Miniature Continuous-Flow Polymerase Chain Reaction: A Breakthrough?

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On August 27th, 1998, the Associated Press released the following notice: "TI announces tech breakthrough: Dallas—Texas Instruments Inc. says it has developed semiconductor technology based on minuscule transistors that could someday allow hearing aids to be built small enough to be directly implanted into the inner ear. The Texas Instruments transistor is so small, more than 400 million of them will fit onto a chip the size of a fingernail, researchers said Wednesday. Texas Instruments said products based on these chips will be smaller, weigh less, consume less power and execute software much faster than existing technology. (…) While Texas Instruments claims the transistor is the 'smallest announced' researchers at Bell Laboratories, the research and development arm of the New Jersey-based Lucent Technologies, announced in November that they had created the 'world's smallest complete metal oxide semiconductor transistors.' (…)"[1] This news is symptomatic of the notion that the human zeal for the creation of giant items, such as buildings, machines, and carriages,—frantically driven by the superpowers dozens of years ago—can seemingly nowadays only be beaten by the fervor to miniaturize devices down to micrometer and nanometer range. But not only so in electronic application sciences; the triumphant advance of this enterprise has conquered chemical and biochemical science as well.

We all want to save time, space, and costs. During the last decade a plethora of miniature systems have been studied and some prototypes developed. In particular, microfabricated devices for microfluidic systems (pumps, valves) and microfiltration systems, micro heat exchangers and microseparators, micromixers, microreaction chambers (for reviews see references [2, 3]), and electric field directed nucleic acid hybridization on microchips[4] have been presented. High-speed capillary electrophoresis (CE)[5] has been further developed successfully into capillary array electrophoresis[6, 7] and also two-dimensional capillary array electrophoresis[8] for DNA analyses on chips. Chip technology and performance also proved suitable for the powerful DNA analysis techniques for ligase chain reaction (LCR)[9] and polymerase chain reaction (PCR).[10] Moreover, microsystems with several functionally integrated features have been elaborated. These include CE with an integrated volume-defined sample injector for an unbiased injection procedure,[11] CE and PCR-coupled-CE with integrated real time monitoring,[12, 13] integrated cell isolation with PCR analysis using silicon microfilter chambers,[14] and even more complex systems, for example combining a microdialysis sampling interface with a microflow manifold and integrated biosensor array for glucose and lactate detection with associated computer software.[15] A new paradigm for genetic analysis are DNA chips with high density arrays of polynucleotide probes for gene discovery, observation of the expression of thousands of genes in concert under defined conditions, as well as for genotyping and DNA sequencing by array hybridization, epifluorescence confocal scanning, and data analysis.[16] These last developments gave rise to the so-called miniaturized total analysis systems (μTAS) that bring to reality the lab-on-a-chip concept (for critical recent overviews and perspectives see references [17–21]). According to Affymetrix (Santa Clara, California), a DNA array pioneer and the best known DNA-chip maker, it is in fact possible today that a plastic cartridge smaller than a credit card carries out seven different experimental procedures after an extracted DNA sample has been injected into a storage chamber. By a computer-controlled system, the sample is bound to a tiny glass wall, rinsed, dissolved again, amplified, converted into RNA, labeled, chopped up, and delivered to one of the company's microarray test.[22]

Compared with traditional "normal size" machines, miniature systems come off quite impressively. Processes are ultrafast (ranging from some seconds to some minutes,[12, 23] the sensitivity of detection of the analyzed substances touches the order of attomoles (10^{-18} moles)[12] and even zeptomoles (10^{-21} moles)[24], and the resolving power of the microdevices is similar to those of conventional systems.[5, 25] In summary, fabrication of micromachines, although miles apart from the minute dimensions of their electronic trailblazers, realize reproducible, low-cost manufacturing,[26] a very small sample volume (usually <10 μL), reduction of reagents, excellent thermal conductivity, a fast response, automated assays, and are suitable for high-throughput analysis through parallel sample processing. Evidently, these new analytical tools are useful in chemical and medical analysis, for example, disease diagnosis and research, and also meet the needs of the pharmaceutical industry in drug development.

The promising virtues of the biochemical micromachines led to a summit of enthusiasm as expressed by the bold and rousing announcement last May by J. Craig Venter, president

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of The Institute for Genomic Research (Rockville, Maryland), to sequence the human genome at a fraction of the time and the costs anticipated for the US governmental endeavor.[27] He focuses on miniaturized, nonstop working "pocket DNA sequencers" that will be delivered by Perkin–Elmer, the world's largest manufacturer of automated sequencing machines.[22] Although Perkin–Elmer's CE-microfluidics are still in the testing stage and doubts about their suitability have been expressed,[28] David Burke and co-workers of the University of Michigan in Ann Arbor, provided a convincing foretaste towards the realization of this direction. These researchers described recently a DNA analysis chip that integrates and performs at the nanoliter scale most of the steps needed in DNA amplification, restriction endonuclease digestion, or DNA sequencing, and detection of discrete DNA products with virtually no human intervention.[26]

However, specific ploblems show up with microfluidics that are not encountered with normal size machines. For example, handling and processing of microsized samples are still challenging, and have a series of attendant problems, such as loss of sample on the walls of transfer devices, loss by evaporation, loss of components from the sample during manipulation and processing, and obtaining a representative sample from a nonhomogeneous specimen. A further difficulty is that the expected concentration of the analyte restricts the scale of miniaturization of the sample.[2] Miniaturization inevitably leads to a reduction of the surfaces needed for efficient chemical or enzymatical reactions, which, however, can be successfully controlled by the use of porous silicon.[29] In addition, care has to be taken with respect to the compatibility of the new materials, other than polyethylene tubes, with the desired reaction, such as PCR, to take place in fabricated micromachines on chips.[30] Obviously, there's still a lot to do.

Additional, nontechnical problems may also jeopardize or slow down a rapid advent and a large-scale application of microfluidics. In particular, DNA arrays are still very expensive, and for medical use, for example, in pinpointing the onset of diseases or tailoring medication to patients with specific genetic makeups, they have in addition "to wind their way through clinical trials and regulatory approval".[31] Moreover, patent battles and questions of proprietary rights over key aspects of technology and gene sequences in arrays could bring this new gold rush to a halt. However, as a matter of fact these legal fights of companies and the fear of being defeated also nurture the burgeoning of novel ideas and the invention of new principles.

In this context, a significant new development in the world of miniature systems was reported by Kopp et al. (Zeneca/SmithKline Beecham, UK) in the May 15, 1998 issue of *Science*.[32a] These authors described a PCR chip for continuous-flow DNA fragment amplifications. The glass microchip was fabricated at the Alberta Microelectronic Centre, Canada, and is about 1 mm thick and 4 cm wide and long. The PCR mixture together with the DNA sample to be amplified is introduced by a precision syringe pump under simultaneous and constant buffer flow through a tiny hole drilled into the cover plate into a single channel (40 μm deep, 90 μm wide, 2.2 m long) etched into the glass chip (Figure 1 A). The

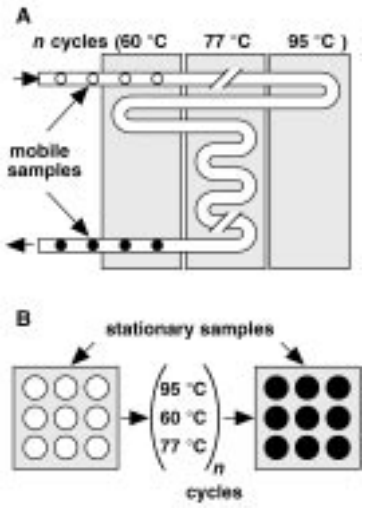

Figure 1. Schematic presentation of the continuous-flow PCR (A) and the classical batch process (B). ○ = input DNA (template); ● = product DNA (amplicates).

channel passes twenty times through three temperature zones that are kept at 95 °C, 60 °C, and 77 °C by controlled thermostated copper blocks, thus defining a thermal cycling process of 20 identical cycles in total. The sample (10 μL) is hydrostatically pumped through the channel and the PCR product (also 10 μL) is collected at an outlet capillary. Analysis is performed by standard slab-gel electrophoresis. By varying the flow rates of this set up, the authors demonstrated that they could achieve satisfactory amplifications, that is several nanograms final product of a 176 bp fragment, after only 3–4 min of total cycling time, and starting with approximately 10^8 copies of the template. The range of the flow rates was between 5.8 and 72.9 nL s^{-1}, which corresponds to between 18.8 and 1.5 min total amplification time for the 20 cycles.

For a critical evaluation of this report the presented results must be compared not only to the declared goals of the work, but also to existing alternatives and must be judged with regard to opening the gate for further developments as well. Kopp et al. claim to have presented an example of a "chemical amplifier". The latter is defined as a system that should increase a weak signal in a *continuous manner* (continuous input and output of material) and by a large *constant* factor, which means independently of the signal's initial size or quantity. As opposed to electronic amplifiers, which allow weak signals to be increased with virtually no time delay, chemical amplification does require time because of both diffusion-limited mass transport and dependence on the specific reactivities of the substances. Following the authors' requirements for a "true" chemical amplifier, however, amplification should also occur with the *same* time dependence, again regardless of the signal's initial magnitude.

To my knowledge, a micromachined, continuous-flow PCR has been presented here for the first time. This device in fact amplifies the input DNA in a continuous manner. This has been accomplished by a time–space conversion, that is by keeping the temperatures constant at different locations and moving the sample to the different temperature zones. In

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general, conventional thermocyclers alternately heat and cool the stationary samples[33] (Figure 1 B). In addition, they perform the amplification of the target DNA in a batch process, precluding them from being "true" chemical amplifiers and, more importantly, from the potential to be coupled with other, continuous-flow systems (see below). As a consequence of the fact that cross-contamination is not a major problem, the continuous-flow PCR chip may be used for serial injection of different samples to be analyzed. By only one-step intervening buffer washings, the samples can be introduced one after the other and give rise to a very high throughput, which in other microdevices is accomplished by an array set-up of the elements (see above).

However, with regard to the required constancy of the factor and invariable time dependence of the amplification, the achievements of the manufactured microdevice are not so clear. Neither feature has been shown in this report experimentally. Moreover, the amplification factor of an input DNA target is by no means determined solely by the (constant) number of PCR cycles. The theoretical value of a PCR amplification is shown in Equation (1), with N being the

$$N = N_0 2^n \quad (1)$$

number of amplifiedmolecules (product), N_0 the initial number of molecules before amplification (input), and n the number of the cycles. In this ideal formula the number of molecules in fact is doubled in each cycle. However, in practice the outcome is better described by Equation (2),

$$N = N_0 (1 + E)^n \quad (2)$$

where E is the efficiency of the reaction and can be any value between 0 and 1. Experimental values of $E = 0.8$ and $E = 0.9$ have been found for an optimized PCR.[34] Moreover, at the final stages of a PCR, E is subject to a vast decline because of exhaustion of ingredients, polymerase inactivation, etc. Therefore, an amplification above about 10^{13} molecules in the final product of one PCR is usually not possible. As a result of the nonlinear response of amplification, methods for determining and correcting these deviations have been sought in the last few years ("quantitative", "competitive" PCR).[35]

The continuous-flow PCR on a chip shares several features with existing microdevices, such as a small sample volume, rapid thermal conductivity (heating and cooling times less than 100 ms), and a very fast performance. In some aspects, however, thermocyclers of conventional size and batch processing are in fact superior to the presented continuous-flow PCR microdevice. For example, up to 12 primer-annealing temperatures can be checked in a single experiment with a commercial machine (RoboCycler from Stratagene) thereby increasing the rate of successful amplification. Another apparatus on the market (LightCycler from Boehringer Mannheim) combines not only high speed analysis (15 – 30 min for 30 cycles) and a reasonably small volume (5 – 20 μL) of up to 32 samples, but also on-line and real-time detection of the amplicate by a kinetic approach.

What is the significance of this work then? First of all, for Zeneca/SmithKline Beecham this new invention may help to avoid legal scuffles by eluding restrictions of existing patents. Besides, in my opinion two—more scientific—points must also be emphasized. First, the demonstration of the feasibility of continuous-flow PCR. PCR is known to be a highly sensitive method that will amplify traces of template and is thus prone to false amplifications because of contaminations. To me, it is very surprising—pleasing though!—that the described PCR chip obviously operates without this shortcomming. Probably, the process of mutual mixing and contaminating of the consecutive analyte peaks is efficiently restricted by the low-dispersion characteristics of the capillary-shaped chemical reactor in combination with the silanized surface of the channel walls and the use of a non-ionic surfactant in the buffer. However, it would have been interesting to examine, whether or not a *different* template injected after the first, a much longer template and, finally, less than 10^8 copies of a template would have led to similar satisfactory results. Second, the potential of further development of total analysis and synthesis systems by means of incorporation of the continuous-flow microamplifier is very high. For example, one can imagine linking the microamplifier to microfabricated devices for cell isolation,[14] volume-defined sample injectors,[11] and on-line sensors[6, 12] to elaborate a μTAS for a fast, integrated, and continuous DNA analysis of cell populations. For this purpose, all modular parts should operate in a continuous manner similar to the microamplifier in the report of Kopp et al. Indeed, as pointed out by the authors, continuous-flow microsystems are already available.[32b] These combinations would allow the continuous-flow PCR microamplifier to be useful not only in fast and reliable routine clinical diagnosis, but also versatile enough to challenge research laboratories as well. In conclusion, does the report of Kopp et al. mean a breakthrough in PCR techology? Perhaps no, but it is a (good) step in the right direction.

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[1] See under "http://www.usatoday.com:80/life/cyber/tech/ctd352.htm".
[2] "Microsystem Technology In Chemistry And Life Science": J. Cheng, L. J. Kricka, E. L. Sheldon, P. Wilding, *Top. Curr. Chem.* **1998**, *194*, 215 – 231.
[3] "Microsystem Technology In Chemistry And Life Science": W. Ehrfeld, V. Hessel, H. Lehr, *Top. Curr. Chem.* **1998**, *194*, 233 – 252.
[4] C. F. Edman, D. E. Raymond, D. J. Wu, E. Tu, R. G. Sosnowski, W. F. Butler, M. Nerenberg, M. J. Heller, *Nucleic Acids Res.* **1997**, *25*, 4907 – 4914.
[5] A. T. Woolley, R. A. Mathies, *Anal. Chem.* **1995**, *67*, 3676 – 3680.
[6] A. T. Woolley, G. F. Sensabaugh, R. A. Mathies, *Anal. Chem.* **1997**, *69*, 2181 – 2186.
[7] P. C. Simpson, D. Roach, A. T. Woolley, T. Thorsen, R. Johnston, G. F. Sensabaugh, R. A. Mathies, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2256 – 2261.
[8] H. Becker, K. Lowack, A. Manz, *J. Micromech. Microeng.* **1998**, *8*, 24 – 28.
[9] J. Cheng, M. A. Shoffner, K. R. Mitchelson, L. J. Kricka, P. Wilding, *J. Chromatogr. A* **1996**, *732*, 151 – 158.
[10] J. Cheng, M. A. Shoffner, G. E. Hvichia, L. J. Kricka, P. Wilding, *Nucleic Acids Res.* **1996**, *24*, 380 – 385.

  1433-7851/99/3808-1057 $ 17.50+.50/0

[11] C. S. Effenhauser, A. Paulus, A. Manz, H. M. Widmer, *Anal. Chem.* **1994**, *66*, 2949 – 2953.
[12] A. T. Woolley, K. Lao, A. N. Glazer, R. A. Mathies, *Anal. Chem.* **1998**, *70*, 684 – 688.
[13] A. T. Woolley, D. Hadley, P. Landre, A. J. de Mello, R. A. Mathies, M. A. Northrup, *Anal. Chem.* **1996**, *68*, 4081 – 4086.
[14] P. Wilding, L. J. Kricka, J. Cheng, G. Hvichia, M. A. Shoffner, P. Fortina, *Anal. Biochem.* **1998**, *257*, 95 – 100.
[15] R. Freaney, A. McShane, T. V. Keaveny, M. McKenna, K. Rabenstein, F. W. Scheller, D. Pfeiffer, G. Urban, I. Moser, G. Jobst, A. Manz, E. Verpoorte, M. W. Widmer, D. Diamond, E. Dempsey, D. V. F. Saez, M. Smyth, *Ann. Clin. Biochem.* **1997**, *34*, 291 – 302.
[16] "Microsystem Technology In Chemistry And Life Science": R. C. Anderson, G. McGall, R. J. Lipshutz, *Top. Curr. Chem.* **1998**, *194*, 117 – 129.
[17] C. S. Effenhauser, G. J. Bruin, A. Paulus, *Electrophoresis* **1997**, *18*, 2203 – 2213.
[18] "Microsystem Technology In Chemistry And Life Science": C. S. Effenhauser, *Top. Curr. Chem.* **1998**, *194*, 51 – 82.
[19] "Microsystem Technology In Chemistry And Life Science": S. Shoji, *Top. Curr. Chem.* **1998**, *194*, 163 – 188.
[20] "Microsystem Technology In Chemistry And Life Science": A. van den Berg, T. Lammerink, *Top. Curr. Chem.* **1998**, *194*, 21 – 49.
[21] G. Blankenstein, U. D. Larsen, *Biosens. Bioelectron.* **1998**, *13*, 427 – 438.
[22] R. F. Service, *Science* **1998**, *282*, 399 – 401.
[23] F. von Heeren, E. Verpoorte, A. Manz, W. Thormann, *Anal. Chem.* **1996**, *68*, 2044 – 2053.
[24] C. S. Effenhauser, G. Bruin, A. Paulus, M. Ehrat, *Anal. Chem.* **1997**, *69*, 3451 – 3457.
[25] D. E. Raymond, A. Manz, H. M. Widmer, *Anal. Chem.* **1996**, *68*, 2515 – 2522.
[26] M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. H. Mastrangelo, D. T. Burke, *Science* **1998**, *282*, 484 – 487.
[27] E. Marshall, E. Pennisi, *Science* **1998**, *280*, 994 – 995.
[28] R. F. Service, *Science* **1998**, *280*, 995.
[29] J. Drott, K. Lindstrom, L. Rosengren, T. Laurell, *J. Micromechan. Microeng.* **1997**, *7*, 14 – 23.
[30] M. A. Shoffner, J. Cheng, G. E. Hvichia, L. J. Kricka, P. Wilding, *Nucleic Acids Res.* **1996**, *24*, 375 – 379.
[31] R. F. Service, *Science* **1998**, *282*, 396 – 399.
[32] a) M. U. Kopp, A. J. Mello, A. Manz, *Science* **1998**, *280*, 1046 – 1048; b) footnotes 8 – 11 in reference [32a]
[33] There is one notable exeption to this rule known to me: the RoboCycler sold by Stratagene, which also moves the tube samples to the different temperature blocks.
[34] G. Haberhausen in *Bioanalytik* (Eds.: F. Lottspeich, H. Zorbas), Spektrum, Heidelberg, **1998**, pp. 673 – 703.
[35] S. Diviacco, P. Norio, L. Zentilin, S. Menzo, M. Clementi, G. Biamonti, S. Riva, A. Falaschi, M. Giacca, *Gene* **1992**, *122*, 313 – 320.

Heterogenization of Metallocene Catalysts for Alkene Polymerization**

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Metallocenes, when used in combination with suitable activating agents, are extremely active catalysts for alkene polymerization.[1] At present, research in this area has reached such a level of sophistication that new metallocenes and activating agents can be more or less designed in a rational way. Current work focuses on controlling the stereoselectivity of alkene polymerization and, increasingly, minimizing competing insertion and chain-transfer reactions as well as determining the mechanisms that account for catalyst deactivation.

In developing large-scale industrial processes for gas-phase and slurry polymerization of alkenes it is extremely important to heterogenize the essentially homogeneous metallocene/activator catalyst systems. Without heterogenization of metallocenes many new polyolefins could not be produced at all on an industrial scale! The reason is that although homogeneous polymerization catalysts form at best finely divided polymer powders, a heterogeneous catalyst can control the morphology of the polymer formed. As a result the formed polymer beads are enlarged duplicates of the catalyst particles (Figure 1).[2]

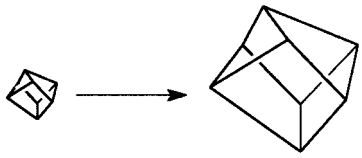

Figure 1.

Due to the complexity of the systems, catalyst immobilization is difficult to approach at the molecular level. Common supports, such as high-surface-area silica and alumina have received the most attention and have proved to be very successful for the commercial-scale production of polyolefins.[3] Yet despite their apparent simplicity, these supports have undesirable or ill-exploited properties that can lead to multiple active sites, catalyst deactivation, and leaching. Clearly, support characteristics such as microstructure, surface functionalities, and polarity must be further investigated, and catalyst-anchoring techniques including, for instance, grafting, tethering, physisorption, and electrostatic immobilization must be devised. This highlight describes some approaches to developing new catalyst supports and the corresponding methods to best exploit valuable metallocene catalysts.

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