Such benefits have spawned a rapidly increasing number of studies of cell culturing for tissue growth that address different aspects of the technology. Wu et al. [204] were interested in developing a perfusions-based micro-3D platform for rapid screening of potential drugs against liver and kidney malfunctions. Korin et al. [194], on the other hand, focused on long-term culturing of human foreskin fibroblasts to understand the relationship between design parameters and cell behavior. In another study, Wu et al. [205] developed a microbioreactor with a PDMS-treated surface to understand the optimum conditions for long-term stable cultures of chondrocytes.

Among other notable instigations, Chin et al. [206] developed a microbioreactor array for high throughput monitoring of stem cell proliferation. Lee et al. [207] reported a novel 3D direct printing technique to construct hydrogel scaffolds containing fluidic microchannels. Bettinger and associates [208] fabricated a microfluidic platform from poly(glycerol sebacate) (PGS) that stacked layers of microfluidic structures to form a 3D network of scaffolds to promote the adhesion and proliferation of hepatocyte (HepG2) cells. The efficacy of their design was demonstrated by the production of albumin by the HepG2 cells.

Lee et al.'s [207] printing technique points to the possibility of using microcontact printing and templating to synthesize artificial microtissue structures without requiring scaffolding. This is an important development because scaffold-free synthesis is simpler than with scaffolds, and natural assembly forces do not interfere with cell regulation, thus establishing conditions where tissue growth is not hundred by limitations in the supply of nutrients and oxygen [209].

In addition, scaffold biodegradation (which is desirable) can lead to (undesirable) inflammatory reaction [210].

Recognition of the benefits of scaffold-free synthesis has led to many recent studies with different objectives and different systems. Ando and coworkers have described two such experiments. In the first one they generated mesenchymal stem cell (MSC)-based tissue-engineered constructs (TECs) for *in vivo* repair of porcine chondral defects. The TECs exhibited high expression of glycosaminoglycan and chondrogenic marker genes, and could initiate repair with a chondrogenic like tissue that became biologically integrated to the adjacent tissue. Their follow-up work [212] generated TECs with human synovial mesenchymal stem cells. TECs developed in a chondrogenic culture medium containing ascorbic acid-2-phosphate showed high stiffness, resistance to load and expression of chondrogenic marker genes, suggesting promising applications for cartilage repair.

Hayes et al.'s [213] interest was in the mechanistic aspects of the macromolecular organization of tissue-engineered neocartilage grafts, their *in vitro* development and the effect of chondrocyte differentiation. With such mechanistic information, current work is more directly oriented toward applications. Hadidi and Athanasiou [214] concentrated on repair of the knee meniscus fibrocartilage (KMF) since this gets frequently injured (about 1 million cases a year in the US and Europe). They reported a self-assembling process to synthesize fibrocartilage, which is free of scaffolds and relies on cell-to-cell interactions. On treatment with signaling phospholipid lysophosphatidic acid (LPA), the TECs displayed increased tensile properties, collagen organization and cytoskeleton reorganization, thus making them potential candidates to augment damaged KMF. Another application, from Liu et al. [215], describes the construction of scaffold-free bilayered tissue-engineered skin containing a capillary network. This skin exhibited a stratified epidermis after 7 days, which was promoted by the epithelium. Transmission election microscopy showed the capillaries to be microblood vessels, indicating the likely suitability of such tissue-engineered skin for grafting on to damaged areas. The commercial value of such application-oriented work is underlined by U.S. Patent # 20080004713A1 [216], which describes a process to make implantable synthetic tissue for surgical applications. The tissue is claimed to provide a therapy and medicament to repair and/or regenerate damaged tissue with biological compatibility with the surrounding tissue.

Miniaturizing the microbioreactor: liquid droplets as nanoreactors

The theory and applications considered above pertain mainly to single-phase homogeneous systems. While inter-phase mass transfer has been accounted for in some instances, the reactions still occur in the bulk liquid phase. Lately, however there is a discernible shift toward microreactions in two-phase emulsions. The reactants diffuse inward from the continuous phase to the dispersed phase, where the reactions take place. The products may either be retained within the dispersed droplets or they may be discharged into the continuous phase.

The use of liquid droplets offers a number of advantages over continuous phase microbioreactors. The surface-to-volume ratios of droplets being much larger than those of reactors, heat and mass transfer times and diffusion resistances are much shorter. Since many microfluidic reactions are diffusion-controlled [14-17], these improvements can greatly speed up the reaction. Moreover, the method allows for independent control of individual droplets, thus effectively generating hundreds or thousands of nanoreactors in one microreactor, each of which can be transported, mixed in a prescribed manner and analyzed [217, 218]. Typically, water-in-oil droplets for cellular systems are controlled in such a manner as to ensure that statistically each droplet has just one gene. This ensures that each droplet functions as an independently controllable nanoreactor.

The discussion above should suggest that the droplets are extremely small. Indeed it has been reported [219, 220] that droplets can be of the sizes of bacteria, i.e. with diameters ~1 μm

and volumes less than a femtoliter. Considering that about 50 of a reaction mixture can be dispersed into $>10^{10}$ individual droplet nanoreactors, this technology is ideally suited for high-throughput processes for the production of high-volume drugs as well as for rapid screening of biological or chemical reactions or products.

Two key issues determine the success of droplet based microreactors. One issue is the rapid generation and sustenance of uniform size droplets. Current methods can produce emulsions with < 3% polydispersity [221] at rates up to 10,000 droplets per second [222], but improvements in these benchmarks will speed up and economize these processes even further. The second issue is ensuring good micromixing inside the droplets. While this is physically different from micromixing in the bulk of a microbioreactor, laminar flow prevails in both situations, mixing at both levels are diffusion dominated, and both active and passive mixing methods are available in both situations. Active mixing in droplets is generally controlled by regulating electric fields that induce this kind of mixing. This technique has been demonstrated for droplets composed of water buffers and biological liquids in both air and oil [223, 224]. Electrowetting on a dielectric is an effective electrical method for both controlled mixing and controlled droplet generation [225].

Passive mixing does not use external intervention and relies instead on the design of the microchannel. As in the case of the bulk fluid, chaotic advection is also a prime method to promote mixing inside microdroplets, the degree of mixing being determined by the channel length and the number of reorientations a droplet undergoes from the inlet to the outlet [226]. However, winding channels may not always be the best design, as investigations with straight channels with internal protrusions have been observed to generate much better mixing inside droplets than with winding channels [227]. So it is possible that the protrusions help intra-droplet circulation by thinning the oil on one side of the droplet and increasing interfacial stresses [230].

Another benefit of carrying out reactions inside emulsion droplets is that nonspecific interactions between individual molecules and their immediate surroundings are favored more inside droplets than in microchannels *per se*. This enhancement may be attributed to the greater closeness between the reacting molecules and their surroundings in small droplets. It has been reported [231] that repulsive interactions enhance the rate and extent of macromolecular interactions whereas attractive interactions promote the association of molecules to adsorbing surfaces. This feature has been exploited by using cell sized water droplets coated by a phospholipid layer in mineral oil as nonreactors for two enzymatic reactions: calcein production and green fluorescence protein expression [232].

The many advantages of carrying out biological reactions reactions inside liquid droplets have generated a large number of recent applications. ATP synthesis using microbubbles [233], protein expression in emulsions [219], yellow fluorescent protein expression by *E. coli* [234] and DNA amplification [235] illustrate the variety of biological processes that are feasible in emulsion droplets. The scope of droplet based microreactors has been widened further by current reports [236, 237] of integrated multi-functional droplet-fused microreactors for the room temperature synthesis of needle shaped hyroxyapatite. Commercial interest in such reactors is exemplified by a U.S. Patent [238], which describes a microreactor comprising at least one ionic liquid that can be used for carrying out chemical and biochemical reactions. Ionic liquids offer the benefit of low volatility, easy miscibility and the facility to move them by electrowetting on dielectric and similar methods [239].

Even as *lab-on-a-chip* applications of droplet-based microdevices are getting adequately understood and applied, research has moved further into *chip-in-a-lab* applications. Explaining the difference, Streets and Huang [240] have pointed out that *lab-in-a-chip* technologies provide elegant solutions to practical problems whereas *chip-in-a-lab* applications offer innovative solutions to foreseeable needs (or "non-practical problems" [240]) by providing fundamental

insight into new life science avenues. Microfluidic droplets enable suitable miniaturizations for implementation of the new tools and techniques of *chip-in-a-lab* processes. In the context of the speed and widening scope of such microfluidic reactors, it is heartening but difficult to predict the enormously promising future of microreactors and other microdevices for biological and medical applications. Silic et al.'s [241] recent survey adds substance to these possibilities.

Concluding observations

Microbioreactors are swiftly emerging as a preferred device for high-throughput screening of cell cultures as well as for their large-scale cultivation for the synthesis of high-value biological products. The entry of commercial applications into the market even while research continues into the weaker aspects of these reactors underscores the usefulness of microbioreactors *vis-à-vis* conventional macrobioreactors.

Being of more recent origin than macroreactors, some of the limitations of microbioreactors are still being analyzed to obtain workable solutions. Fluid mixing is one major area that has been studied by many investigators but difficulties still remain. A key question to be answered is: what is the optimum level of micromixing required for a particular application and best may this be achieved under the laminar flow conditions of microbioreactors? The answer may emerge from a better appreciation and understanding of the physics of fluid flow through microtubes, especially under reacting conditions, which has received much less attention than the engineering aspects.

Optimization of microbioreactor performance depends on the formulation of mathematical models that have a strong physical foundation to be sufficiently reliable and are still simple enough to be implemented on a commercial scale. Optimization also depends on the availability of fast on-line microscale measurements, both of the reactor as a stand-alone unit and in conjunction with other microdevices. This implies that the success of a microbioprocess depends ultimately on the successful integration and on-line optimization of different inter-connected microdevices on a single chip. This requirement poses both bodeling and technological challenges, some of which have been discussed here.

Even though microbioreactor-based processes for cell cultures are still being perfected, their huge potential benefits are evident from the steady emergence of commercial applications. The understanding offered by recent research and by industrial experience with microbioreactors has led to radical shifts even in our perspective of microbioreactors *per se*; now liquid droplets and even living cells are being employed as nanoreactors. The presence of millions of such nanoreactors operating simultaneously and possibly interacting with one another in one microbioreactor, and the incorporation of many such reactors on to a single chip indicates the tremendous processing power of such microdevices within much smaller volumes and costs than are possible with conventional macroreactors.

References

1. Betts J. I., F. Beganz (2006). Miniature bioreactors. Current practices and future opportunities, Microbial Cell Factories, 5, 21.
2. Schapper D., M. N. H. Z. Alam, N. Szita, A. E. Lantz, K. V. Gernaey (2009). Application of microbioreactors in fermentation process development: A review, Analytical and Bioanalytical Chemistry, 395, 679-695.
3. Pasirayi G., V. Auger, S. M. Scott, R. K. S. M. Rahman, M. Islam, L. O' Hare L., Z. Ali (2011). Microfluidic bioreactors for cell culturing: A review, Micro and Nanosystems, 3, 137-160.
4. Hegab H. M., A. El Mekaway, T. Stakenborg (2013). Review of microfluidic microbioreactor technology for high throughput submerged microbiological cultivation, Biomicrodevices, 7, 021502.
5. Szita N., P. Boccazzi, Z. Zhang, P. Boyle, A. J. Sinskey, K. F. Jensen (2005). Development of a multiplexed microbioreactor for high-throughput processing, Lab on a Chip, 5, 819-826.

6. Zhang Z., P. Bocazzi, Z. Zhang, P. Boyle, A. J. Sinskey, K. F. Jensen (2006). Microchemostat-microbial continuous culture in a polymer-based instrumented micro bioreactor, Lab on a Chip, 6, 906-913.
7. Buchenauer A., M. C. Hofmann, M. Funke, J. Buchs, W. Mokwa, U. Schnakenborg (2009). Micro-bioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices, Biosensors and Bioelectronics, 24, 1411-1416.
8. Sotowa K. I., S. Sugiyama, K. Nakagawa (2009). Flow uniformity in deep microchannel reactor under high throughput conditions, Organic Process Research & Development, 13, 1026-1031.
9. Walker G. M., H. C. Zeringue, D. J. Beebe (2004). Microenvironment design considerations for cellular scale studies, Lab on a Chip, 4, 1-97.
10. Weibel D. B., G. M. Whitesides (2006). Applications of microfluidics in chemical biology, Current Opinion in Chemical Biology, 10, 584-591.
11. Erickson D., D. Li (2004). Integrated microfluidic devices, Analytica Chimica Acta, 507, 11-26.
12. Patnaik P. R. (2011). Multi-unit integration in microfluidic processes: Current status and future horizons, International Journal Bioautomation, 15(2), 77-84.
13. Ottino J. M., S. Wiggins (2004). Introduction to mixing in microfluidics, Philosophical Transactions of the Royal Society London, Series A, 362, 923-935.
14. Roberge D. M., M. Gottsponer, M. Eyholzer, N. Kockmann (2009). Industrial design, scale-up, and use of microreactors, Chemistry Today, 27, 8-11.
15. Hong J. W., S. R. Quake (2003). Integrated nanoliter systems, Nature Biotechnology, 21, 1179-1183.
16. Beebe D. V., G. A. Mensing, G. M. Walker (2002). Physics and applications of microfluidics in biology, Annual Reviews of Biomedical Engineering, 4, 261-286.
17. Squires T. M., S. R. Quake (2005). Microfluidics: Fluid physics at the nanoliter scale, Reviews of Modern Physics, 77, 977-1026.
18. Chou H. P., M. A. Unger, S. R. Quake (2001). A microfabricated rotary pump, Biomedical Microdevices, 3, 323-330.
19. Aref H. (2002). The development of chaotic advection, Physics of Fluids, 14, 1315-1325.
20. Stroock A. D., G. J. McGraw (2004). Investigation of the staggered herringbone mixer with a simple analytical model, Philosophical Transactions of the Royal Society, London, Series A, 362, 971-986.
21. Williams M. S., K. J. Longmuir, P. Yager (2008). A practical guide to the staggered herringbone mixer, Lab on in Chip, 8, 1121-1129.
22. Gu H., M. H. G. Duits, F. Mugele (2011). Droplets formation and merging in two-phase flow microfluidics, International Journal of Molecular Science, 12, 2572-2597.
23. Braun D., N. L. Goddard, A. Libchaber (2003). Exponential DNA replication by laminar convection, Physical Review Letters, 91, 158103.
24. Gad-el Hak M. (1999). The fluid mechanics of microdevices – The Freeman Scholar lecture, Journal of Fluids Engineering, 121, 5-33.
25. Buchs J. (2001). Introduction to advantages and problems of shaken cultures, Biochemical Engineering Journal, 7, 91-98.
26. Humphrey A. (1998). Shake flask to fermenter: What have we learned?, Biotechnology Progress, 14, 3-7.
27. Tucker K. G., C. R. Thomas (1994). Inoculum effects on fungal morphology: Shake flasks *vs* agitated bioreactors, Biotechnology Techniques, 8, 153-156.
28. Anderlei T., J. Buchs (2001). Device for sterile online measurement of the oxygen transfer rate in shaking flasks, Biochemical Engineering Journal, 7, 157-162.
29. Muller N., P. Girard, D. L. Hacker, M. Jordan, F. M. Wurm (2005). Orbital shaker technology for the cultivation of mammalian cells in suspension, Biotechnology and Bioengineering, 89, 400-406.
30. Isett K., H. George, W. Herber, A. Amanullah (2007). Twenty-four well plate miniature bioreactor high-throughput system: Assessment for microbial cultivations, Biotechnology and Bioengineering, 98, 1017-1028.

31. Elmahdi I., F. Baganz, K. Dixon, T. Harrop, D. Sugden, G. J. Lye (2003). pH control in microwell fermentations of *S. erythraea* CA340: Influence of biomass growth kinetics and erythromycin biosynthetics, Biochemical Engineering Journal, 16, 299-310.
32. Ferraria-Torrs C., M. Micleletti, G. J. Lye (2005). Microscale process evaluation of recombinant biocatalyst libraries: application to Bayer-Villiger monooxygenase catalyzed lactone synthesis, Biosystems and Bioengineering, 28, 83-93.
33. Lye G. J., P. A. Shamlou, F. Baganz, P. A. Dalby, J. M. Woodley (2003). Accelerated design of bioconversion processes using automated microscale processing techniques, Trends in Biotechnology, 21, 29-37.
34. Micheletti M., T. Barrett, S. D. Doig, E. Baganz, M. S. Levy, J. M. Woodley, G. J. Lye (2006). Fluid mixing in shaken bioreactors: Implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures, Chemical Engineering Science, 61, 2939-2949.
35. Hermann R., M. Lehmann, J. Buchs (2003). Characterization of gas-liquid mass transfer phenomena in microtiter plates, Biotechnology and Bioengineering, 81, 178-186.
36. http://www.sigmaaldrich.com/labware/labware-products.html/ (Last access 11 Mart 2015).
37. Zimmermann H. F., G. T. John, H. Trauthwein, U. Dingerdissen, K. Huthmacher (2003). Rapid evaluation of oxygen and water permeation through microplate sealing tapes, Biotechnology Progress, 19, 1061-1063.
38. De Jesus M. J., P. Girard, M. Bourgeois, G. Baumgartner, B. Jacko, H. Amstutz, F. M. Wurm (2004). TubeSpin satellites: A fast track approach for process development with animal cells using shaking technology, Biochemical Engineering Journal, 17, 217-223.
39. Puskeiler R., K. Kaufmann, D. Weuster-Botz (2005). Development, parallelization, and automation of a gas-inducing milliliter-scale bioreactor for high-throughput bioprocess design (HTBD), Biotechnology and Bioengineering, 89, 512-523.
40. Szita N., P. Boccazzi, Z. Zhang, P. Boyle, A. Sinskey, K. Jensen (2005). Development of a microplexed microbioreactor system for high-throughput bioprocessing, Lab on a Chip, 5, 819-826.
41. Schapper D., S. M. Stocks, N. Szita, E. A. Lantz, K. V. Gernaey (2010). Development of a single-use microbioreactor for cultivation of microorganisms, Chemical Engineering Journal, 160, 891-898.
42. Holcomb R. E., L. J. Mason (2011). Culturing and investigation of stress-induced lipid accumulation in microalgae using microfluidic device, Analytical and Bioanalytical Chemistry, 400, 245-253.
43. Lee K. S., P. Boccazzi, A. J. Sinskey, R. J. Ram (2011). Microfluidic chemostat and turbidostat with flow rate, oxygen and temperature control for dynamic continuous culture, Lab on a Chip, 11, 1730-1739.
44. Lamping S. R., H. Zhang, B. Allen, P. Ayazi Shamlou (2003). Design of a prototype miniature bioreactor for high throughput automated bioprocessing, Chemical Engineering Science, 58, 747-758.
45. Ge X., M. Hanson, H. Shen, Y. Kostor, K. A. Bronson, D. D. Fry, A. R. Moreira, G. Rao (2006). Validation of an optical sensor-based high-throughput bioreactor system for mammalian cell culture, Journal of Biotechnology, 22, 293-306.
46. Harms P., Y. Kostov, J. A. French, M. Soliman, M. Anjanappa, A. Rao, G. Rao (2006). Design and performance of a 24-station high-throughput microbioreactor, Biotechnology and Bioengineering, 93, 6-13.
47. Weuster-Botz D. (2005). Parallel reactor systems for bioprocess development, Advances in Biochemical Engineering/Biotechnology, 92, 125-143.
48. Doig S. D., A. Diep, F. Baganz (2005). Characterization of a novel miniaturized bubble column bioreactor for high throughput cell cultivation, Biochemical Engineering Journal, 23, 97-105.
49. Kantarci N., F. Borak, K. O. Ulgen (2005). Bubble column reactors, Process Biochemistry, 40, 2263-2283.
50. Hessel V., S. Hardt, H. Lowe (2004). Chemical Microprocess Engineering. Fundamentals, Modelling and Reactions, Wiley-VCH, Weinheim, Germany.
51. Villena G. K., M. Gutierrez-Correa (2006). Production of cellulose by *Aspergillus niger* biofilms developed on polyester cloth, Letters in Applied Microbiology, 43, 262-268.

52. Jensen K. F. (2001). Microreaction engineering – is small better? Chemical Engineering Science, 56, 293-303.
53. McMullen J. P., K. F. Jensen (2011). Rapid determination of reaction kinetics with an automated microfluidic system, Organic Process Research & Development, 15, 398-407.
54. Hessel N., H. Lowe, F. Schonfeld (2009). Micromixers – a review on passive and active mixing, Chemical Engineering Science, 60, 2479-2501.
55. Jayaraj S., S. Kang, Y. K. Suh (2007). A review on the analysis and experiment of fluid flow and mixing in microchannels, Journal of Mechanical Science & Technology, 21, 536-548.
56. Suh Y. K., S. Kang (2010). A review on mixing in microfluidics, Micromachines, 1, 82-111.
57. Lee C.-Y., C.-L. Chang, Y.-N. Wang, L. M. Fu (2011). Microfluidic mixing: A review, International Journal of Molecular Sciences, 12, 3263-3287.
58. Wu Z., D. Li (2008). Micromixing using induced-charge electrokinetic flow, Electrochemica Acta, 53, 5827-5835.
59. Lee S. H., D. Van Noort, S. Y. Lee, B. T. Zhang, T. H. Park (2009). Effective mixing in a microfluidic chip using electrokinetic particles, Lab on a Chip, 9, 479-482.
60. Liu R. H., J. Yang, M. Z. Pindera, M. Athavale, P. Grodznisk (2002). Bubble-induced acoustic micromixing, Lab on a Chip, 2, 151-157.
61. Mashimo T., R., Shibuya, K. Terashima (2012). Piezoelectric micromixer using a swirling motion, Proc. of 16th International Conference on Miniaturized Systems for Chemistry and Life sciences, Okinawa, Japan, 1840-1842.
62. Ahmed D., X. Mao, B. K. Juluri, T. J. Huang (2009). A fast microfluidic mixer based on acoustically driven sidewall-trapped microbubbles, Microfluidics and Nanofluidics, 7, 727-731.
63. Luong T-D., V-N. Phan, N.-T. Nguyen (2011). High-throughput micromixers based on acoustic streaming induced by surface acoustic wave, Microfluidics and Nanofluidics, 10, 619-625.
64. Deval J., P. Tabeling, C-M. Ho (2002). A dielectrophoretic chaotic mixer, Proc. of 15th IEEE Conference on Microelectromechanical Systems, Las Vegas, USA, 36-39.
65. Campisi M., D. Accoto, F. Damiani, P. Dario (2011). A soft-lithographed chaotic electrokinetic micromixer for efficient chemical reactions in lab-on-chips, Journal of Micro- and Nano-Mechanics, 5, 69-76.
66. Wu Z., D. Li (2008). Micromixing using induced-charge electrokinetic flow, Electrochemicl Acta, 53, 5827-5835.
67. Chen C-K., C-C. Cho (2008). Electrokinetically driven flow mixing utilizing chaotic electric fields, Microfluidics and Nonofluidics, 5, 785-793.
68. Lim C. Y., Y. C. Lam, C. Yang (2010). Mixing enhancement in microfluidic channel with a contraction under periodic electro-osmotic flow, Biomicrofluidics, 4, 014101.
69. Kang J., H. S. Heo, Y. K. Suh (2008). LbM simulation on mixing enhancement by the effect of heterogenous zeta-potential in a microchannel, Journal of Mechanical Science and Technology, 22, 1181-1191.
70. Shin S. M., I. S. Kang, Y.-K. Cho (2005). Mixing enhancement by using electrokinetic instability under time-periodic electric field, Journal of Micromechanics and Microengineering, 15, 455.
71. Chun H., H. C. Kim, T. D. Chang (2008). Ultrafast active mixer using polyelectrolyte ion extractor, Lab on a Chip, 8, 764-771.
72. Zhang Z., C.-H. Yim, M. Lin, X. Cao (2008). Quantitative characterization of micromixing simulation, Biomicrofluidics, 2, 034104.
73. Du Y., Z. Zhang, C.-H. Yim, M. Lin, X. Cao (2010). A simplified design of the staggered herringbone micromixer for practical applications, Biomicrofluidics, 4, 024105.
74. Gleeson J. P., J. West (2002). Magnetohydrodynamic micromixing, Nanotechnology, 1, 318-321.
75. Wang Y., J. Zhe, B. T. F. Chung, P. Dutta (2008). A rapid magnetic particle driven micromixer, Microfluidics and Nanofluidics, 4, 375-389.
76. Wen C.-Y., C.- P. Yeh, C.-H. Tsai, L.-M. Fu (2009). Rapid magnetic microfluidic mixer utilizing AC electromagnetic field, Electrophoresis, 30, 4179-4186.
77. Niu X., Y. K. Lee (2003). Efficient spatio-temporal chaotic mixing in microchannels, Journal of Micromechanics and Microengineering, 13, 454-462.
78. Pradere C., M. Joanicot, J. C. Batsale, J. Toutain, C. Gourdon (2006). Processing of temperature field in chemical microreactors with infrared thermography, QIRT Journal, 3, 117-135.

79. Xu B., T. N. Wong, N.-T. Nguyen, Z. Che, J. C. K. Chai (2010). Thermal mixing of two miscible fluids in a T-shaped microchannel, Biomicrofluidics, 4, 044102.
80. Mashimo T., R. Shibuya, K. Terashima (2012), Piezoelectric micromixer using a swirling motion, Proc. of 16th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Okinawa, Japan, 1840-1842.
81. Martinez-Lopez J. I., S. Ozuna-Chacon, L. D. Garza-Gacia, E. Garia-Lopez, M. M. Alvarez, V. M. Coello-Cardenas, H. R. Siller-Carillo, C. A. Rodriguez-Gonzalez, A. Elias-Zuniga (2012). Design and evaluation of a piezoelectric micropump perfusion system for a CHO microbioreactor, available at http:/www.bartels-microtechnik.de/images/stries/components/download/promed2012%20isreal%20martinez.pdf (Last access 11 March 2015).
82. Kongthon J., S. Devasia (2013). Iterative control of a piezoactuator for evaluating biomimetic, cilia-based micromixing, IEEE/ASME Transactions on Mechatronics, 18, 944-953.
83. Ukita Y., T. Asano, K. Fujiwara, K. Matsui, M. Takeo, S. Negoro, T. Kanie, M. Katayama, Y. Utsumi (2008). Application of vertical microreactor stack with polystylene microbeads to immunoassay, Sensors and Actuators A, 145-146, 449-455.
84. Eisner T. (2005). For the Love of Insects, Harvard University Press.
85. Srinivasan B., J. S. Lee, J. Hohnbaum, S. Tung, J.-W. Kim (2010). Performance evaluation of a pneumatic-based micromixer for bioconjugation reaction, Proc. of 5th IEEE International Conference on Nano/Micro Engineered and Molecular Systems (NEMS), Xiamen, China, 810-814.
86. Zhu X., T. Bersano-Begey, Y. Kamotani, S. Takayama (2006). Microbioreactors. Encyclopedia of Biomedical Devices and Instrumentations, Wiley & Sons, New York.
87. Jensen K. H., M. N. Alam, B. Scherer, A. Lambrecht, N. A. Mortensen (2008). Slow-light enhanced light-matter interactions with applications in gas sensing, Optical Communications, 281, 5335-5339.
88. Perez-Arllano I., G. Pere-Martinez (2003). Optimization of the green fluorescent protein (GFD) expression from a lactose-inducible promoter in *Lactobacillus casei*, FEMS Microbiology Letters, 222, 123-127.
89. Su W. W. (2005). Fluorscent protein as tools to aid protein production, Microbial Cell Factories, 12, 1-6.
90. Zanzotto A., P. Boccazzi, N. Gorret, T. K. Van Dyk, A. J. Siniskey, K. F. Jensen (2006). *In situ* measurement of bioluminescence and fluorescence in an integrated microbioreactor, Biotechnology and Bioengineering, 93, 40-47.
91. Bower D. M., K. S. Lee, R. J. Ram, K. L. J. Prather (2012). Fed-batch microbioreactor platform for scale down and analysis of a plasmid DNA production process, Biotechnology and Bioengineering, 109, 1976-1986.
92. Krommenhoek E. E., M. Van Leeuwen, H. Gardeniers, W. M. Van Gulik, A. Van den Berg, X. Li, M. Ottens, L. A. M. Van der Wielen, J. J. Heijnen (2008). Lab-scale fermentation tests of microchip with integrated electrochemical sensors for pH, temperature, dissolved oxygen and viable biomass concentration, Biotechnology and Bioengineering, 99, 884-892.
93. Zanzotto A., N. Szita, P. Boccazzi, P. Lessard, A. J. Sinskey, K. F. Jensen (2004). Membrane-aerated microbioreactor for high-throughput bioprocessing, Biotechnology and Bioengineering, 87, 243-254.
94. Ding L., D. Du, X. Zhang, H. Ju (2008). Trends in cell-based electrochemical biosensors, Current Medicinal Chemistry, 15, 3160-3170.
95. Xiang G. X., L. B. Pan, L. H. Huang, Z. Y. Yu, X. D. Song, J. Cheng, W. L. Xing, Y. X. Zhou (2007). Microelectrode array-based system for micropharmacological application with cortical neurons cultured *in vitro*, Biosensors and Bioelectronics, 22, 2478-2484.
96. Liu Q.J., H. R. Huang, H. Cai, Y. Xu, Y. Li, R. Li, P. Wang (2007). Embryonic stem cells as a novel cell source of cell-based biosensors, Biosensors and Bioelectronics, 22, 810-815.
97. De Jong J. (2008). Application of membrane technology in microfluidic devices, PhD Thesis, University of Twente, Twente, The Netherlands.
98. Maharbiz M. M., W. J. Holtz, R. T. Howe, J. D. Keasling (2004). Microbioreactor arrays with parametric control for high-throughput experimentation, Biotechnology and Bioengineering, 85, 376-381.

99. Krommenhoek E. E., J. G. E. Gardeniers, J. G. Bomer, X. Li, M. Ottens, G. W. K. Van Dedem, M. Van Leeuwen, W. M. Van Gulik, L. A. M. Van der Wielen, J. J. Heijnen (2007). Integrated electrochemical sensor array for online monitoring of yeast fermentations, Analytical Chemistry, 79, 5567-5573.
100. Boccazzi P., Z. Zhang, K. Kurosawa, N. Szita, S. Bhattacharya, K. F. Jensen, A. J. Sinskey (2006). Differential gene expression profiles and real-time measurements of growth parameters in Sacchamomyces cerevisiae grown in microliter-scale bioreactors equipped with internal stirring, Biotechnology and Bioengineering, 22, 710-717.
101. Mengaud V., J. Josserand, H. H. Girault (2002). Mixing processes in a zig-zag micochannel: Finite element simulations and optical study, Analytical Chemistry, 74, 4279-4286.
102. Liu Y. Z., B. J. Kim, H. J. Sung (2004). Two-fluid mixing in a microchannel, International Journal of Heat and Fluid Flow, 25, 986-995.
103. Jen C. P., C. Y. Wu, Y. C. Lin, C. Y. Wu (2003). Design and simulation of a micromixer with chaotic advection in microchannels, Lab on a Chip, 3, 77-81.
104. Kim D. S., S. W. Lee, T. H. Kwon, S. S. Lee (2004). A barrier embedded chaotic micromixer, Journal of Micromechanics and Microengineering, 14, 798-805.
105. Funke M., A. Buchenauer, U. Schnakenberg, W. Mokwa, S. Diederichs, A. Mertens, C. Muller, F. Kensy, J. Buchs (2010). Microfluidic biolector-microfluidic bioprocess control microtiter plates, Biotechnology and Bioengineering, 107, 497-505.
106. Bower D. M., K. S. Lee, R. J. Ram, K. L. J. Prather (2012). Fed-batch microbioreactor platform for scale down and analysis of a DNA production process, Biotechnology and Bioengineering, 109, 1976-1986.
107. Fonseca L. P., U. C. B. Martins, P. P. Freitas (2011). Microreactors and microdevices for analytical and biosensor applications, Proc. of 1st Portuguese Meeting in Bioengineering (ENBENG), Lisbon, Portugal, 1-4.
108. Balagadde F. K., L. You, C. L. Hansen, F. H. Arnold, S. R. Quake (2005). Long-term monitoring of bacteria undergoing programmed population control in a microthermistat, Science, 309, 137-139.
109. Hamber J. E. (2012). Bioreactor design and bioprocess controls for industrialized cell processing, Bio Process International, 10(6), 22-33.
110. Roberge D. M., M. Gothsponer, M. Eyholzer, N. Kochmann (2009). Industrial design, scale-up and use of microreactors, Chemistry Today, 27, 8-11.
111. Buchenauer A., Microbioreactors and microfluidic control, http://www.iwe1.rwth-aachen.de/englisch/4-forschung/2-projekte/mrsbio.pdf (Last access 11 March 2015).
112. Zhang Z., N. Szita, P. Boccazzi, A. J. Sinskey, K. F. Jensen (2003). Monitoring and control of cell growth in fed-batch microbioreactors, Proc. of 7th International Conference on Miniaturized Chemical and Biochemical Analysis Systems. Squaw Valley, California, 765-768.
113. Howell P. B., D. R. Mott, S. Fertig, C. R. Kaplan, J. P. Golden, E. S. Oram, F. S. Ligler (2005). A microfluidic mixer with grooves placed on the top and bottom of the channel, Lab on a Chip, 5, 524-530.
114. Comesasca M., M. Kaufman, I. Manas-Zloczower (2006). Staggered passive micromixer with fractal surface patterning, Journal of Micromechanics and Microengineering, 16, 2298-2311.
115. Stroock A. D., S. K. W. Dertinger, A. Ajdari, I. Mezic, H. A. Stone, G. M. Whitesides (2002). Chaotic mixer for microchannels, Science, 295, 647-651.
116. Doku G. N., W. Veerboom, D. N. Reinhoudt, A. Van den Berg (2005). On-chip multiphase chemistry − a review of microreactor design principles and reagent contacting modes, Tetrahedron, 61, 2733-2742.
117. Tofteberg T., M. Skilmowski, E. Andreassen, O. Greschke (2010). A novel passive micromixer: lamination in a planar channel system, Microfluidics and Nanofluidics, 2, 209-215.
118. Buchegger W., C. Wagner, B. Lendl, M. Kraft, M. Vellekoop (2011). A highly uniform lamination micro mixer with wedge shaped inlet channels for fine resolved infrared spectroscopy, Microfluidics and Nanofluidics, 10, 889-897.
119. Buchegger W., A. Haller, S. Van den Drieche, M. Kraft, B. Lendl, M. Vellekoop (2012). Studying enzymatic bioreactions in a millisecond microfluidic flow mixer, Biomicrofluidics, 6, 012803.

120. Lee C. Y., C. F. Lin, M. F. Hung, C. H. Tsai, L. M. Fu (2006). Experimental and numerical investigation into mixing efficiency of micromixer with different geometric barriers, Materials Science Forum, 505-507, 391-396.
121. Yang J.-T., K.-J. Huang, K.-Y. Tung, I.-C. Hu, P.-C. Lyu (2007). A chaotic micromixer modulated by constructive vortex agitation, Journal of Micromechanics and Microengineering, 17, 2084-2092.
122. Kim D. S., S. H. Lee, T. H. Kwon, C. H. Ahn (2005). A serpentine laminating micromixer combining splitting/recombination and advection, Lab on a Chip, 5, 739-747.
123. Liu R. H., M. A. Stremler, K. V. Sharp, M. G. Olsen, J. G. Santiago, R. J. Adrian, H. Aref, D. J. Beebe (2000). Passive mixing in a three-dimensional selective microchannel, Journal of Microelectromechanical Systems, 9, 190-197.
124. Kang T. G., M. K. Singh, P. D. Anderson, H. E. H. Meijer (2009). A chaotic serpentine mixer efficient in the creeping flow regime: From design concept to optimization, Microfluidics and Nanofludics, 7, 783-794.
125. Neerinex P. E., R. P. S. Denteneer, S. Peelen, H. E. H. Meijer (2011). Compartmental mixing using multiple splitting, stretching and recombining flows, Macromolecular Materials and Engineering, 296, 349-361.
126. Moon D., K. B. Migler (2010). Forced assembly and mixing of melts via planar polymer micromixing, Polymer, 51, 3147-3155.
127. Cha J., J. Kim, S.-K. Ryu, J. Park, Y. Jeong, S. Park, S. Park, H. C. Kim, K. Chun (2006). A highly efficient 3D micromixer using soft PDMS bonding, Journal of Micromechanics and Microengineering, 16, 1778-1782.
128. Hardt S., H. Pennemann, H. Schonfeld (2006). Theoretical and experimental characterization of a low-Reynolds number split-and-recombine mixer, Microfluidics and Nanofludics, 2, 237-248.
129. Ansari M. A. A., K.-Y. Kim, K. Anwar, S. M. Kim (2010). A novel passive micromixer based on unbalanced splits and collisions of fluid streams, Journal of Micromechanics and Microengineering, 20, 055007.
130. Jen C. P., C. Y. Wu, Y. C. Lin, C. Y. Wu (2003). Design and simulation of the micromixer with chaotic advection in microchannels, Lab on a Chip, 3, 77-81.
131. Hashimoto M., G. M. Whitesides (2010). Formation of bubbles in a multi-section flow-focusing junction, Small, 6, 1051-1059.
132. Kim D. S., S. W. Lee, T. H. Kwon, S. S. Lee (2004). A barrier embedded chaotic micromixer, Journal of Micromechanics and Microengineering, 14, 798-805.
133. Kim D. S., S. W. Lee, T. H. Kwon, D. W. Cho (2004). A barrier embedded Kenics micromixer, Journal of Micromechanics and Microengineering, 14, 1294-1301.
134. Singh M. K., P. D. Anderson, H. E. H. Meijer (2009). Understanding and optimizing the SMX static mixer, Micromolecular Rapid Communications, 30, 362-376.
135. Tsai R.-T., C.-Y. Wu (2011). An efficient micromixer based on multidirectional vortices due to baffles and channel curvature, Biomicrofluidics, 5, 014103.
136. Sotowa K-I., A. Yamamoto, K. Nakagawa, S. Sugiyama (2011). Indentations and baffles for improving mixing rate in deep microchannel reactors, Chemical Engineering Journal, 167, 490-495.
137. Sotowa K.-I., K. Takagi, S. Sugiyama (2008). Fluid flow behavior and the rate of an enzymatic reaction in deep microchannel reactor under high-throughput condition, Chemical Engineering Journal, 135S, S30-S36.
138. Chen C.-K., C.-C. Cho (2008). A contrived active/passive scheme for enhancing the mixing efficiency of microfluidic devices, Chemical Engineering Science, 63, 3081-3087.
139. Guichardon P, L. Falk (2000). Characterization of micromixing efficiency by the iodide-iodate reaction system. Part I. Experimental procedure, Chemical Engineering Science, 55, 4233-4243.
140. Falk L., J. M. Commenge (2010). Performance comparison of micromixers, Chemical Engineering Science 65, 405-411.
141. Ottino J. M., W. E. Ranz, C. W. Macosko (1979). A lamellar model for analysis of liquid-liquid mixing, Chemical Engineering Science, 34, 877-890.
142. Kashid M., A. Renken, L. Kiwi-Minsker (2011). Mixing efficiency and energy consumption for five generic microchannel designs, Chemical Engineering Journal, 167, 436-443.

143. Schonfeld F., V. Hessel (2004). An optimized split-and-recombine micromixer with uniform chaotic mixing, Lab on a Chip, 4, 65-69.
144. Jain M., K. Nandakumar (2010). Novel index for micromixing characterization and comparative analysis, Biomicrofluidics, 4, 031101.
145. Chang C. C., R. J. Yang (2007). Electrokinetic mixing in microfluidic systems, Microfluidics and Nanofluidies, 3, 501-525.
146. Wu Z. M., D. Q. Li (2008). Micromixing using induced-charge electrokinetic flow, Electrochimica Acta, 53, 5827-5835.
147. Jain M., A. Yeung, K. Nadakumar (2009). Efficient micromixing using induced-charge electroosmosis, Journal of Microelectromechanical Systems, 18, 376-384.
148. Tian F. Z., B. M. Li, D. W. Kwok (2005). Trade-off between mixing and transport for electroosmotic flow in heterogeneous microchannels with nonuniform surface potentials, Langmuir, 21, 1126-1131.
149. Coleman J. T., D. Sinton (2005). A sequential injection microfluidic mixing strategy, Microfluidics and Nanofluidics, 1, 319-327.
150. Aoki N., S. Hasebe, K. Mae (2004). Mixing in microreactors: effectiveness of lamination segment as a form of feed on product distribution for multiple reactions, Chemical Engineering Journal, 101, 323-331.
151. Bailey R., F. Jones, B. Fisher, B. Elmore (2005). Enhancing design of immobilized enzyme microbioreactors using computational simulation, Applied Biochemistry and Biotechnology, 122, 639-652.
152. Li X., G. Van der Steen, G. W. K. Van Dedum, L. A. M. Van der Wielen, M. Van Leeuwen, W. M. Van Gulik, J. J. Heijnen, E. E. Krommenhoek, J. G. E. Gardeniers, A. Van den Berg, M. Ottens (2008). Improving mixing in microbioreactors, Chemical Engineering Science, 63, 3036-3046.
153. Li X., G. Van der Steen, G. W. K. Van Dedem, L. A. M. Van der Wielen, M. Van Leeuwen, W. M. Van Gulik, J. J. Heijnen, M. Ottens, E. E. Krommenhoek, J. G. E. Gardeniers, A. Van den Berg (2009). Application of direct fluid flow oscillations to improve mixing in microbioreactors, AIChEJ, 53, 2725-2736.
154. Chen Y. T., W.-F. Fang, Y.-C. Liu, J.-T. Yang (2011). Analysis of chaos and FRET reaction in split-and-recombine microreactors, Microfluidics and Nanofluidics, 11, 339-352.
155. Fernandes R. L., U. Kruhe, I. Nopens, A. D. Jensen, K. V. Gernaey (2012). Multi-scale modeling for prediction of distributed cellular properties in response to substrate spatial gradients in a continuously run microreactor, Computer-aided Chemical Engineering, 31, 545-549.
156. Schapper D., R. L. Fernandes, A. E. Lantz, F. Okkels, H. Bruis, K. V. Gernaey (2011). Topology optimized microbioreactors, Biotechnology and Bioengineering, 108, 786-796.
157. Kaul H., Z. Cui, Y. Ventikos (2013). A multi-paradigm modeling framework to simulate dynamic reciprocity in a bioreactor, PLoS ONE, 8, e59671.
158. Paulsson J. (2005). Models of stochastic gene expression, Physics of Life Reviews, 2, 157-175.
159. Patnaik P. R. (2009). External, extrinsic and intrinsic noise in cellular systems: Analogies and implications for protein synthesis, Biotechnology and Molecular Biology Reviews, 1, 123-129.
160. Wilkinson D. J. (2009). Stochastic modeling for quantitative description of heterogeneous biological systems, Nature Reviews Genetics, 10, 122-133.
161. Julien C., W. Whitford (2007). Bioreactor monitoring, modeling and simulation, BioProcess International, Supplement, 10-17.
162. Mellin J., S. R. Quake (2007). Microfluidic large-scale integration: the evolution of design rules for biological automation, Annual Reviews of Biophysical and Biomolecular Structures, 36, 231-231.
163. Hua Z. S., Y. M. Xia, O. Srivannavit, J. M. Rouillard, X. Zhou (2006). A versatile microreactor platform featuring a chemical-resistant microvalve array for addressable multiplex syntheses and assays, Journal of Micromechanics and Microengineering, 16, 1433-1443.
164. Liu J., C. Hansen, S. R. Quake (2003). Solving the "world-to-chip" interface problem with a microfluidics matrix, Analytical Chemistry, 75, 4718-4723
165. Marcus J. S., W. F. Anderson, S. R. Quake (2006). Microfluidic single cell mRNA isolation and analysis, Analytical Chemistry, 78, 3084-3089.

166. Liu J., M. Enzelberger, S. R. Quake (2002). A nanoliter rotary device for polymerase chain reaction, Electrophoresis, 23, 1531-1536.
167. West J., B. Karmata, J. R. Lillis, J. Glesson, J. Alderman, J. K. Collins, W. Lane, A. Mathewson, H. Berney (2002). Application of magnetohydrodynamic actuation to continuous flow chemistry, Lab on a Chip, 2, 224-230.
168. Yuen P. K., L. J. Kricka, P. Fortina, N. J. Panaro, T. Sakazume, P. Wilding (2001). Microchip module for blood sample preparation and nucleic acid amplification reactions, Genome Research, 11, 405-412.
169. Legally E. T., P. C. Simpson, R. A. Mathies (2000). Monolithic integrated DNA amplification and capillary electrophoresis analysis system, Sensors and Actuators B, 63, 138-146.
170. Khandurina J., T. E. McKnight, S. C. Jacobson, L. C. Waters, R. S. Foote, J. M. Ramsey (2000). Integrated system for rapid PCR-based DNA analysis in microfluidic devices, Analytical Chemistry, 72, 2995-3000.
171. Rodriguez I., M. Lesaicherre, Y. Tie, Q. Zou, C. Yu, J. Singh, L. T. Meng, S. Uppili, S. F. Y. Li, P. Gopalakrishnakone, Z. E. Selvanayagam (2003). Practical integration of polymerase chain reaction amplification and electrophoretic analysis in microfluidic devices for genetic analysis, Electrophoresis, 24, 172-178.
172. Hong J. W., T. Fujii, M. Seki, T. Yamamoto, I. Endo (2001). Integration of gene amplification and capillary gel electrophoresis on polydimethylsiloxane-glass hybrid microchip, Electrophoresis, 22, 328-333.
173. Lee C. C., A. Eliazov, C. J. Shu, Y. S. Shin, A. N. Dooley (2005). Multi-step synthesis of a radiolabeled imaging probe using integrated microfluidics, Science, 310, 1793-1796.
174. Losey M. W., R. J. Jackman, S. L. Firebaug, M. A. Schmidt, K. F. Jensen (2002). Design and fabrication of microfluidic devices for multiphase mixing and reaction, Micro electromechanical systems, 11, 709-717.
175. Brivio M., R. H. Fokkens, W. Verboom, D. N. Reinhoudt, N. R. Tas, M. Goed-Bloed, A. Van den Berg (2002). Integrated microfluidic system enabling biochemical reactors with on-line MALDI-TOF spectrometry, Analytical Chemistry, 74, 3972-3976.
176. Lee H. L. T., P. Boccazzi, R. J. Ram, A. J. Sinskey (2006). Microbioreactor arrays with integrated mixers and fluid injectors for high-throughput experimentation with pH and dissolved oxygen control, Lab on a Chip, 6, 1229-1235.
177. Buchenauer A., M. C. Hofman, M. Funke, J. Buchs, W. Mokwa, U. Schnakenberg (2009). Microbioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices, Biosensors and Bioelectronics, 24, 1411-1416.
178. Pohar A., P. Znidarsic-Plazl, I. Plazl (2012). Integrated system of a microbioreactor and a miniaturized continuous separator for enzyme catalyzed reactions, Chemical Engineering Journal, 189-190, 376-382.
179. Thorsen T., S. J. Maerkl, S. R. Quake (2002). Microfluidic large-scale integration, Science, 298, 580-584.
180. Yeo L. Y., H.-C. Chang, P. P. Y. Chan, J. R. Friend (2011). Microfluidic devices for bioapplications, Small, 7, 12-48.
181. Hessel V., C. Knobloch, H. Lowe (2008). Review on patents in microreactor and microprocess engineering, Recent Patents on Chemical Engineering, 1, 1-16.
182. Weigl B., G. Domingo, P. Labarre, J. Gerlach (2008). Towards non- and minimally instrumented microfluidics-based diagnostic devices, Lab on a Chip, 8, 1999-2014.
183. Zhao W., M. M. Ali, S. D. Aguirre, M. A. Brook, Y. Li (2008). Paper-based bioassays using gold nanoparticle colorimetric probes, Analytical Chemistry, 80, 8431-8437.
184. Reis N., C. N. Goncalves, A. A. Vicente, J. A. Teixeira (2006). Proof-of-concept of a novel microbioreactor for fast development of industrial bioprocesses, Biotechnology and Bioengineering, 95, 744-753.
185. Edlich A., V. Magdanz, D. Rasch, S. Demming, S. A. Zadech, R. Segura, C. Kahler, R. Radespiel, S. Buttgenbach, E. Franco-Lara, R. Krull (2010). Microfluidic reactor for continuous cultivation of *Saccharomyces cerevisiae*, Biotechnology Progress, 26, 1259-1270.
186. Gepstein L. (2002). Derivation and potential applications of human embryonic stem cells, Circulation Research, 91, 866-876.

187. Van Noort D., S. M. Ong, C. Zhang, S. Zhang, T. Arooz, H. Yu (2009). Stem cells in microfluidics, Biotechnology Progress, 25, 52-60.
188. Gupta K., D. H. Kim, D. Elison, C. Smith, A. Kundu, J. Tuan, K. Y. Suh, A. Levchenko (2010). Lab-on-chip devices as an emerging platform for stem cell biology, Lab on a Chip, 10, 2019-2031.
189. Chung B. G., L. A. Flanagan, S. W. Rhee, P. H. Schwartz, A. P. Lee, E. S. Monuki, N. L. Jeon (2005). Human neural stem cell growth and differentiation in a gradient generating microfluidic device, Lab on a Chip, 5, 401-406.
190. Kim L., M. D. Vahey, H. Y. Lee, J. Voldman (2006). Microfluidic arrays for logarithmically perfused embryonic stem cell culture, Lab on a Chip, 6, 394-406.
191. Park J. Y., S. K. Kim, D. H. Woo, E. J. Lee, J. H. Kim, S. H. Lee (2009). Differentiation of neural progenitor cells in a microfluidic chip-generated cytokine gradient, Stem Cells, 27, 2646-2654.
192. Korin N., A. Bransky, U. Dinnar, S. Levenberg (2009). Periodic "flow-stop" perfusion microchannel bioreactors for mammalian and human embryonic stem cell long-term culture, Biomedical Microdevices, 11, 87-94.
193. Wang D. Y., S. C. Wu, S. P. Lin, S. H. Hsiao, T. W. Chung, Y. Y. Huang (2011). Evaluation of transdifferentiation from mesenchymal stem cells to neuron-like cells using microfluidic patterned co-culture system, Biomedical Microdevices, 13, 1-10.
194. Korin N., A. Bransky, U. Dinnar, S. Levenberg (2006). The culture of human embryonic stem cells in microchannel perfusion bioreactors, Proc. of the SPIE6416, Biomedical Applications of Micro-and Nono-Engineering III, 64160N.
195. Chung B. G., L. Kang, A. Khademhosseini (2007). Micro- and nano-scale technologies for tissue engineering and drug discovery applications, Expert Opinion in Drug Discovery, 2, 1653-1668.
196. Coutinho D., P. Costa, N. Neves, M. E. Gromes, R. L. Reis (2011). Micro- and nanotechnology in tissue engineering, Chapter 1, In: Pallua N., C. U. Suschek (Eds.), Tissue Engineering, Springer-Verlag, Berlin, Heidelberg.
197. Nakamishi J., T. Takada, K. Yamaguchi (2008). Recent advices in cell micropatterning techniques for bioanalytical and biomedical sciences, Analytical Science, 24, 67-72.
198. http://www.wisegeek.org/what-are-chondrocytes.htm©2003-2013, Conjecture Corporation, Sparks, NV, USA (Last access 11 March 2015).
199. Barbucci R., P. Toricelli, M. Fin (2005). Proliferative and re-differentiative effects of photo-immobilized micro-patterned hyaluronan surfaces on chondrocyte cells, Biomaterials, 26, 7596-7605.
200. Petersen E. F., R. G. S. Spencer, E. W. McFarland (2002). Microengineering neocartilage scaffolds, Biotechnology and Bioengineering, 78, 801-804.
201. Barber R. W., D. R. Emerson (2008). Optimal design of microfluidic networks using biologically inspired principles, Microfluidics and Nanofluidics, 4, 179-191.
202. Borenstein J. T., H. Terai, K. R. King (2002). Microfabrication technology for vascularized tissue engineering, Biomedical Microdevices, 4, 167-175.
203. Khetani S. R., S. N. Bhatia (2008). Microscale culture of human liver cells for drug development, Nature Biotechnology, 26, 120-126.
204. Wu W.-H., S. B. Huang, Z. Cui (2008). Development of perfusion-based micro 3-D cell culture platform and its application for high throughput drug testing, Sensors and Actuators B, 129, 231-240.
205. Wu M. H., J. P. Urban, Z. Cui (2006). Development of PDMS microbioreactor with well-defined and homogeneous culture environment for chondrocyte 3-D culture, Biomedical Microdevices, 8, 331-340.
206. Chin V. I., P. Taupin, S. Sanga, J. Scheel, F. H. Gage, S. N. Bhatia (2004). Microfabricated platform for studying stem cell fates, Biotechnology and Bioengineering, 88, 399-415.
207. Lee W., V. Lee, S. Polio, P. Keegan, J. Lee, K. Fischer, J. Park, S. Yoo (2010). On-demand three-dimensional freeform fabrication of multi-layered hydrogel scaffold with fluidic channels, Biotechnology and Bioengineering, 105, 1178-1186.
208. Bettinger C. J., J. T. Borenstein, R. S. Langer (2005). Nanoscale materials science in biology and medicine: Biodegradable microfluidic scaffolds for vascular tissue engineering, Proc. of the Materials Research Society Symposium, Boston, MA, USA, 845, 25-30.

209. Kelm J. M., M. Fusenegger (2004). Microscale tissue engineering using gravity-enhanced cell assembly, Trends in Biotechnology, 22, 199-202.
210. Shimizu T., M. Yamato, A. Kikuchi, T. Okano (2003). Cell sheet engineering for myocardial tissue reconstruction, Biomaterials, 24, 2309-2316.
211. Ando W., K. Tateishi, D. A. Hart, D. Katakai, Y. Tanaka, K. Nakata, J. Hashimoto, H. Fujie, K. Shino, H. Yoshikawa, N. Nakamura (2007). Cartilage repair on in vitro generated scaffold-free tissue-engineered construct derived from porcine synovial mesenchymal stem cells, Biomaterials, 28, 5462-5470.
212. Ando W., K. Tateishi, D. Katakai, D. A. Hart, C. Higuchi, K. Nakata, J. Hashimoto, H. Fujie, K. Shino, H. Yoshikawa, N. Nakamura (2008). *In vitro* generation of a scaffold-free tissue-engineered construct (TEC) derived from human synovial mesenchymal stem cells: Biological and mechanical properties and further chondrogenic potential, Tissue Engineering Part A, 14, 2041-2049.
213. Hayes A. J., A. Hall, L. Brown, R. Tubo, B. Caderson (2007). Macromolecular organization and *in vitro* growth characteristics of scaffold-free neocartilage grafts, Journal of Histochemistry and Cytochemistry, 55, 853-866.
214. Hadidi P., K. A. Athanasiou (2013). Enhancing the mechanical properties of engineered tissue through matrix remodeling via the signaling phospholipid lysophosphatidic acid, Biochemical and Biophysical Research Communications, 433, 133-138.
215. Liu Y., H. Luo, X. Wang, A. Takemura, Y. R. Fang, Y. Jin, F. Suwa (2013). *In vitro* construction of scaffold-free bilayered tissue-engineered skin containing capillary networks, BioMedical Research International, Article ID 561410.
216. Nakamura N., H. Yoshikawa, W. Ando (2008). Scaffold-free self-organized 3D synthetic tissue, U.S. Patent 20080004713 A1.
217. Fair R. B. (2007). Digital microfluidics: is a true lab-on-chip possible?, Microfluidics and Nanofluidics, 3, 245-281.
218. Link D. R., E. Grasland-Mongram, A. Duri, F. Sarrazin, Z. D. Cheng, G. Cristobal, M. Marquez, D. A. Weitz (2006). Electric control of droplets in microfluidic devices, Angewandte Chemistry International Edition, 45, 395-402.
219. Griffths A. D., D. S. Tawfik (2006). Miniaturizing the laboratory in emulsion droplets, Trends in Biotechnology, 24, 395-402.
220. Taly V., B. T. Kelly, A. D. Griffiths (2004). Droplets as microreactors for high-throughput biology, Chem BioChem, 8, 263-272.
221. Anna S. L., N. Bontoux, H. A. Stone (2003). Formation of dispersions using "flow focusing" in microchannels, Applied Physics Letters, 82, 364-366.
222. O' Hare H. M., K. Johnsson (2005). The laboratory in a droplet, Chemical Biology, 12, 1255-1257.
223. Chatterjee D., B. Hetaythin, A. R. Wheeler, D. J. King, R. L. Garrell (2006). Droplet-based microfluidics with nonaqueous solvents and solutions, Lab on a Chip, 6, 199-206
224. Wheeler A. R., H. Moon, C. J. Kim, J. A. Loo, R. L. Garrell (2004). Electrowetting-based microfluidics for analysis of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry, Analytical Chemistry, 76, 4833-4838.
225. Paik P., V. K. Pamula, R. B. Fair (2003). Rapid droplet mixers for digital microfluidic systems, Lab on a Chip, 3, 253-259.
226. Bringer M. R., C. J. Gerdts, H. Song, J. D. Tice, R. F. Ismagilov (2004). Microfluidic systems of chemical kinetics that rely on chaotic mixing in droplets, Pilosophical Transactions of the Royal Society London Series A, 362, 1087-1104
227. Sarrazin F., L. Prat, N. DiMiceli, G. Cristabal, D. R. Link, D. A. Weitz (2007). Mixing characterization inside microdroplets engineered on a microcoalescer, Chemical Engineering Science, 62, 1042-1048.
228. Liau A., R. Karnik, A. Majumdar, J. H. D. Cate (2005). Mixing crowded biological solutions in milliseconds, Analytical Chemistry, 77, 7618-7625.
229. Cabral J. T., S. D. Hudson (2006). Microfluidic approach for rapid multi-component interfacial densitometry, Lab on a Chip, 6, 427-436.

230. Teh S.-H., R. Liu, L.-H. Hung, A. P. Lee (2008). Droplet micro fluidics, Lab on a Chip, 8, 198-220.
231. Minton A. P. (2006). How can biochemical reactions within cells differ from those in test tubes?, Journal of Cell Science, 199, 2863-2869.
232. Hase M., A. Yamada, T. Hamada, D. Baigl, K. Yoshikawa (2007). Manipulation of cells-sized phospholipid-coated microdroplets and their use as biochemical microreactors, Langmuir, 23, 348-352.
233. Choi H. J., C. D. Montemagno (2006). Biosynthesis within a bubble architecture, Nanotechnology, 17, 2198-2202.
234. Huebner A., M. Srisa-Art, D. Holt, C. Abell, F. Hollfelder, A. D. Demello, J. B. Edel (2007). Quantitative detection of protein expression in single cells using droplet microfluidics, Chemical Communications, 12, 1218-1220
235. Wang W., Z. X. Li, R. Luo, S. H. Lu, A. D. Xu, Y. J. Yang (2005). Droplet-based micro oscillating-flow PCR chip, Journal of Micromechanics and Microengineering, 15, 1369-1377.
236. Liu K., J. Qin (2013). Droplet-fused microreactors for room temperature synthesis of nanoscale needle-like hydroxyapatite, Nanotechnology, 24, 125602.
237. Shum H. C., A. Bandopadhyay, S. Bose, D. A. Weitz (2009). Double emulsion droplets as microreactors for synthesis of mesoporous hydroxyapatite, Chemistry of Materials, 24, 5548-5555.
238. Marchand G., F. Yinet, G. Delappiere, F. Hassine, S. Gmouth, M. Vaultier (2008). Droplet Microreactor, U.S. Patent 20080124252 A1.
239. Dubois P., G. Marchand, Y. Foillet, J. Berthier, T. Douki, F. Hassine, S. Gmouth, M. Vaultier (2006). Ionic liquid droplet as e-microreactor, Analytical Chemistry, 78, 4909-4917.
240. Streets A. M., Y. Huang (2013). Chip-in-a-lab: microfluidics for next generation life science research, Biomicrofluidics, 7, 011302.
241. Salic A., A. Tusek, B. Zelic (2012). Application of microreactors in medicine and biomedicine, Journal of Applied Biomedicine, 10, 137-153.
242. Kamholz A. E., B. H. Weigl, B. A. Finlayson, P. Yager (1999). Quantitative analysis of molecular interaction in a microfluidic channel, Analytical Chemistry, 71, 5340-5347.
243. Ismagilov R. F., A. D. Stroock, P. J. A. Kenis, G. Whitesides, H. A. Stone (2000). Experimental and theoretical laws for transverse diffusive broadening in two-phase laminar flows in microchannels, Applied Physics Letters, 72, 2376-2381.

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