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Miniaturized Electrospraying as a Technique for the Production of Microarrays of Reproducible Micrometer-Sized Protein Spots

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Electrospraying in a stable cone-jet mode at <400 μm above a substrate is shown to be a powerful technique to produce arrays of identical micrometer-sized spots consisting of biologically active substances. Aqueous solutions with a surface tension of 0.04 N m^{-1} and conductivities ranging from 0.04 to 2.2 S m^{-1} were sprayed at ultralow flow rates ranging from 100 to 300 pL s^{-1}. The charged jet that emanates from the cone tip breaks up into a spray of charged droplets that are deposited in the form of a uniform spot of 130−350 μm in diameter by spraying during 0.5−3 s at 220−400 μm above a substrate, respectively. After a spot was deposited, spraying was stopped instantaneously by increasing the distance between the capillary tip and the substrate by an additional 100 μm using a computer-controlled x-y-z table. This was immediately followed by a rapid shift of the substrate 400 μm sideways and 100 μm upward, thus causing spraying to resume instantaneously because of the increased electric field strength, which resulted in the deposition of the next spot. It is shown here that spraying of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6P-DH), and pyruvate kinase (PK) on a liquid layer resulted in the complete preservation of their activities despite the high solution conductivity of 3.3 S m^{-1} and high currents ranging from 300 to 500 nA. LDH and PK activities were fully preserved after spraying onto dry aluminum by adding 0.05 M buffer and 0.5 and 1 wt % of trehalose, respectively, to the spray solutions. Electrospraying allows for accurate dispensing of liquid volumes as small as 50 pL. Enzymatic activities of LDH and PK are fully preserved after spraying.

Currently, a strong tendency is observed toward extreme miniaturization of (bio)chemical assays[1−3] that are used for quality management in biotechnology, medical diagnostics, environmental monitoring, combinatorial chemistry, and high throughput biochemical screening. Miniaturization of assays requires special techniques to deposit picoliters (pL) of reagent solutions (enzymes, antibodies, etc.) on well-defined positions of suitable substrates. In particular, when multiple analytes from a single sample need to be simultaneously determined for quantitative studies, a device is needed that allows for accurate, reproducible, and parallel dispensing of small liquid volumes while avoiding cross-contamination. Available liquid deposition techniques are based on direct-contact or noncontact deposition (dispensing). For example, the "pin-ring" technique (GMS 417 arrayer, Westburg BV) and stamp microcontact technique[4] allow for a direct-contact deposition of dots of identical composition. Consequently, the deposition of multiple substances on a single spot requires additional washing and drying steps to avoid cross-contamination, which makes these techniques time-consuming. The noncontact piezoelectric technique, on the other hand, allows for fast dispensing of monodisperse droplets. Lemmo et al.[5] used this technique in combination with a 48 × 48 array of 8-μL wells for high-speed, parallel microdispensing. However, liquids containing surfactants, and viscous liquids are very difficult to handle with piezoelectric dispensing, and cross contamination is likely to occur because of splashing that is caused by the impact of the droplets on the substrate. Furthermore, because of the relatively large dispensing heads (>3 mm) it is not possible to dispense multiple substances simultaneously on target areas that are <3 mm apart (center to center) in a straightforward manner.

We have shown previously[6] that miniaturized electrospraying of liquids containing ethylene glycol in a stable cone-jet mode allows for noncontact, accurate, and reproducible dispensing of ultrasmall liquid volumes. Spraying in a stable cone-jet mode[7−9]

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at typical nozzle−substrate distances on the order of centimeters is a powerful technique to produce an aerosol of small droplets that are more or less monodisperse. Because the charged droplets repel each other, substrates are covered with a thin layer of material, as is used for the production of very thin homogeneous layers of polymer,[10] paint,[11] and semiconducting ceramics.[12] This technique was applied by us in a strongly miniaturized form to produce micrometer-sized spots of biologically active substances. The miniaturization is based on the spraying of ultrasmall amounts of liquid, typically on the order of a few hundred picoliters, at distances of 220−400 μm between a capillary tip and the target area. In addition to the spraying of liquids containing 40−70% v/v ethylene glycol, aqueous solutions containing additives, such as surfactant, trehalose, and electrolytes having liquid conductivities up to 2.2 S m^{-1}, can be sprayed in a stable cone-jet mode. In contrast to our technique, Morozov and Morozova[13,14] used "nanoelectrospray ion sources"[15,16] to deposit the single spray droplets containing protein or DNA onto various conductive substrates using capillary−substrate distances on the order of 10−15 mm. Deposition was spatially controlled by guiding the droplets using a dielectric mask or illuminated areas of a photoconductive layer; however, their liquid composition was restricted to conductivities <0.03 S m^{-1} to avoid discharging, which means that practically no buffer can be added to stabilize the solution pH. Furthermore, the authors do not report whether spraying occurs in a specific spraying mode, like the cone-jet mode, which is required to produce monodisperse droplets and allows the prediction of the droplet size[17] as a function of the liquid properties. In contrast to this, our technique is applicable for quantitative as well as qualitative studies, because the reduced distance between the capillary tip and the substrate allows deposition of an accurate amount of liquid on a specified target area of a suitable substrate without the need for immobilization techniques. In addition, the reduced distance opens possibilities for parallel spraying of multiple solutions using capillaries or channels that are positioned at a typical distance of 400−600 μm apart.

In this paper, optimal conditions for spraying in a stable cone-jet using water as solvent containing additives such as the surfactant Brij 35, trehalose, and electrolytes are reported. An optimal production of arrays of spots is achieved by combining precision liquid delivery with precision positioning of the substrate at <400 μm below a capillary. We have shown previously[6] that the enzymes glucose oxidase and horseradish peroxidase (dissolved in 70% v/v aqueous ethylene glycol) remain active upon electrospray dispensing (ESD) in aluminum wells. In this paper, we use a similar method to analyze in a quantitative manner the effect of electrospraying on activities of the relatively labile enzymes lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6P-DH), and pyruvate kinase (PK) that are currently used in the analysis of primary metabolites in yeasts. To exclude activity loss due to mechanical and drying effects, the enzyme solution was electrosprayed onto a liquid film consisting of 3 μL of reagent solution, and enzymatic activity was determined using luminescence detection. To determine loss of LDH and PK activities as a result of impact combined with drying of the spray droplets, LDH and PK solutions containing trehalose and Brij 35 were sprayed onto a dry aluminum well bottom.

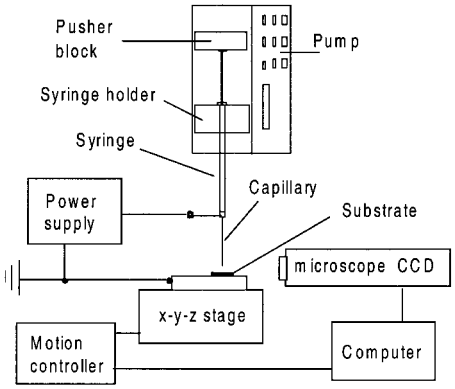

Figure 1. Schematic diagram of the ESD system.

EXPERIMENTAL SECTION

Chemicals. Ethylene glycol (99+%) was from Aldrich (Milwaukee, WI). Brij 35, D(+) trehalose, phospho(enol)pyruvate (PEP) and pyruvate were from Sigma (St. Louis, MO). NADP, NADH, ADP, G6P-DH (1250 U/mL), LDH from beef heart (1250 U/mL), PK from rabbit muscle (2000 U/mL), and the ATP Bioluminescence assay kit CLS II (cat. 1699695) were from Boehringer Mannheim (Germany). All enzyme suspensions contained 3.2 M $(NH_4)_2SO_4$. In all cases, MilliQ-purified water was used.

Apparatus. *Electrospray Device.* In Figure 1, a schematic diagram of the ESD setup is shown. The piston of a Hamilton syringe (cat. 802 SNE, Bonaduz, Switzerland) is displaced by the pusher block of a Harvard PHD 2000 precision displacement pump (minimum plunger travel rate, 0.18 μm min^{-1}) from ANTEC Leyden (Leiden, The Netherlands). The piston displaces liquid through the syringe and through the connected bendable stainless steel capillary that has an i.d. of 60 μm, and an o.d. of 160 μm. The metal part of the syringe is connected to the positive output of a DC power supply (Air Parts, HCN 12500, Alphen aan den Rijn, The Netherlands). The aluminum plate that fits on a grounded metal holder (6 × 7 cm) is accurately positioned and moved relative to the capillary using an x-y-z table that is moved by high-speed precision actuators (4.7 mm s^{-1} travel speed) that are controlled by a Newport motion controller (all from Newport, CT). ESD was followed and recorded by a CCD camera (VC 3031) mounted on a microscope (1629 Z-90, objective 2 ×, C mount 20 ×) using fiber-optic light sources, all from Euromex (Arnhem, The Netherlands). A framegrabber (AGP-V2740 driver) was purchased from Asus Computer International (Newark, CA).

Substrates. Silicon substrates of 2 × 1 cm with a 50-nm-thick silicon nitride top layer were made at the Delft Institute of Microelectronics and Submicron Technology (DIMES).

Aluminum wells that are 1 mm deep, 3 mm in diameter, and 3.5 mm apart (center to center) were used to quantify enzymatic

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activities. The wells were drilled in an aluminum plate that is 12.7 × 6.5 and 1 cm high, which conforms to standard 96-well plate dimensions.

Pipetting. A single-channel Proline electronic pipet with a maximum pipetting volume of 10 μL (cat. 710520) from Beun De Ronde (Abcoude, The Netherlands) was used to add the cofactor and substrate solutions to the aluminum wells.

Luminescence. Fluorescence was measured on a Perkin-Elmer HTS 7000+ microtiter plate reader (Nieuwerkerk a/d IJssel, The Netherlands) that allows for scanning and optimal readout of the aluminum wells. Bioluminescence was measured on a PhL mediator purchased from Mediators Diagnostica GmbH, (Vienna, Austria).

Surface Tension. Liquid surface tensions were measured on a Krüss tensiometer (Hamburg, Germany).

Conductivity. Liquid conductivities were measured on a Consort K610 conductometer (Turnhout, Belgium).

Safety. To avoid the current's flowing from the syringe piston to the electrical parts of the pump, the metal pusher block was replaced by Delrin. The aluminum plate was moved using the motion controller, which allowed the performance of the experiments without the risk of touching electrical parts. When an experiment was completed, the power supply was turned off.

METHODS

Miniaturized Spraying of Identical Spots. *ESD Procedure.* The required distance between the capillary tip and the substrate was accurately set using the motion-controlled x-y-z-table. After flushing the capillary for 1 min at 100 μL hr^{-1} to remove residual air bubbles, the desired flow rate was set. Excess liquid was removed from the capillary tip to avoid discharging during the cone-formation period. A potential difference was rapidly applied until a stable cone was formed. A positive polarity was used to spray in a stable cone-jet mode at a wider range of potential difference.[18]

ESD Characteristics. The effect of liquid conductivity and potential difference on the spraying behavior at ultralow flow rates and ultrasmall distances between the capillary tip and the silicon nitride surface was visually observed. Brij 35 and TEA buffer were added to the aqueous ESD solutions to lower the surface tension and increase the conductivity, respectively.

During stable spraying on a gold electrode, the current was measured, using a lab-made potentiostat, as a function of the flow rate, liquid conductivity, and potential difference.

ESD of Arrays of Spots. Solutions containing Brij 35, TEA buffer, trehalose, and LDH were sprayed in the form of spots by repeatedly moving the substrate 100 μm downward (spraying stops), 400 μm sideways (to the next target area in <150 ms), and 100 μm upward (spraying resumes), as is shown in Figure 2 panels A to C, respectively. During spraying of arrays of spots, neither the potential difference nor the flow rate was changed. The spot diameter was determined as a function of flow rate, liquid conductivity, potential difference, spraying time, and distance between the capillary tip and silicon nitride surface. Spot diameters were measured using a ruler from 180× magnified frames that were obtained by the microscope-CCD camera.

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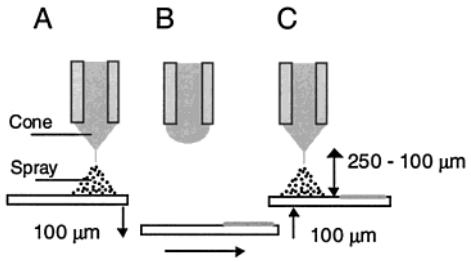

Figure 2. (A) Schematic view of the cone-jet mode. (B) Spraying stops due to an increase of the distance between the tip and substrate. (C) Spraying resumes.

Quantification of Enzymatic Activities. To evaluate the way enzyme activities are affected by the electrospray process alone, solutions of LDH, G6P-DH, and PK were sprayed on top of 3 μL of cofactor solution that was pipetted into an aluminum well.

Reaction Protocols. Activities of LDH and G6P-DH were determined according to Bergmeyer[19,20] by measuring the decrease in NADH and increase in NADPH concentrations with time, respectively. PK activity was determined according to the coupled reaction

$$\mathrm{PEP} + \mathrm{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \mathrm{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \mathrm{AMP} + \text{oxyluciferin} + \text{light} \quad (1)$$

The conversion of PEP into pyruvate was performed according to Bergmeyer,[21] and the coupled conversion of ATP into AMP under emission of green light was performed according to the prescription supplied with the ATP assay kit CLS II. At the prescribed optimal assay volume of 50% v/v of luciferase reagent solution, 0.5% of the formed ATP is converted per minute for a working range between 10^{-5} and 10^{-11} M ATP. This means that the light signal is constant with time at a defined ATP concentration and linearly related to the amount of converted PEP and ATP with time.

Reagent Solutions. All LDH, G6P-DH, and PK solutions contained 0.1/0.01 M TEA/EDTA, pH 7.6; 0.1/0.01 M TRIS/EDTA, pH 7.8; and 0.1 M TEA, pH 7.6, respectively. The LDH cofactor and substrate solutions contained 1.3 mM NADH and 2 mM pyruvate, respectively. The G6P-DH cofactor and substrate solutions contained 1.3 mM NADP and 2 mM G6P, respectively. The PK cofactor solution contained 53 μM ADP, and 67% v/v luciferase assay solution. The PK substrate solution contained 80 μM PEP.

In control experiments, the above-mentioned buffered cofactor solutions contained 2 mM NADH, 2 mM NADP, and 80 μM ADP in 100% v/v luciferase assay solution, respectively. The compositions of the substrate solutions were similar to the above-mentioned compositions.

Enzyme Solutions. LDH, G6P-DH, and PK were diluted in the above-mentioned buffer solutions. A nonionic surfactant was added to all ESD solutions, because ionic surfactants may cause a rapid loss of LDH activity, depending on the LDH isoenzyme and

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surfactant.[22] Brij 35 was chosen because it is easy to handle and stable. Brij concentrations of 0.5, 1, and 2 wt % were used, depending on the enzyme.

For each ESD experiment, the syringe was filled with fresh enzyme solution that had been stored for no longer than 2 h at 4 °C. The amount of electrosprayed enzyme solution was calculated by multiplying the calibrated flow rate[6] by the dispensing time.

Determination of Enzymatic Activity. At the start of each experiment, 3 μL of the corresponding cofactor solution was pipetted into a single aluminum well (assay volume, 4 μL). This was immediately followed by spraying 0.015−0.2 μL of the corresponding enzyme solution into the center of the cofactor solution at relatively high flow rates of 550−1100 pL s^{-1} for 20−120 s. Finally, 1 μL of substrate solution was pipetted to start the reaction, which was immediately followed by coverage of the well with a glass slide to prevent evaporation during the luminescence measurements. In control experiments, 1 μL of enzyme solution (0.25−2 U/mL), 2 μL of cofactor solution, and 1 μL of substrate solution were pipetted in a single well. Before detection, initial pyruvate and NADH concentrations were 0.5 mM and 1.0 mM, respectively. Initial G6P and NADP concentrations were 0.50 mM and 1.0 mM, respectively. Initial PEP, ADP, and luciferase assay solution concentrations were 20 μM, 40 μM, and 50% v/v, respectively.

The decrease in NADH and the increase in NADPH concentrations were followed in time by measuring their fluorescence (λ_{exc} = 340 nm, λ_{em} = 465 nm) every 20 s for 2−5 min, depending on the enzyme concentration. Bioluminescence at 563 nm was measured every 10 s for 1−3 min, and the signal was integrated for 1 s.

ESD of LDH and PK upon Dry Aluminum. To evaluate the activities of LDH and PK upon impact of the spray droplets with the aluminum surface, appropriate LDH and PK solutions containing trehalose and Brij 35 were sprayed onto a dry aluminum well bottom at a flow rate of 280 pL s^{-1} for 12 and 6 min, respectively. In control experiments, the LDH solution was injected directly at the well bottom at a flow rate of 280 pL s^{-1} for 12 min. The liquid was distributed adequately by moving the well bottom continuously below the capillary during spraying as well as during injection. The deposited 0.2 μL of LDH solution was left to dry for 10 min at 22 °C. In control experiments, 0.1 μL of the PK solution was rapidly injected by the capillary onto the well bottom to avoid drying.

Calculation of Reaction Rates. The reaction rates (signal in arbitrary units min^{-1} per 4 μL of assay volume) were determined from the slopes of the progress curves. The reaction rates were converted to nanomoles of converted pyruvate min^{-1} per well and nanomoles of converted glucose-6-phosphate min^{-1} per well by measuring the NADH and NADPH signals from known amounts of the fully converted corresponding substrates. The reaction rates for PK were calculated indirectly by dividing the calculated reaction rates by the reaction rate for luciferase, that is, a conversion of 0.5% of the ATP formed.

RESULTS

Miniaturized Spraying of Identical Spots. *ESD at Ultralow Flow Rates.* Spraying of aqueous solutions requires a surface tension lower than 0.055 N m^{-1} to avoid discharging that is caused by ionization phenomena[18,23] that results in loss of cone stability. Addition of 0.1−10 wt % of Brij 35 resulted in a surface tension of 0.044 N m^{-1}, which allowed for spraying using flow rates ranging from 1100 to 100 pL s^{-1}. However, in the range of 150−100 pL s^{-1}, a rounded pulsating cone was obtained instead of a stable point-shaped cone, as is shown in Figure 3. By adding at least 0.005 M TEA buffer, enough surface charges were generated to spray in the cone-jet mode using flow rates ranging from 140 pL s^{-1} to 280 pL s^{-1} at potentials ranging from 0.95 to 1.13 kV at a spray distance of 220 μm and up to 1.00−1.30 kV at a spray distance of 300 μm. At the extremes of the voltage range, a pulsating cone tip or discharging was observed. Spraying was not influenced by the addition of 0.5−10 wt % of trehalose. To spray with flow rates higher than 300 pL s^{-1}, liquid conductivities should be lower than 0.9 S m^{-1}, preferably lower than 0.2 S m^{-1}, to reduce the risk of discharging, and a Brij 35 concentration of >0.4 wt % is preferred to spray at less critical potential differences. At flow rates <140 pL s^{-1}, the cone was still rounded instead of pointed, probably because of the fast evaporation. Evaporation is also an explanation for the required minimum flow rate, which was higher than the previously reported[6] minimum flow rate of 50 pL s^{-1} obtained with a solution consisting of 0.3 M NaCl and 70% v/v ethylene glycol.

The currents measured during spraying at a distance of 400 μm and a potential of 1.38 kV are listed in Table 1 as a function of flow rate and conductivity. The current shows good linearity with flow rate and reasonable linearity with conductivity. In the conductivity range of 0.06−0.6 S m^{-1}, the current does not increase significantly with an increase in potential of 0.07 kV. At a very high conductivity of 3.3 S m^{-1}, the current increases strongly from 300 nA to 600 nA by increasing the potential by 0.07 kV at a constant spray-distance of 400 μm.

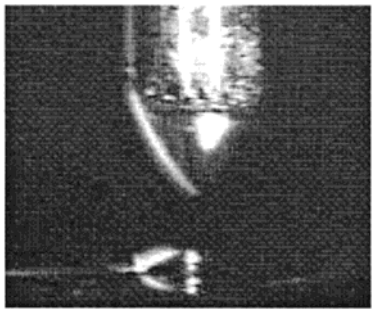

Figure 3. ESD of a small spot 130 μm in diameter. The distance between the capillary tip and the substrate is ~220 μm. The cone-length is 130 μm.

Table 1. Current as a Function of Conductivity and Flow Rate

flow rate, pL s^{-1}	current, nA[a]			
166	7 (0.055)	8 (0.070)	50 (0.68)	90 (1.57)
220	(0.055)	(0.070)	75 (0.68)	125 (1.57)
280	14 (0.055)	14 (0.070)	90 (0.68)	170 (1.57)
333	18 (0.055)	(0.070)	105 (0.68)	(1.57)

[a] Conductivity, S m^{-1}, given in parentheses.

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ESD of Arrays of Spots. Stable spraying at a flow rate of 100 pL s^{-1} at spray distances of 400 and 220 μm, and at potential differences of 1.37−1.43 kV and 1.04−1.08 kV, respectively, resulted in spot diameters of 350 and 130 μm, respectively. However, as is shown in Figure 3, a cone length of 130 μm is obtained at a spray distance of 220 μm, meaning that only 90 μm of space is left for the jet and the spray of droplets. As a consequence, a liquid dot is obtained instead of a thin liquid layer.

A spray-distance of 300 μm is regarded by us as an optimal distance to obtain homogeneous spots of acceptable size using a capillary o.d. of 160 ± 10 μm. In Figure 4A,B, arrays of such uniform spots are shown that were obtained by the spraying of a liquid consisting of 20 U/mL of LDH, 0.5 wt % of trehalose, 0.5 wt % of Brij 35, and 0.05 M TEA using a typical spray time of 1 s and a potential difference of 1.3 kV. Cone formation and retraction were practically instantaneous at a potential difference of 1.2−1.3 kV, which is preferred to obtain identical spots. No splashing was observed during the spraying experiments. Spot diameters of 220, 230, and 260 μm were obtained using flow rates of 140 pL s^{-1}, 185 pL s^{-1}, and 280 pL s^{-1}, respectively. The spot diameter was not influenced by a change in potential difference (1.20−1.30 kV), dispensing time (0.5, 1, 2, or 3 s), or liquid conductivity (0.1−1.6 S m^{-1}). Spraying for longer than 3 s or at high humidity resulted in an increasing amount of liquid that was pushed outward and enlarged the spot height and diameter.

Quantification of Enzymatic Activities after ESD. *ESD of Enzyme Solutions.* The enzyme solutions listed in Table 2 were sprayed onto 3 μL of cofactor film at flow rates of 550−1100 pL s^{-1} to obtain adequate enzyme concentrations in the wells in a relatively short dispensing period of 20−120 s. Liquid conductivities of 1.5, 3.3, and 3.6 S m^{-1} were used to investigate the enzyme activity at relatively high currents.

To avoid discharging, spray-distances >400 μm were used, and before every ESD start-up, the potential was applied prior to the start of the liquid feed. However, this resulted in a start-up period of 1−3 s during which some air bubbles were released occasionally. If the capillary tip is not cleaned properly before the ESD start-up, crystallization of salt at the capillary tip may occur, which may result in a multicone or spindle cone formation. The enzyme solution compositions listed in Table 2 allowed for critical but stable spraying onto the cofactor film at the indicated ESD parameters.

Enzymatic Activity. The calculated reaction rates and standard deviations obtained from ESD and control experiments are listed in Table 3. From direct comparison, we conclude that no significant loss of the enzyme activities occurred as a result of either the spraying process that was performed under the conditions listed in Table 2, electrochemical destruction, or the impact of the droplets upon the cofactor film. Standard deviations are due to pipetting errors, errors caused by the difficult ESD start-up, and evaporation of the liquid film during spraying.

The ratio of reaction rate (nanomoles of converted substrate min^{-1} per well) and enzyme concentration (milliunits per well) should be equal to 1. However, nominal ratios of 0.65, 0.77, and 1.3 were calculated for LDH, G6P-DH, and PK, respectively, which showed good linearity, so this is probably due to a systematic error resulting from either the enzyme suspensions, which may have had concentrations that were different from the concentrations reported by the supplier, or a systematic error resulting from the conversion of the luminescence signals into concentrations by fully converting known amounts of substrates.

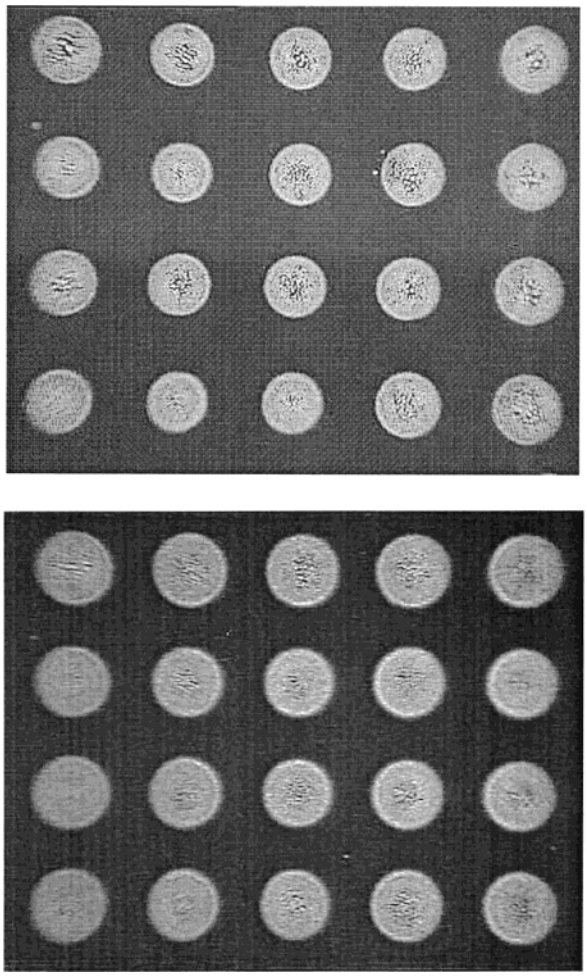

Figure 4. LDH spots that are sprayed 400 μm apart (center to center) using a distance between the capillary tip and substrate of 300 μm; (A) flow rate, 140 pL s^{-1}; (B) flow rate, 280 pL s^{-1}.

Table 2. Composition of Enzyme Solutions and ESD Parameters

spray-solution composition[a]				ESD parameters	
enzyme	conc U/mL	cond S m^{-1}	Brij wt %	flow pL s^{-1}	potential kV
LDH	4	1.5	0.5	1100	1.5−1.8
LDH	20	3.3	0.5	1100	1.8
LDH, dry	5	0.65	0.5	280	1.35
G6P-DH	20	3.6	1	830	1.5−1.6
G6P-DH	20	3.6	2	830	1.55
PK	20	3.3	1	550	1.37 or 1.77
PK	20	3.3	2	550	1.33−1.45
PK, dry	4	0.60	1	280	1.30

[a] All solutions except the LDH and PK solutions that are sprayed onto dry aluminum (dry) contain 0.1 M NH_4Cl.

Spraying a PK solution containing 1 wt % instead of 2 wt % of Brij 35 at a potential difference of 1.37 kV resulted in a relative activity loss of 40%. This can be explained by spraying close to discharging at a slightly higher required electric field strength and higher current flow of 600 nA instead of 400 nA. Another explanation for the activity loss is that PK may interact with the

Table 3. Comparison of Reaction Rates of ESD and Control Experiments[a]

enzyme conc.mU/well	ESD	Nr[b]	controls	Nr
	LDH			
0.48	0.31 ± 0.03	2	0.29 ± 0.02	2
1.00			0.68 ± 0.02	2
1.20			0.75	1
1.44	0.91 ± 0.06	3		
	LDH dry			
1.00	0.48 ± 0.02	5	0.46 ± 0.02	5
	G6P-DH			
1.00	0.75 ± 0.08	3	0.82 ± 0.05	2
1.28	0.98	1	1.09 ± 0.05	2
2.00	1.57 ± 0.11	2	1.44 ± 0.03	2
	PK			
1.30	1.58	1	1.55	1
1.7	2.20 ± 0.14	2		
2.5	3.15 ± 0.08	2	3.25 ± 0.18	3
3.6			4.98	1
	PK dry			
0.4	0.52 ± 0.04	5	0.49 ± 0.03	5

[a] Reaction rates in nmol of converted substrate min^{-1} per well. [b] Number of experiments.

surface of the spray droplet and this might be suppressed by addition of extra Brij 35, as reported by Adler and Lee[24] for an LDH-polysorbate system.

Spraying a PK solution containing 2 wt % of Brij 35 at a potential difference of 1.77 kV and a spray distance of 800 μm resulted in a relative activity loss of 70%, although spraying at this distance was less critical, and comparable currents of 350−400 nA were measured. So PK activity is probably lost during the longer flight time of the droplets, which exposes them to a longer evaporation period, resulting in higher salt and charge concentrations.

ESD of LDH and PK upon Dry Aluminum. ESD of the above-mentioned enzyme solutions upon dry aluminum under the above-mentioned conditions resulted in 98−100% loss of LDH and G6P-DH, as well as PK activity. Extra addition of 0.5−1 wt % of the protectant trehalose to the spray solutions was not effective; hence, loss of enzyme activity was not caused by drying of the enzyme deposits during and after spraying. Therefore, it is most likely that activities are lost upon the landing of the highly charged droplets on the aluminum surface. To reduce high charge densities and high salt concentrations, the conductivities of the LDH and PK solutions were lowered to 0.7 and 0.6 S m^{-1} by adding only 0.05 M buffer and 5 U/mL LDH and 4 U/mL PK, respectively. In addition, 0.5 wt % Brij 35 and trehalose, and 1 wt % Brij 35 and trehalose were added to the LDH and PK solutions, respectively, to preserve their activities upon drying. In five experiments, LDH and PK solutions were sprayed in a stable cone-jet mode at a spray distance of 450 μm by applying potential differences of 1.35 and 1.30 kV, resulting in currents of 85 and 75 nA, respectively. During visual observation of the spraying, it appeared that the solutions were deposited in the form of a nearly dry film of material.

The calculated reaction rates and standard deviations obtained from ESD (dry) and control experiments performed with 1 mU of LDH and 0.4 mU of PK are listed in Table 3. From direct comparison, we conclude that LDH and PK activities were preserved completely during the spraying process and the impact of the droplets upon the aluminum surface. Compared to the reaction rate of 0.68 nanomoles of pyruvate min^{-1} obtained from pipetted LDH (Table 3), we conclude that the 30% loss of LDH activity is entirely due to drying.

DISCUSSION

Our miniaturized electrospray technique allows the production of arrays of uniform spots of 130−350 μm in diameter without splashing. In contrast to the relatively complicated and expensive piezoelectric technique in which enzyme solutions are exposed to high shear rates, liquid is displaced at ultralow flow rates through a capillary and sprayed delicately onto a substrate in a controlled and reproducible manner without discharging. Spraying of highly conductive LDH, G6P-DH, and PK solutions resulted in severe loss of their activities that can only be ascribed to the landing of the droplets on the aluminum surface combined with evaporation, thus resulting in very high salt concentrations and high charge densities. To avoid this, buffer concentrations on the order of 0.05 M should be used. In addition, Brij 35 appears to be an excellent protectant in combination with trehalose, because LDH and PK activities were preserved completely and reproducibly. If required, >10 wt % of trehalose can be added to the spray solution to preserve the enzyme activities for longer periods after procedures such as drying and freeze-drying, as reported by Miller[25] and Jang[26] for LDH systems.

Spraying characteristics, such as spot diameter, are not influenced by changing the solution conductivity from 0.1 to 1.6 S m^{-1}, meaning that enzyme solutions can be sprayed without the need to measure or reduce their conductivities. Preliminary experiments have shown that it is possible to spray enzyme solutions having different conductivities in parallel with identical capillaries (o.d. = 160 μm) that are positioned 600 μm apart (center to center) and 250−400 μm above a substrate. If this is extended to an array of capillaries, different enzyme solutions can be sprayed simultaneously within 1 s in the form of arrays of spots.

In contrast to our controlled ESD technique, the spraying technique developed by Morozov and Morozova[14] requires solution conductivities <0.02−0.03 S m^{-1} to avoid discharging, which means that a buffer cannot be added to the spray solution to stabilize the enzymes and the solution pH. Moreover, instead of simultaneous spraying of different substances, they have to spray the different solutions in a sequential way, thereby exposing the enzyme spots to different and longer drying times. Furthermore, the dot volumes cannot be fully controlled because the number of droplets and the droplet sizes differ along the cross section of the conical spray.

Our ESD technique can be optimized in different ways. Spraying with a relatively large capillary o.d. of 160 μm resulted in a cone length of 120−130 μm, which is large, as compared to the distance between the cone-tip and substrate of 100−250 μm. A capillary o.d. of 50−100 μm will result in a cone-length of 50−100 μm, allowing deposition of even smaller spots at higher densities. In addition, a smaller capillary o.d. allows for spraying at flow rates <100 pL s^{-1}, as is used for mass spectral analysis.[27−29]

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The reproducibility of the spraying of arrays of spots can be improved by moving the substrate at a higher speed below the capillary between spraying. Especially when an array of capillaries is used to spray large numbers of identical arrays of spots, ultrahigh-speed actuators are required to move the substrates accurately below the capillaries in a fraction of a second. In addition, the electric field can be changed more rapidly and efficiently by turning the potential difference on and off instead of moving the substrate up and down.

CONCLUSIONS

It is shown here that miniaturized electrospraying in the cone-jet mode is a powerful and promising technique for producing microarrays of reproducible active enzyme spots. LDH and PK activities were completely preserved after spraying at moderate current using trehalose and Brij 35, and these conditions seem to be very promising for preserving the activities for a wide range of enzymes, thus allowing the performance of not only qualitative but also, in particular, quantitative studies. In contrast to piezoelectric dispensing, our ESD technique is easily extended to a parallel dispensing device that allows for spraying of a wide variety of enzyme solutions in (sub)nanoliter wells. Currently we are developing a method that combines fast sample addition to the wells with evaporation control during kinetic measurements. This will serve as a platform for a cost-effective and high-speed quantification of multiple analyte concentrations and enzyme levels.

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