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Integrated Microfluidic System Enabling (Bio)chemical Reactions with On-Line MALDI-TOF Mass Spectrometry

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A continuous flow micro total analysis system (μ-TAS) consisting of an on-chip microfluidic device connected to a matrix assisted laser desorption ionization [MALDI] time-of-flight [TOF] mass spectrometer (MS) as an analytical screening system is presented. Reaction microchannels and inlet/outlet reservoirs were fabricated by powderblasting on glass wafers that were then bonded to silicon substrates. The novel lab-on-a-chip was realized by integrating the microdevice with a MALDI-TOFMS standard sample plate used as carrier to get the microfluidic device in the MALDI instrument. A novel pressure-driven pumping mechanism using the vacuum of the instrument as a driving force induces flow in the reaction microchannel in a self-activating way. Organic syntheses as well as biochemical reactions are carried out entirely inside the MALDI-MS ionization vacuum chamber and analyzed on-line by MALDI-TOFMS in real time. The effectiveness of the μ-TAS system has been successfully demonstrated with several examples of (bio)chemical reactions.

The introduction of chip-based technologies and the corresponding design strategies has given an enormous impetus to progress in the field of fluidic devices, mainly for analytical applications. As reported in some excellent reviews, these include integrated chip-based separation methods,[1−5] biochemical application of microsystems,[6] and the clinical potential of chip-based analysis systems.[7] Over the past decade, this trend has been driven by the need of rapid, on-line measurements at low concentration in the fields of chemical synthesis, DNA sequencing, drug discovery, pharmaceutical screening, medical diagnostic, and environmental analysis.[6−10] A large number of applications clearly demonstrate fundamental advantages of miniaturized analysis systems, as compared to lab-scale instrumentation.[11−15]

Small microfabricated devices have excellent mass and heat transfer properties as well as uniform flow patterns and residence time distributions.[16] Miniaturization represents a promising way to decrease time and costs of performed processes.[11] Significant enhancements in efficiency of mixing and separation[17,18] and

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increased selectivity and safety[19] in the use of microreactors for chemical synthesis and biochemical reactions[16] are important aspects. Research into the fundamental and practical advantages of micrometer-scale reactors for chemical and biochemical applications is growing,[20] but application of the integrated lab-on-a-chip concept to synthetic chemistry[21,22] is still in its infancy. The introduction of on-line analysis of reaction products by integration of a microreactor and an analytical instrument would provide in-real-time information about the reaction.[22] This is of interest for high-throughput reaction screening and automated product library synthesis.

A key issue for the efficiency of the lab-on-a-chip approach to chemical and biochemical microfluidics manipulation and analysis is the interfacing of a chip-sized fluidic device to a sensitive analytical instrument.[16]

Because of the extremely small detection volumes and analyte concentrations, sensitive detection in microfluidic analytical devices is a challenge. Several recent papers address this issue, for example, by spectroscopic and electrochemical techniques.[23]

In the past decade, mass spectrometry (MS) has become one of the most powerful detection techniques used in liquid-phase analyses,[24] mainly as a result of its facile interfacing with separation techniques, such as capillary electrophoreses (CE)[25] and high-performance liquid chromatography (HPLC).[26]

Electrospray ionization (ESI)-MS[27] and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF)-MS[28] allow the analysis of extremely small sample volumes and low concentrations and flow rates (nanoliters to microliters per minute)[26] that closely match those encountered in many microfluidic chip systems. Consequently, these are useful tools for the structural identification of analytes processed on a lab-on-a-chip device.

On-line chip-MS[29,30] has mainly been restricted to the electrospray ionization method because of the easy interfacing between the electrospray capillary and microfluidic chips; in addition it operates at atmospheric pressure. The ionization process in MALDI-TOFMS is mainly carried out in a vacuum. This requires special precautions for integration with a microfluidic chip.

In this manuscript, we describe the first example of the coupling of a microfluidic device to a MALDI-TOF mass spectrometer by integrating an on-chip microreaction unit onto a MALDI-TOF standard sample plate. This allows (bio)chemical reactions to take place in the MALDI-TOF instrument. The flow control is pressure-driven because of the vacuum in the ionization chamber of the MALDI-TOF, and this avoids wires and tubes for feed and flow control.

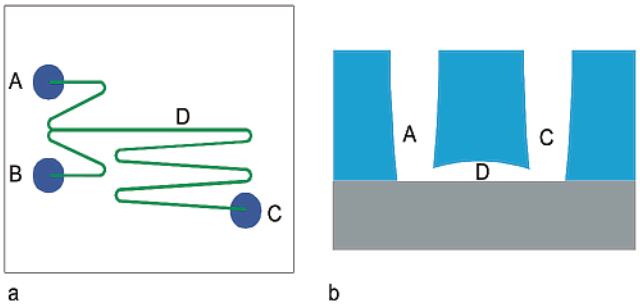

Figure 1. (a) Schematic top view of the microfluidic chip with the inlet (A and B) and the outlet (C) cups and the reaction microchannel (D); (b) side view of the micromachined top borofloat wafer with one of the two inlets (A), outlet (C), and microchannel (D) anodically bonded with the bottom silicon substrate.

A variety of examples ranging from simple biochemistry, organic, and polymer chemistry will illustrate the effectiveness and versatility of our chip-MS system.

EXPERIMENTAL SECTION

Microreactor Fabrication Process and Design. The microfluidic device used in the experiments (Figure 1) was fabricated in the cleanroom facilities of the MESA$^+$ Research Institute.

The microreactor was designed to study on-line MALDI-TOFMS analyses of products of simple chemical and biochemical reactions (of the type A + B = C). Therefore, the microreactor consists of two inlet reservoirs (A and B) for the injection of a maximum volume of 5 μL of reagent solutions and a 5-μL volume outlet pocket (C) where the analyte solution is collected and analyzed. The reaction microchannel in which the reagents react is 80 mm long, 200 μm wide, and 100 μm deep and has a volume of 1.2 μL. Microchannels and inlet/outlet holes were realized in a top borofloat wafer (1.1 mm thick) by high-resolution powder-blast micromachining.[31] This relatively new microfabrication technique[32] is faster but less precise than isotropic HF[33] etching and has the advantage of avoiding underetching. A great advantage of this technique is the possibility of realizing an unlimited number of vertical feedthroughs in one process step of ~30 min. The top borofloat wafer was anodically bonded (400 °C, 800 V) to a silicon wafer (500 μm thick).

Inlets Filling Procedure. The inlet reservoirs were filled, using a micropipet, one-by-one and by taking care that both the second inlet and the outlet holes were closed. Filling the inlet pockets without carefully following the procedure would cause the capillary force to drive the liquid out from the inlet reservoir into the channel. Closing the outlet and one of the inlet cups while loading the liquid in the other one causes a counterpressure in the channel that keeps the liquid in the inlet pocket being filled. During the filling procedure, both the outlet and the inlet hole not being filled were closed by means of adhesive tape. After the loading of each inlet was completed, the inlet cups were sealed by applying a piece of glass fixed to the surface of the chip by means of adhesive tape.

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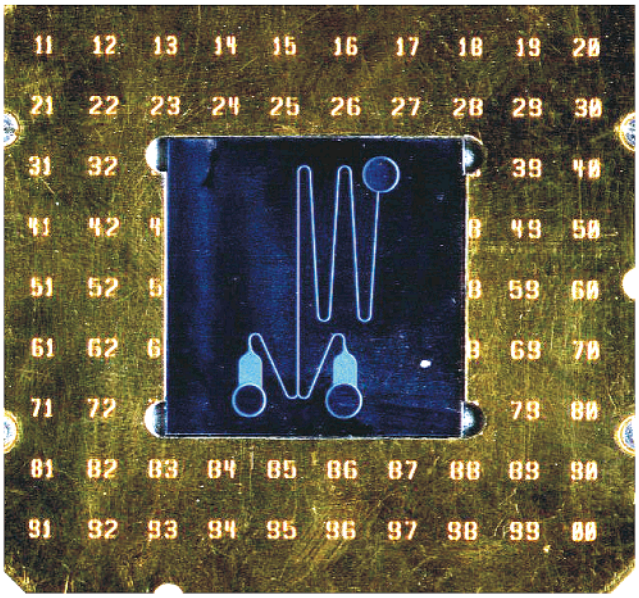

Figure 2. Picture of the microfabricated fluidic device integrated with a standard MALDI-TOF sample plate. Because of the self-activating character of the microfluidic device, the system can be introduced into the MALDI ionization chamber without any wire or tube for the feeding and the flow control.

Chip-MALDI Sample Plate Integration. To perform the on-line experiments, the glass−silicon chip was integrated into the MALDI-TOF sample plate (Figure 2).

The integration of the microreaction and detection unit was achieved by placing the chip in a hole (1.35 mm deep) made in the sample plate by milling. Subsequently, the system (chip and sample plate) was introduced into the MALDI-TOF instrument by the loading procedure.

Activation Method. Just before loading the system, the tape that closed the outlet pocket was removed. At this stage, the liquid is kept in the inlet cup by the atmospheric pressure coming from the opened outlet. Once the MALDI target with the integrated microchip enters the vacuum ionization chamber, the counter-pressure from the outlet disappears and the air in both the inlet pockets pushes the liquid through the channel to the outlet pocket.

A second driving force that might affect the fluidic flow is the capillary pressure $2\gamma/r$ (where r is the radius of the tube, and γ is the surface tension of the liquid in the channel).[34] However, in the current chip, in which the channel diameter is of the order of 100 μm, the capillary pressure (1.44×10^3 Pa for water) is small compared to the pneumatic driving force (10^5 Pa).

Detection. The reaction products formed in the microreactor channel and collected in the chip outlet were in real time identified by MALDI-TOFMS using a Voyager-DE-RP MALDI-TOF mass spectrometer (Applied Biosystems/PerSeptive Biosystems, Inc., Framingham, MA) equipped with delayed extraction[35,36] and a 337-nm UV nitrogen laser producing 3-ns pulses. The mass spectra were obtained in the linear and reflectron mode. To avoid fast crystallization of the reaction products at the outlet reservoirs of the microchip, the vacuum pressure in the ionization chamber of the mass spectrometer was reduced while introducing the chip-MALDI sample plate. The pressure of the time-of-flight analyzer was kept at 1×10^{-8} Torr (1.33×10^{-6} Pa).

Testing the Chip via Schiff's Base Formation. Reagents were obtained from Aldrich Chemicals, The Netherlands. Samples were used as supplied commercially without further purification. No matrix was added for MALDI detection of the aromatic product of these systems because of their strong absorption at the laser wavelength (λ = 337 nm). Three Schiff's base reactions were performed in the μ-TAS system and the products analyzed on-line.

In the first reaction, inlet A was filled with 3 μL of a solution of 0.2 mM aniline in ethanol; 3 μL of a solution of 0.2 mM benzaldehyde in ethanol was injected into inlet B.

In the second reaction, inlet A was filled with 3 μL of a solution of 0.2 mM 4-*tert*-butylaniline in ethanol, and inlet B was filled with a solution of 0.2 mM 4-*tert*-butylbenzaldehyde in ethanol.

In the third reaction, inlet A was filled with 3 μL of a solution of 0.2 mM 4-*tert*-butylaniline in ethanol, and inlet B was filled with 3 μL of a solution of 0.4 mM 4-*tert*-butylbenzaldehyde in ethanol.

Testing the Chip with Various Polymers. Reagents were obtained from Aldrich Chemicals, The Netherlands and were used as supplied commercially without further purification. Dihydroxybenzoic acid (DHB) was used as the matrix.

A 0.3-μL portion of polystyrene (PS) ($M_{w,ave}$ = 3500 amu) in THF/water and DHB as matrix was injected into inlet A; 0.3 μL of poly(methyl methacrylate) PMMA ($M_{w,ave}$ = 3500 amu) in THF/water and DHB as matrix was injected into inlet B.

Testing the Chip for Oligonucleotide Sequencing. The Sequazyme (PerSeptive Biosystems, Framingham, USA) oligonucleotide sequencing kit was used for sequencing synthetic deoxyoligonucleotides by partial exonuclease digestion followed by MALDI-TOFMS. The method was applied to a 41-base oligodeoxynucleotide in which a nucleobase was replaced by a hydrogen atom.

Since oligonucleotides strongly bind trace amounts of salts, sample deionization via cation exchange is required. Therefore, the sample was transferred to Parafilm and mixed with the cation exchange beads provided with the kit. Since the investigated oligonucleotide is longer than 10 bases, desalting is performed using spin column purification with a QuickSpin C-25 column (Boehringer Mannheim, Germany). Inlet A was used for the introduction of 0.3 μL of the oligonucleotide at a concentration of 0.2 mM in high-grade water.

The pH optimum for snake venom phosphodiesterase (SVP) digestion experiments is basic, and therefore, 0.3 μL of ammonium citrate buffer was mixed with 1 μL of SVP dilution. SVP hydrolyzes in the 3′-to-5′ direction of the oligonucleotide. A 0.3-μL portion of supernatant SVP, free of beads, was transferred to the inlet position B of the chip system. Additionally, 0.2 μL of a saturated solution of 3-hydroxypicolinic acid (HPA, matrix) in water was injected into inlet B.

Testing the Chip for Peptide Sequencing. The Sequazyme (PerSeptive Biosystems, Framingham) C-peptide sequencing kit enables peptide digestion followed by analysis of sequentially truncated peptides using MALDI-TOFMS.

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Before digesting and analyzing unknown samples of the peptide (adrenocorticotropic hormone), the activity of the enzyme carboxypeptidase Y CPY was checked by dilution experiments containing citrate buffer at pH 6.1, CPY dilution and the peptide standard angiotensin I.

The adrenocorticotropin ACTH (18−39) fragment (0.3 μL) as a solution of 0.05 mM in a mixture of water/acetonitrile 50/50% was transferred to inlet A.

Inlet B contained 0.3 μL of a solution of 0.1 mM CPY in HLPC-grade water and ammonium citrate (at pH 6.1) and 0.2 μL of the matrix α-cyano-4-hydroxycinnamic acid in water/acetonitrile 50/50% and 0.1% trifluoroacetic acid (TFA). Immediately after the introduction of the ACTH 18−39 peptide, the CPY dilution was transferred to inlet B including the matrix.

RESULTS AND DISCUSSION

The chemical and biochemical reactions were successfully carried out in the microfluidic device, and the product was analyzed on-line.

An important novelty is the possibility to analyze the reaction products by MALDI-TOFMS immediately after the reaction has taken place. After the chip is placed in the MALDI-TOF vacuum chamber and as soon as the reaction product leaves the outlet, the laser beam ionizes the molecules, which are detected by the time-of-flight analyzer. This technology offers the opportunity to analyze reaction products in situ as soon as they are formed. A design with multiple outlet sample ports would allow a quasicontinuous monitoring of the reaction products in time, ideal for kinetic studies of reactions.

Schiff's Base Formation. The Schiff base reaction, in which primary amines react with aldehydes to give imines, was chosen as a primary study of the microreaction unit and its coupling with MALDI TOFMS, because it is a straightforward reaction, and the products are obtained in high yields.

The pH control required for the Schiff's base formation[37] turned out to be not necessary under lab-on-a-chip conditions as a consequence of the high surface-to-volume ratio that characterizes fluidic microreactors. The MALDI mass spectra (Figure 3a,b) show that the two reagents injected into the inlets of the microchip for both experiments 1 and 2 reacted quantitatively in the microfabricated channel, since signals of reactants are not observed in the mass spectrum. Confirmation of the reaction product structure for reaction 1 was obtained using postsource decay experiments[38] of the mass-selected ions, as shown in Figure 3a.

Further confirmation of the effectiveness of the MALDI-chip system is given by the result of the third reaction. When reacting two equivalents of aldehyde with one equivalent of amine, signals of both product and excess of reagent are detected (Figure 3c).

Polymers. To prove the versatility of the system as a platform for different applications, we tested its ability to separate mixtures of polymers. MALDI mass spectra show two different polymer distributions separated in time (Figure 4). The two different polymer distributions can be easily assigned to the injected polymers by the peak-to-peak separation due to the different molecular weight of the monomers. The first pattern recorded in time was assigned to polystyrene (Figure 4A), and the second, to poly(methyl methacrylate) (Figure 4B); the differences in mass to adjacent peaks were 104 and 100 mass units, respectively.

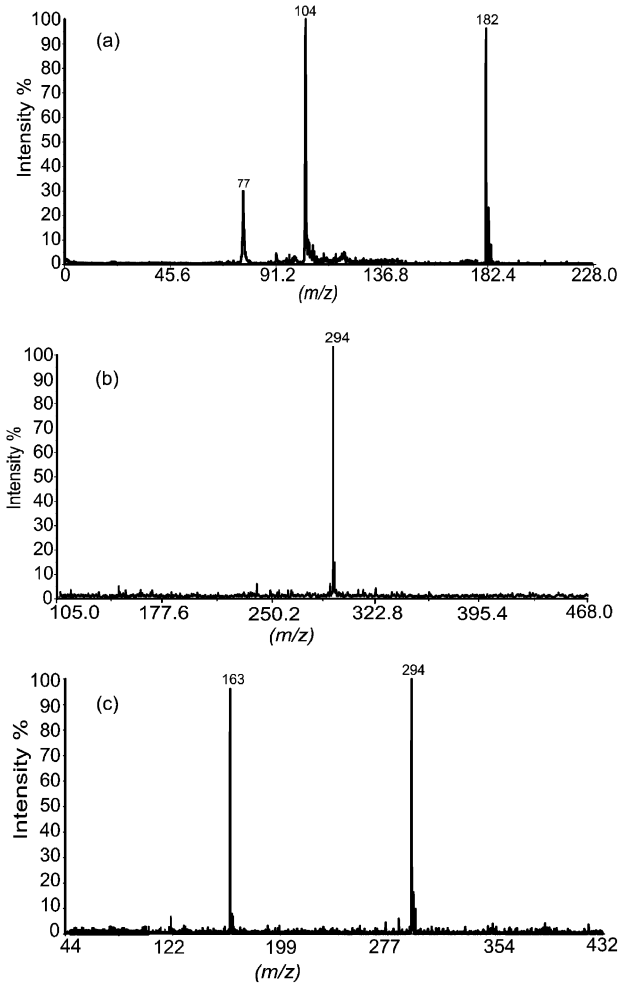

Figure 3. MALDI-TOF PSD mass spectrum of the Schiff base reaction products (a) $[M + H]^+ = 182$ Da formed in the reaction microchannel in experiment 1, (b) $[M + H]^+ = 294$ Da formed in the reaction microchannel during experiment 2, and (c) $[M + H]^+ = 294$ Da formed in the reaction microchannel during experiment 3 and the excess of reagent $[M + H]^+ = 163$ Da.

This result shows that the chip is capable of separating the two polymers on the basis of different polarities. This is probably due to the high viscosity of the polymeric solutions, giving rise to a longer residence time in the microchannel. These results suggest the possible application of the here-presented lab-on-a-chip as a separation tool, comparable to gel permeation chromatography.[39]

Oligonucleotides. Mass spectrometry is an outstanding technique for the characterization and sequence determination of oligonucleotides generated via solution-phase chemical reactions.[40] The DNA sequence can be determined by the mass difference between the oligonucleotide fragments in (partial) digests from either end of the DNA molecule. Oligonucleotide digestion was performed directly on the microfluidic chip by mixing the oligonucleotide (substrate) with snake venom phos-

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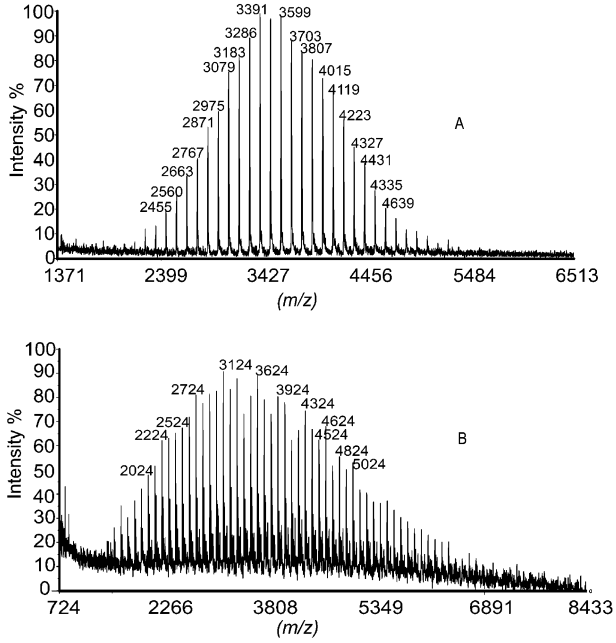

Figure 4. MALDI-TOF mass spectra of the two different polymer distributions separated in arrival time at the outlet reservoir and assigned to PS (A) and PMMA (B).

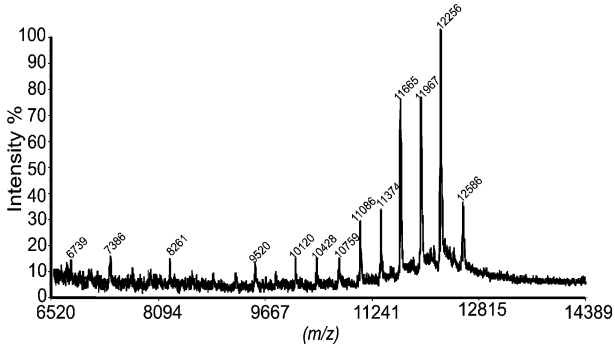

Figure 5. MALDI-TOF mass spectrum of the oligonucleotide $[M + H]^+ = 12\,586$ Da and the oligonucleotide residue average masses of the enzymatic digestion. Individual bases' average masses: A = 313.2, C = 289.2, G = 329.2, and T = 304.2 Da, and sequence GCTCTAGACT.

phodiesterase [SVP] that hydrolyzes in the 3′-to-5′ direction. The mass difference between adjacent pairs of peaks in the MALDI-TOF mass spectrum identifies each nucleotide base in the sequence. In Figure 5, the oligonucleotide residue average masses are shown, from which it is possible to derive the oligonucleotide sequence.

This way to analyze exonuclease ladders seems to be a particularly promising tool to determine rapidly the sequence of oligonucleotides. This offers the advantage of saving time and material. In addition, it reduces the risks encountered when manipulating biomolecules.

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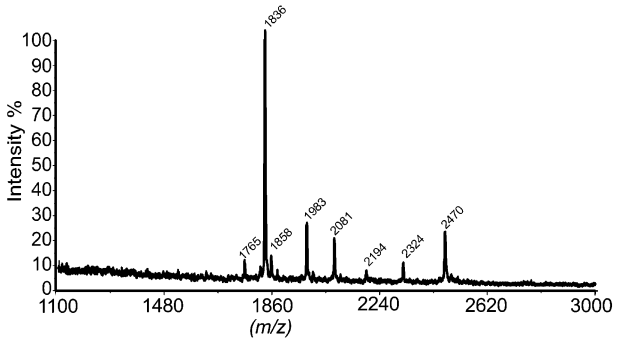

Figure 6. MALDI-TOF mass spectrum of the ACTH peptide $[M + H]^+ = 2470$ Da and the amino acids residues of enzymatic digestion. The sequence resulting from the partial on-chip digestion is Phe-Glu-Leu-Pro-Phe-Ala.

Peptide-Sequencing. Carboxypeptidase Y (CPY) sequentially hydrolyzes the C-terminal residues of peptides,[41] which can be analyzed by mass spectrometry within less than a couple of minutes. The sequence from the peptide fragments can be determined from the mass differences between the adjacent peaks in the mass spectrum.

In our approach, peptide digestion and product analysis are performed directly on the chip, minimizing sample handling, sample loss, and method development time. In the lab-on-a-chip methods, indeed, only a few picomoles of total peptides is required, and the analysis can be made immediately after digestion in situ, avoiding sample transferring to the mass spectrometer. After data acquisition and mass calculation, the mass differences between the adjacent peaks in the ladders were used to determine the C-terminal sequence of the peptide (see the amino acid assignments in Figure 6).

CONCLUSIONS

To the best of our knowledge, a novel lab-on-a-chip system that allows chemical syntheses, separations, and biochemical processes on a microscale and in real time monitored by MALDI-TOFMS was developed for the first time. The effectiveness of the system was illustrated for a variety of systems ranging from simple synthetic chemistry to polymer analysis and enzymatic digestion of peptides and oligonucleotides. This result was achieved by realizing a self-activating chip, which avoids the need of placing wires and tubes into the ionization chamber of the MALDI TOF instrument.

Further reactions using this methodology are in progress. By a proper design of the fluidic circuit, a direct monitoring of product formation in-real-time during the reaction will become feasible, opening the way to kinetics studies.

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