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Liquid lid for biochemical reactions in chip-based nanovials

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Abstract

The present paper describes a new technique to suppress evaporation of solvent from very small volumes. Vials (15 nl) on a silicon microchip were filled with water, and covered with a thin, flowing film of a volatile liquid (e.g., octane). Water evaporation was greatly reduced. At 37°C, no appreciable loss of water could be observed over a period of 90 min. At 95°C, most of the water sample was left in the vial for more than 3 min. The applicability of the method is demonstrated by a tryptic digest, where the resulting peptide fragments were analyzed by capillary electrophoresis. In addition, a discussion of the possibilities and limitations of some alternative methods is included in the paper, as well as an outlook on future developments of the liquid lid concept.

Keywords: Liquid lids; Chip-based nanovials

1. Introduction

Miniaturization is a strong on-going trend in clinical chemistry and drug development due to an increased need for throughput [1–4]. The standard plate format for 96 wells has been modified for work with smaller reaction volumes and is now available with 384- as well as 864-wells [5] and even a new platform with 9600-wells [4] has been presented. As the reaction volume is decreased, the evaporation of solvent from the wells becomes a crucial problem. Working in a chamber with increased humidity can reduce the evaporation [4] but this can lead to problems, including mal-performance of electronic components in the humid environment. Other possible remedies have been suggested, e.g., mechanical lids [6] or covering tape [5], but these concepts also lead to problems when using very small vials.

Other strategies to avoid the evaporation of solvent are a coverage of the solution with mineral oil [7] or the addition of a non-volatile matrix to the reaction mixture [6,8,9]. However, such substances are difficult to remove and can be detrimental in the subsequent analysis or sample work-up. In this context, the use of a volatile substance, e.g., heptane [10,11], is more attractive since it is easier to remove.

We have earlier reported on technologies for performing chemistry in open chip-based nanoliter sized volumes. Different approaches, such as working in a humid atmosphere [12] or continuous addition of water to compensate for the evaporation of water from the sample [13], were used. This report discusses these technologies and describes a novel method based on covering the reaction vials with a thin layer of a volatile liquid, where the evaporation of the covering solvent is continuously compensated for during the reaction in the vials. In this way, the evaporation of water from the sample to the surrounding atmosphere is greatly reduced,

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while full accessibility to the vials is maintained before, during and after the reaction. The applicability of the technology is evaluated by performing an enzymatic degradation of myoglobin using trypsin followed by electrophoretic separation of the reaction products.

2. Experimental

2.1. Positioning equipment

The experiments were performed using a computer controlled precision robot developed in our laboratory. A schematic of the equipment is shown in Fig. 1 and the setup consists of the following parts: a *X*–*Y* translation table (TIXY200, Newport, Irvine, CA, USA) with a control unit (MM4000, Newport) and three vertically mounted (*z*-direction) motor-driven micro-positioners (MM-3M-EX-2, part Nos. 25100-07 and 25100-21 with a multi-axis Servo-controller MC-3SA-3, part No. 25100-29, Fine Science Tools, Heidelberg, Germany). All positioning devices in the *z*-direction were controlled using the LabView (National Instruments, Austin, TX, USA) environment and the VI library for pcControl (part No. 25100-26, Fine Science Tools).

2.2. Chip-based vials

The vials (15 nl) used in the present study were fabricated by anisotropic etching of monocrystalline silicon and were gold coated by sputtering [14]. The chip was positioned onto an aluminum holder in order to be able to create the liquid barrier over the chip (Fig. 2). The holder was temperature controlled using a proportional, integrational and derivational (PID) regulator (CAL3200, CAL Controls, Libertyville, IL, USA) and a heating element (Thorin Thorin, Hindås, Sweden) as well as a temperature sensor (PT100 Type 16, TemFlow, Stockholm, Sweden). The temperature at the top of the chip was also measured using a bimetal thermometer (CIE Model 307, Rapid-Tech, Australia) kept in surface contact with the chip. The liquid barrier was guided over the surface of the chip through the inlet channel of the holder and kept at constant thickness via a draining hole. The thickness of the liquid layer over the chip surface was determined by adjusting the height of a PTFE (Teflon) tube positioned inside the drainage hole (Fig. 2).

The vials were monitored by using a microscope (Questar, New Hope, PA, USA) equipped with a charged coupled device (CCD) camera (C2400-75i, Hamamatsu Photonics K.K., Japan) and an image analysis system (Argus-20, Hamamatsu Photonics K.K.). Light was guided via the optics of the microscope (FLQ1500, Hund Wetzlar, Germany) and an additional fiber-optic ring-light system (KL1500 electronics, Schott, Germany) positioned between the microscope and the chip holder. To minimize influences of vibration, the entire equipment was mounted onto an optical table (TMC, Peabody, MA, USA).

2.3. Tryptic digest of myoglobin

The dosing of reagent into the vials was performed via a fused-silica capillary (40 μm I.D.×105 μm O.D., Part No. TSP040105, Polymicro Technologies, Phoenix, AZ, USA) which was mounted on one of the micro-positioners. The inlet side of the capillary was passed through a rubber membrane into a Pyrex vial (2 ml), with an insert of a plastic Eppendorf tube. The insert contained ca. 4 μl of a mixture of native myoglobin (4 mg/ml) and reaction buffer (4 m*M* NH_4HCO_3, pH 7.9). The sample flow through the capillary was controlled by applying an (part No. 8607-2-A-1-1-2, Brooks Instrument, Veenendaal, The Netherlands) air overpressure (0.2–0.4 bar) into the Pyrex vial [13] and the myoglobin–buffer mixture was dosed in consecutive order in a row of eight individual vials (vial volume 15 nl). In order to reduce concentration changes of the protein due to surface adsorption onto the capillary wall, the capillary was rinsed with ca. 0.3 μl of the mixture prior to dosing into the first vial.

After sample transfer, the water in the mixture was allowed to evaporate (which occurred in ca. 30 s). *n*-Octane (KEBO, Stockholm, Sweden) was used as a cover fluid and was guided over the dried chip surface from a container by siphoning via the inlet hole in the chip-holder (Fig. 2). When the film of octane had stabilized (ca. 10 s), 15 nl of trypsin dissolved in water (0.2 mg/ml) was added to each of the individual vials using a second (micro-positioner

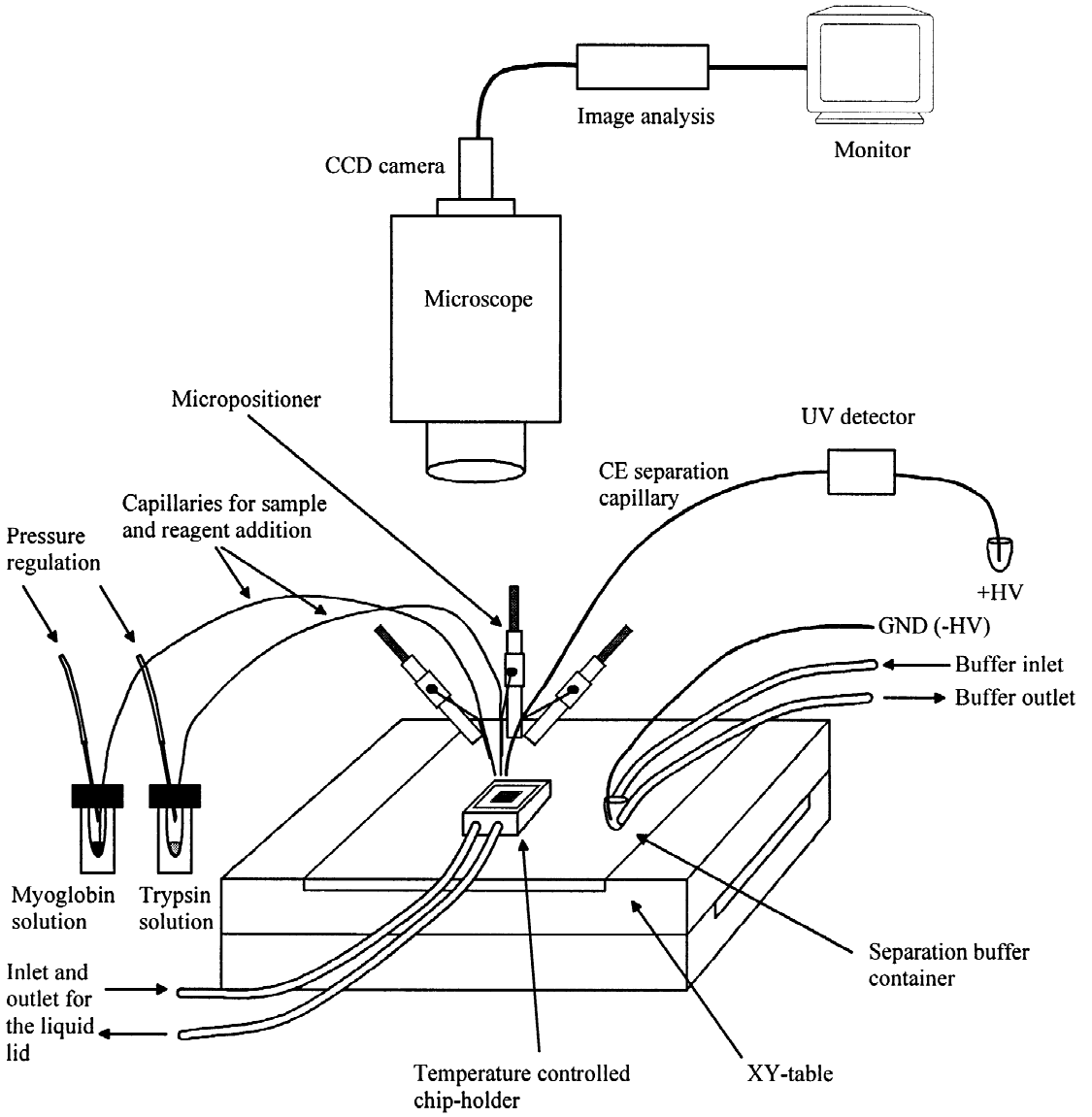

Fig. 1. A schematic of the set-up used for performing tryptic digest reactions where the evaporation of water from the reaction mixture is prevented by the use of a volatile liquid lid. The evaporation of the lid is continuously compensated by addition of the solvent (octane) by siphoning. The thickness of the lid is controlled by a piece of PTFE tubing inserted in the drainage hole of the chip-holder (details of the holder are shown in Fig. 2). The overflow of the cover solvent is removed from the chip-holder by applying a vacuum to the outlet PTFE tubing. Reagents are added to the chip using thin capillaries where the inlet of each capillary is inserted into a pressurized vessel with the relevant components. The injection end of the CE capillary is mounted onto a micro-positioner for high precision positioning into the chip-based vials. After vacuum injection of an aliquot of the reaction mixture, the inlet of the CE separation capillary is repositioned into the separation buffer container (-HV) mounted onto the $X–Y$ table and the analysis of the reaction products is initiated when the high voltage is applied. The separation buffer in the container as well as in the separation capillary is automatically replenished between each run.

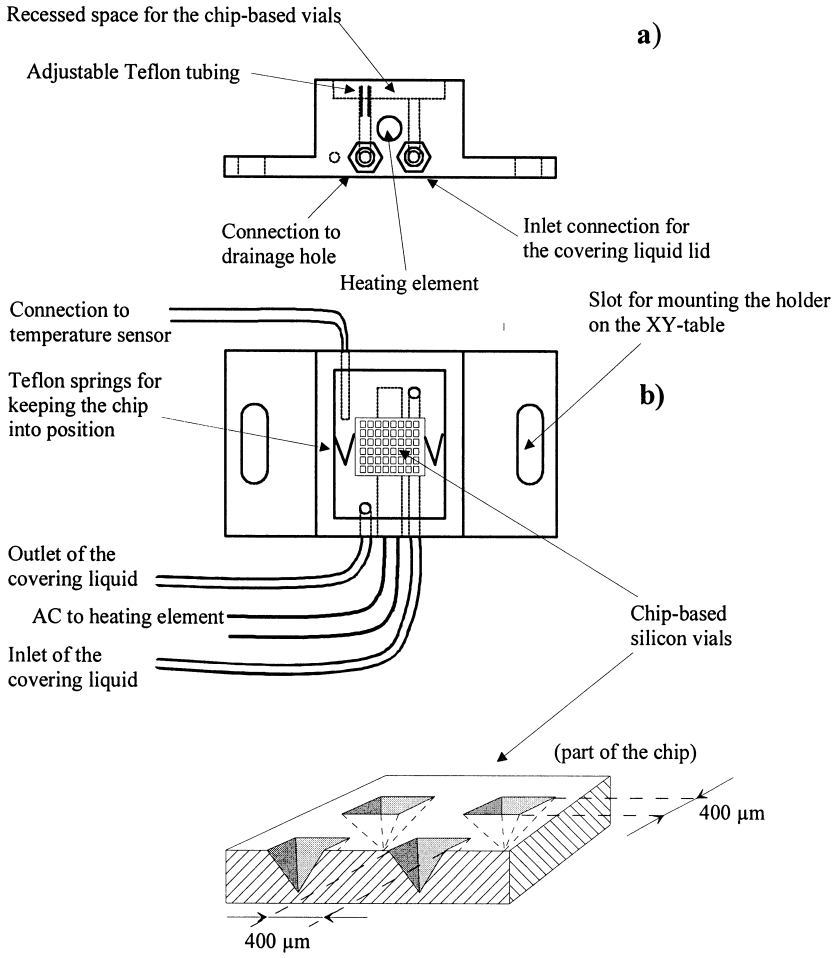

Fig. 2. A side-view (a) as well as a top-view (b) of the chip-holder, made of aluminum, used in the study. The thickness of the liquid lid is determined by the height of the PTFE tubing, inserted into the drainage hole.

controlled) fused-silica capillary (40 μm I.D.×105 μm O.D.) which was passed through the octane film, resulting in a protein/enzyme ratio ~25:1. Thus, in this step, the dried myoglobin and buffer salts attached to the walls of the vials redissolved, and the digestion process was initiated. The chip-holder was then heated to 37°C to optimize the reaction conditions.

2.4. Capillary electrophoresis (CE)

After completion of the enzymatic reaction, a CE analysis of the reaction products was carried out. For this purpose, the injection end of the separation capillary was positioned through the octane layer into the water phase inside the vial (micropositioner/*z*-direction). A sample aliquot of ~5 nl of the reaction mixture was injected by applying vacuum to the detection end of the separation capillary. Thereafter, the injection end of the separation capillary was brought into the buffer container of the CE equipment in order to start the separation. As soon as the analysis was terminated, the content in the subsequent vials were analyzed in the same way in sequential order. All reagents were obtained from Sigma–Aldrich Sweden (Stockholm, Sweden).

The detailed set-up for the CE analysis has been described earlier [12]. In summary, it consists of a high-voltage supply, an on-column variable-wavelength UV detector and arrangements for vacuum injection as well as running buffer replenishment. The cathode buffer container is mounted on the $X–Y$ table while the anode buffer container (detection end) is mounted inside a Plexiglas box with safety interlocks. The buffer was replenished automatically between each run and the separation capillary was flushed with running buffer for 1 min prior to injection of a new sample.

3. Results and discussion

3.1. Miniaturized reactions

During the last few years, the need for miniaturized chemistry has increased dramatically. This need is particularly driven by areas like clinical diagnostics including DNA analysis and drug development (ultra high throughput screening and combinatorial chemistry). Also in other areas such as material science, there is a great interest in combinatorial approaches, since a huge number of experiments can be carried out on relatively small-sized platforms. The use of systems based on microchips is an obvious approach in view of the rational processes (photolithography, wet or dry etching methods, etc.) for fabrication, which are used to obtain a large number of identical functional microstructures positioned at highly defined coordinates.

While the merits of miniaturized systems have been pointed out extensively (e.g., [15,16]), perhaps one particular advantage needs to be highlighted. In situations where chemical reactions are to be performed, and the amount of material is limited, volume reduction is very advantageous, since high sample concentrations can be maintained in this way. Consequently, reaction rates are promoted (e.g., the conversion rate of an enzymatic digest [12]). Moreover, the increased surface area/volume ratio associated with volume reduction can provide an extra benefit, if the solid surface is engaged in the reaction (as a ligand carrier or as a catalyst).

Two basic methodologies can be visualized for miniaturized reactions [17]: flow systems, where the reaction occurs in a capillary tube or chip-based channel, or systems where the reaction occurs in a static environment like a nanovial or a droplet. In the vial system, both open and closed architectures can be utilized. Since the introduction of our concept of chip-based nanovials [14], the number of application areas have expanded rapidly, e.g., in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [18–20].

The choice between flow systems or vial-based systems for performing nanoscale reactions is not always straightforward. Both systems have their particular merits and potentials. The present study refers to the use of open vials. Some of the attractive features of this concept are:

1. A large number of individual reactors (e.g., 500 000) can be manufactured on a single chip (glass, silicon, quartz or plastic).
2. Random access to individual vials is possible, using a precision robotics system. Moreover, open systems can be accessed any time before, during and after a reaction (for e.g., addition of reagents, withdrawal of sample or detection).
3. The sample is kept in a confined space. Sample dispersion and/or dilution, which is an inherent problem in flow systems, is avoided.
4. No carry-over problems are experienced, as is due to dispersion and lagging of sample (adsorption) in sequential flow reactions.

However, there are also drawbacks of the open vial concept, notably:

1. Contamination from the outer environment (dust, including biological material), can be a severe problem.
2. The transfer of small sample volumes, particularly when dealing with a large number of *different* samples, demands for relatively complicated systems, such as arrays of micro-capillaries. However, this sample transfer problem is not trivial in flow systems either.
3. Evaporation of the reaction solvent.

3.2. Methods to prevent solvent evaporation from ultra-small volumes of liquid

Evaporation of the solvent matrix from very small sample volumes (which is usually water in biochemical applications) is a serious problem if the sample is exposed to the atmosphere. Since the evaporation rate is related to the increased surface area/volume ratio, the problem is increased as the sample volume is reduced. In a standard laboratory environment, we found that a water sample of ca. 10 nl evaporates from a chip-vial within 30 s, whereas a 10-pl water sample evaporates in less than a second.

Several strategies have been proposed to counteract the solvent evaporation. An obvious approach is to enclose the sample in a humidified environment [4]. In principle, working at 100% relative humidity would completely prevent an evaporation of water from the sample. However, operation at this humidity level is not possible without introducing severe problems. At a very high humidity level, water starts to condense onto irregularly shaped surface areas, like scratches, dust etc., leading to overflow to adjacent vials and cross contamination. The behavior is related to differences in surface wettability (contact angle), the strive of surfaces to minimize their free energy and the associated well known phenomenon of capillary condensation. Another problem is that the evaporation rate of water from a sample is affected by the presence of salts and other components, which can cause an excessive condensation of water into the sample vials. In practice, the concept is useful if the humidity level is kept at a safe level where condensation does not occur, but a periodic water addition is then necessary [12]. However, operation at elevated temperatures is critically difficult.

Another method for reducing the rate of water evaporation has been suggested, where high-boiling components (e.g., glycerol [8] or triethylene glycol [6]) are added to the reaction mixture in order to reduce the vapor pressure of the water. However, this will still require an occasional addition of solvent, when reactions are carried out under an extended period of time. An additional humidified environment can be beneficial in such circumstances. However, the main drawback of this approach is the possible interference of the additives with the reaction and its analytes. Also, the additives may be detrimental contaminants in subsequent analytical measurements (such as CE separations, MS, etc.).

Instead of reducing the rate of evaporation, another principle can be applied, where water is added continuously to the vials to compensate for evaporation [13]. When a continuous supply of solvent (e.g., via capillaries, connected with the reaction vials) is kept in balance with the evaporation, reactions can be sustained over many hours or even days. However, it is important to utilize very pure solvent, since impurities from the solvent are accumulated in the sample. Recently, we have constructed an array of 32 solvent feeding capillaries with dimensions, suitable for parallel reactions in 1-nl chip-vials, and the fabrication of larger arrays as well as capillaries, designed for smaller reactor volumes, should be quite feasible.

The techniques, discussed thus far are related to work with open vials. The more common strategy (particularly when working in μl-scale or larger) is to use a closed vial. Apart from regular solid lids, plastic sealing tape or film [5,21], a microscope cover slide [6], a liquid lid in the form of a high boiling mineral oil [7,22] or wax [23] have been employed. However, when vial volumes are reduced to nl size, manipulations becomes unfeasible. Part of the sample or condensed solvent, easily adheres to a solid lid and causes sample loss and/or leads to contamination. A mineral oil lid, which is frequently employed in polymerase chain reactions (PCRs), is difficult to remove from very small fluid volumes. Also, it contaminates and obstructs the inlet of micro-pipettes during withdrawal of an aliquot of sample, and disturbs subsequent analysis (CE or MS). Therefore, this method is not an attractive alternative.

The choice of technology for preventing evaporation from very small liquid volumes in open systems is dependent on the required degree of freedom. For some applications, it is desirable to carry out a very large number of parallel-operated reactions, including the possibility to have free access to individual vials or groups of vials (e.g., adding specific reagents) during the reaction. The functional operations of the utilized precision robots should not be impaired by enclosures (such as a humidity chamber). Also, the desired operating temperature limits the

usefulness of some of the approaches, particularly, when the temperature is close to the boiling point of the solvent.

The principles of the discussed methods are schematically depicted in Fig. 3, and in Table 1, a tentative summary of the discussed characteristics of the methods in terms of applicability and usefulness is listed. The characteristics mainly refer to situations, where the liquid volumes are in the low-nl range. Work with extremely small volumes (pl–fl), is increasingly difficult and the applicability of the listed methods will be more limited. Work in these volume domains will require specific, novel solutions.

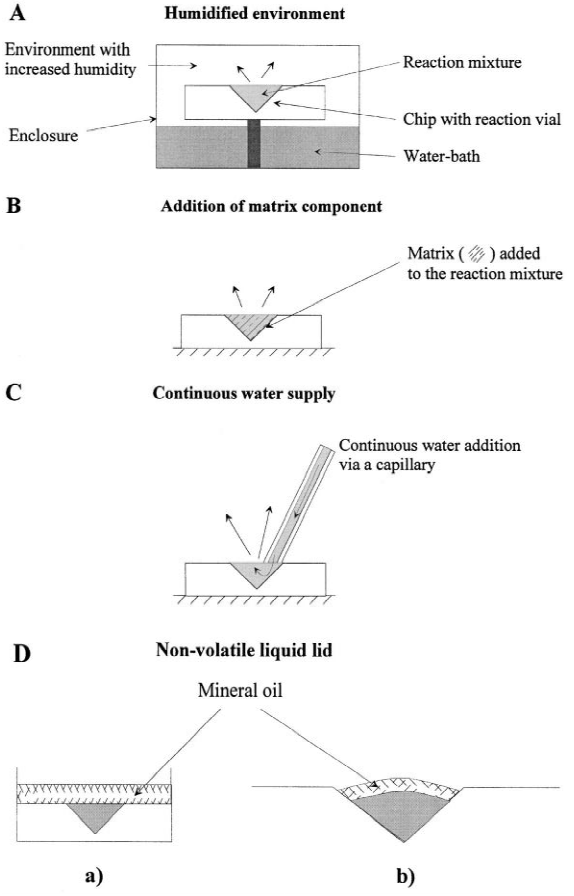

Fig. 3. Four possible solutions to control the evaporation of solvent from a miniaturized chip-based reaction mixture. The solvent evaporation is visualized by the arrows, pointing upwards. (A) Increase of the humidity of the surrounding atmosphere in order to reduce the driving force for the evaporation. The humidity can be increased by the introduction of wet surfaces inside an enclosure or by flushing the chamber with humidified air. (B) A high boiling component is added to reduce the vapor pressure of the solvent in the reaction mixture resulting in decreased evaporation. (C) The rate of evaporation and the supply of water are in balance during the reaction. (D) The evaporation is prevented by covering the reaction mixture with a layer of mineral oil. The schematic shows two different modes: (a) the entire chip is covered (increased risk for cross-contamination with high-density positioned vessels) and (b) a vial with an individual cover.

3.3. Volatile liquid lid

An attractive idea to prevent solvent evaporation is to cover the reaction liquid with a film of a non-miscible volatile liquid. This method was first utilized by Gratzl and Yi [10] who carried out diffusional titrations of pl-sized samples, which were submerged in heptane in a Petri-dish. In our present work, we create a thin layer (0.5–1 mm) of a volatile non-miscible liquid on the surface of chip-based vials. In order to prevent a premature evaporation of the cover liquid, a continuous supply of the liquid is guided over the surface. The level of the liquid is adjusted by means of an overflow outlet. Fig. 4 shows a schematic of the principle.

The advantage of this setup is that a similar degree of accessibility as in open vials is obtained, while the vials are always sealed by the liquid lid. Reagents can be added or sample can be withdrawn any time during or after a reaction, by guiding robot-controlled micro-pipettes or the inlet of a CE column through the covering film close to or into the sample. Contamination of the pipettes or the CE column by adhered covering liquid is not a problem, since this liquid evaporates, as soon as the pipettes or the CE column are exposed to the environment. Moreover, the entire liquid lid (and also the solvent of the sample) will evaporate completely, when the supply of covering liquid is discontinued.

In order to evaluate the loss of matrix solvent over an extended period of time, experiments were carried out where 15 nl of water, kept in a chip-based vial at 37°C was covered with a thin, flowing film of octane (estimated film thickness, ca. 0.5 mm). The meniscus of the water sample was monitored by using the microscope and the video of the robotics equipment. The results are shown in Fig. 5a. Even after 90 min, no loss of water was observed. After 105 min, the

Table 1
A tentative summary of some characteristics of technologies for preventing water evaporation from low-nl scale biochemical reaction mixtures[a]

	Humidity chamber	Matrix addition	Continuous feed of solvent	Solid lid	Lid of mineral oil
Accessibility	+	+++	++	−	+++
Suitability for a large number of vials	+++	+++	+	+++	+++
Suppression of evaporation	0	+	−	+++	+++
Work at elevated temperature	−	0	+	+++	+++
Problems with condensation	−	0	0	−	++
Cross contamination	0	++	++	+	(0)
Possible interference with the reaction	+++	0	+++	++	(−)
Possible interference with the analysis	+++	−	+++	+++	−

[a] Humidity chamber: the reaction is performed in an atmosphere with increased humidity, matrix addition: a high boiling compound is added to the reaction mixture to reduce the water vapor pressure, solvent feed: continuous compensation of evaporated water from the reaction mixture, solid lid: enclosure of the reaction mixture, mineral oil lid: the reaction mixture is covered with a layer of mineral oil. The different characteristics are ranked using the following grades: poor (−), neutral (0), fair (+), good (++) and excellent (+++).

first losses were clearly visible, and after 140 min, losses are on the order of 50%. The experiments were also repeated with water-saturated octane, in order to compensate for possible effects of water dissolution, but we could not note significant improvements. Also, we could not observe a significant dependence of the flow-rate of octane on the loss of water. It seems therefore that the water losses occur primarily via diffusion through the thin layer of the covering solvent.

Also, experiments at higher temperatures were carried out. It showed to be possible to retain most of the water sample in the vial at a temperature close to the boiling point (95°C) for several minutes (Fig. 5b). This performance is unique in the sense that it is well beyond the operating temperature range of the other non-contaminating methods, discussed above.

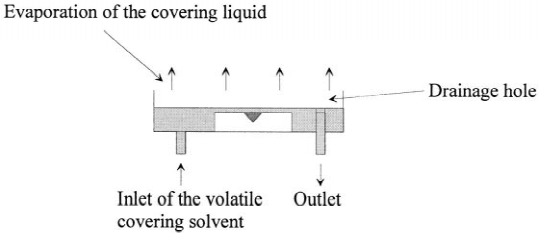

Fig. 4. The water evaporation is reduced by covering the sample with a volatile liquid lid. The evaporation of the lid is continuously compensated by an overflow. The thickness of the lid is determined by the position of the drainage hole.

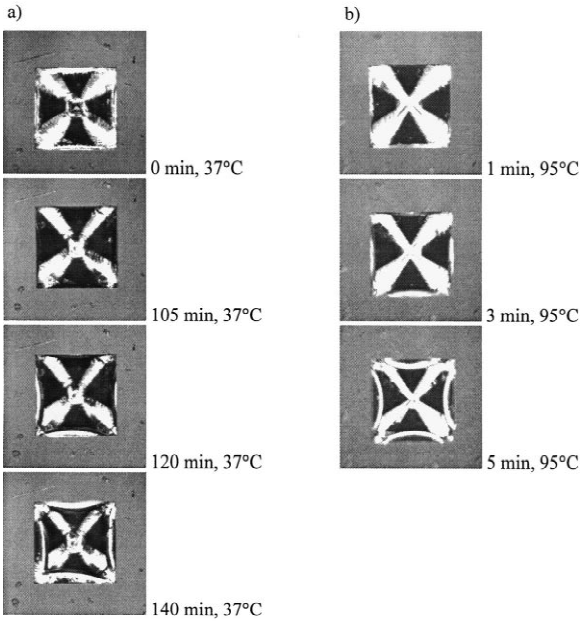

Fig. 5. Micrographs, showing the evaporation vs. time in a chip-based vial (15 nl) filled with water covered with a thin (ca. 0.5 mm) flowing layer of octane. (a) Temperature: 37°C. At 105 min, the beginning of the loss of water from the vial can be observed, by the formation of a small meniscus at the entrance edges of the vial. After 140 min the loss of water is estimated to be on the order of ca. 50%. (b) Temperature: 95°C. No loss of water was observed after 1 min. After 3 min, the formation of a meniscus can be seen and after 5 min, the volumetric loss of water is estimated to be ca. 50%.

In order to test the liquid lid concept in practice, a tryptic digest of myoglobin was performed in a 15 nl vial, covered with a film of octane as described in the Experimental section. Within a time-frame of 90 min, most of the myoglobin was enzymatically converted into peptides. An electropherogram of the peptide map is shown in Fig. 6a.

It is interesting to compare this map to the results obtained from a 15-nl tryptic digest, where the evaporation was counteracted by a continuous supply of water (according to the principle shown in Fig. 3c). The main pattern of the peaks is very similar in both cases. However, in the electropherogram of Fig. 6b, some additional peaks after the last three large peaks are seen. At present, we are not certain about the origin of these peaks. We cannot exclude the possibility that these signals are due to hydrophobic peptides. Such peptides would be extracted or discriminated by the flow of octane.

This is an important possible drawback, which has to be considered when using the liquid lid method.

Thus, the choice of the cover liquid is critical. Undesirable interactions and/or losses of hydrophobic components can occur. On the other hand, there may be situations where the removal of such components is desirable. For example, a comparative study, where both methods, utilized in Fig. 6a and b are used in a side-by-side experiment, may reveal

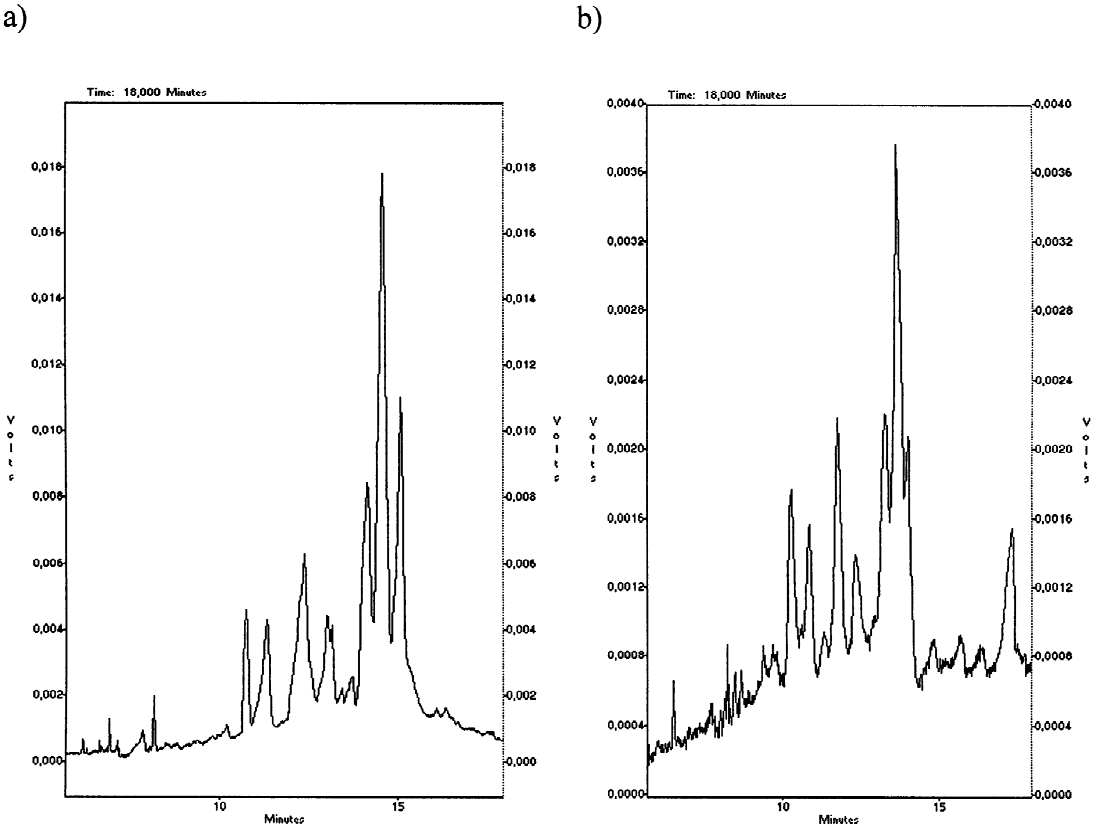

Fig. 6. Two electropherograms showing peptide maps of myoglobin after tryptic digests, performed in chip-based vials (15 nl). (a) The reaction was carried out under a liquid lid of octane. Reaction conditions: 95 min reaction time at 37°C, myoglobin: 4 mg/ml in 4 m*M* NH_4HCO_3, trypsin: 0.2 mg/ml. CE conditions: capillary, 85 cm (60 cm effective length)×50 μm I.D.×150 μm O.D., 0.01 *M* KH_2PO_4, 100 μg FC134/ml, +15 kV, 6 μA. (b) The reaction was carried out by continuous compensation of the evaporating water from the vial (see Fig. 4c). Reaction conditions: 240 min reaction time at 37°C, myoglobin: 8 mg/ml in 8 m*M* NH_4HCO_3, trypsin: 0.2 mg/ml. CE conditions: capillary, 102 cm (72 cm effective length)×50 μm I.D.×150 μm O.D., 0.01 *M* KH_2PO_4, 100 μg FC134/ml, +15 kV, 5 μA.

valuable chemical information of individual components in complex mixtures.

4. Further developments and conclusions

There should be considerable applications for high throughput parallel-operated miniaturized protein digests, particularly in the emerging field of proteomics. Working with the miniaturized volumes is particularly interesting when dealing with weakly expressed proteins, isolated from two-dimensional (2-D) gels, where very little material is available.

The potentials of the liquid-lid technology are critically dependent on the interaction of the covering fluid with the analytes. Hydrophobic or other interactions with analytes or reagents, as pointed out above, should be avoided. In this context, it could be advantageous to utilize perfluorinated solvents. It has been shown that such components have a minimal affinity for biomolecules [24]. In fact, such solvents have even been utilized as a substitute for blood [25].

A particular development of interest is to combine the liquid lid concept with piezo-electrically driven dispensers. In this way, a physical contact between the tip of the dispensing tool and the reaction fluids, including the covering liquid, is omitted. Preliminary investigations showed that pl-sized droplets of fluid can be ''shot'' through the thin layer of covering solvent (Fig. 7). However, in order to prevent the droplet from disintegrating upon impact with the liquid surface, several operating parameters have to be optimized. Factors, affecting the success of this approach are related to the drop-size, the drop-speed and the viscosity, density and thickness of the covering liquid, as well as the distance between the liquid surface and the dispenser nozzle.

Finally, one of the most interesting potentials for the liquid lid concept is in miniaturization of PCR. Low-nl volumes of water can be kept at 95°C for several minutes as shown above, and the possibility to perform extremely rapid temperature cycling is feasible due to the low mass of the system. It should therefore be possible to design a simple system for massive parallel-operated PCR in open vials on a chip. We believe that an interaction of the reaction components with a hydrocarbon-like covering solvent (e.g., octane) is not likely, in view of the fact that mineral oil is already routinely used as a cover in ''macro'' PCR vials. However, the increased surface area/volume ratio, an inherent property of miniaturized systems, can lead to unexpected problems, and therefore, a careful evaluation of the different factors affecting the reaction performance is necessary. This is currently in progress.

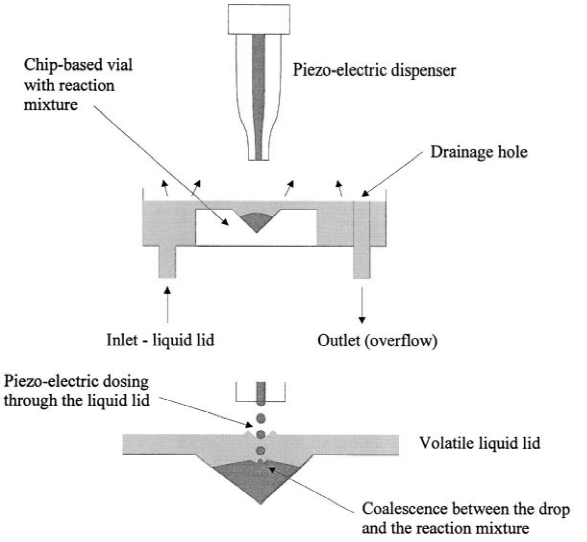

Fig. 7. A schematic of a piezo-electric non-contact dosing of a reagent or a sample through a volatile liquid lid. Under optimized conditions, the entire droplet coalesces with the reaction mixture.

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