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Research Article

Nano-dispensing by electrospray for biotechnology

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Liquid transport of minute amounts of biomaterials is of paramount importance in many biotechnological applications. One of the challenges is the transport of viscous liquids without heating. Electro hydro dynamic atomization or electrospray is a viable method for the controlled transport of nanoliter volume of viscous liquids as shown for PEG400. Experimental results and the design of a novel spraying configuration, which can be incorporated in an optical microscope, are reported.

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1 Introduction

1.1 General/Small volume liquid transport

Modern biotechnological applications often require the transport of minute liquid quantities and numerous advanced robotic methods have been developed in the past decades to achieve this goal. Whereas the scale of biochemical experimentation was in the milliliter range in the 1980s, now volumes in the low nanoliter or even picoliter range need to be transferred in various advanced applications [1, 2]. The human genome project has been a major stimulus for miniaturization in biotechnology, as it was realized very early that high throughput could only be achieved at a reasonable cost by downscaling experimentation. This trend has not yet stopped and is widely spread: the whole field of (bio)analytical chemistry is downscaled and is now highly automated.

Several aspects of liquid transport are crucial during this transition towards lower and lower volumes: the liquid evaporation and the surface properties of construction materials are of great importance [2, 3]. In the field of biotechnology the transfer of fragile, functional proteins and protein complexes has forced the development of more sophisticated methods than the early pin-transfer method that was suitable for the transfer of the more robust DNA molecules.

This work focuses on the prospects of electro hydro dynamic atomization (EHDA) or electrospray as a method to transfer sub-microliter quantities of liquid, and we compare the specific advantages over established methods as piezoelectric dispensing methods. Continuous electrospray ionization has already an enormous impact on biotechnology as it is crucial in automated MS, the core method in proteomic research. Although the current project was born from the need to transport very viscous liquids in the field of protein nanocrystallization, our conclusions should be general for liquid transport of viscous fluids [2–5].

In the physics of any liquid transport system different liquid properties play an important role: viscosity, surface tension, vapor pressure and boiling point. In biotechnological applications the liquid to be transported is usually water or aqueous solutions that have viscosities in the 10–100 mPas range at room or body temperature and ambient pressure. Depending partially on the volume range to be transported, the use of piezoelectric dispensing is a well-developed, highly accurate and reliable method for dispensing. This field has matured by the widespread application of this technology in ink jet printing.

In printing, the use of the bubble jet, where the liquid compound is partly vaporized to produce a propelling escaping gas bubble, is another well-established method to

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Abbreviations: **EHDA**, electro hydro dynamic atomization; **LD**, liquid deposition

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produce very small droplets. Both ink jet and bubble jets can produce small droplets (picoliter to nanoliter range) that can be dispensed in an accurate and controlled way, as demonstrated in many modern households by printing photographs on high quality photographic paper [6].

Notwithstanding the enormous success of the latter two techniques, there are significant problems when liquids other than high definition inks must be dispensed in applications other than printing. In general, it can be stated that any liquid that has a viscosity in the 10–100 mPas range can be dispensed successfully by either piezo dispensing or bubble jet dispensing. When the viscosity is significantly higher than these values all current methods to transport liquids become troublesome, as a significant amount of energy must be transferred to the liquid to produce droplets and to expel them from the liquid body in a container. In most dispensing methods this can (in part) be remedied by heating the liquid to lower the fluid viscosity.

Heating is not an option for labile biomolecules, assemblies and cells [7, 8]. Fortunately, EHDA can be used to dispense highly viscous liquids in a controlled manner as we show.

1.2 Theoretical background

EHDA refers to a process where a liquid jet breaks up into droplets under the influence of electrical forces. It occurs when a liquid is pumped through a capillary at a low constant flow rate with an electric field applied over the droplet hanging down from the capillary, by applying a potential difference between the capillary (nozzle) and a counter electrode. Depending on the strength of the electric stresses in the liquid surface relative to the surface tension stress, and depending on the kinetic energy of the liquid leaving the nozzle, different spraying modes are obtained [9]. For a certain spray configuration, the different modes can be described as a function of the applied potential and the liquid flow rate. The modes can be separated into two general categories: those that do not exhibit a continuous flow of liquid through the meniscus (non-continuous flow modes) and those that exhibit a continuous flow through the meniscus (continuous flow modes). Starting at zero and increasing the applied potential, Φ (Fig. 1), we enter the non-continuous flow modes, with the dripping mode. This mode is characterized by the production of relatively large droplets (usually larger than the capillary diameter at low frequency. The production frequency and the droplet diameter vary directly and inversely, respectively, with the applied potential. At lower flow rates, we enter the micro dripping mode, which produces droplets smaller than the capillary diameter at a higher frequency than the dripping mode. Starting from the dripping mode and increasing the potential difference we enter the pulsating-jet modes: the Spindle and the Intermittent cone-jet mode. These modes

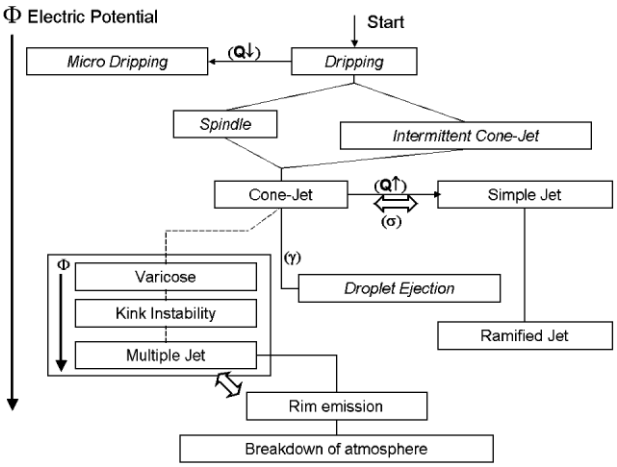

Figure 1. Flow chart of the electric field dependence of different EHDA spray modes (adapted from Fig. 2 in [9]). Modes with a pulsating flow are indicated in italics, continuous flow modes are given in normal text. Probable dependencies of spray mode transitions on physical parameters such as flow, Q, are indicated within parenthesis. Vague transitions are indicated with double arrows.

are transitional modes to the Cone-jet mode and they produce a broad (Intermittent cone-jet mode) or a bimodal (Spindle mode) droplet size distribution.

By increasing the potential difference again, we enter the continuous flow modes, in this case the Cone-jet mode. Due to its superb properties, this is the mode which we have used and describe here in more detail. It should, however, be noted that the micro dripping mode might also be applicable for our purpose, but we are not yet able to use it in a well controlled way. We do not consider the other modes, reached by increasing the potential difference even more and/or increasing the flow rate, as we do not regard them to be applicable for our purpose.

In the cone-jet mode, a liquid is pumped through a nozzle at low flow rate (μL/h to mL/h) [10, 11]. An electric field is applied between the nozzle and some counter electrode. This electric field induces a surface charge in the

Figure 2. EHDA in the cone-jet mode, visualized with reflected white light. Photograph by Philip Broos, Delft Integraal.

growing droplet at the nozzle. Due to this surface charge and to the electric field, an electric stress is created in the liquid surface. If the electric field and the liquid flow rate are in the appropriate range, then this electric stress will overcome the surface tension stress and transform the droplet into a conical shape, the Taylor cone. The tangential component of the electric field accelerates the charge carriers (mainly ions) at the liquid surface towards the cone apex. These ions collide with liquid molecules, so accelerating the surrounding liquid. As a result, a thin liquid jet emerges at the cone apex (Fig. 2).

Depending on the ratio of the normal electric stress over the surface tension stress in the jet surface, the jet will break up due to axisymmetric instabilities, also called varicose instabilities or due to varicose instabilities and also lateral instabilities, called kink instabilities. At a low stress ratio in the varicose break-up mode mono-disperse droplets are produced. The droplets produced carry a high electric charge close to the Rayleigh charge limit, which can be very advantageous for deposition purposes.

To estimate the right conditions and operational parameters to produce droplets of a certain size, scaling laws can be used. We employ the scaling laws as developed by Hartman [10, 11]. For the current scaling for liquids with a flat radial velocity profile in the jet, which is appropriate here because of the high viscosity of the solution, he derived the following relation:

$$I = b(\gamma K Q)^{\frac{1}{2}}$$

where Q is the flow rate (m^3/s), I is the current through the liquid cone (A), γ is the surface tension (N/m). K is conductivity (S/m), and b is a constant, which is approximately 2.

The droplet diameter for the varicose break-up mode is given by:

$$d_{d,\,v} = c\left(\frac{\rho\varepsilon_0 Q^4}{I^2}\right)^{\frac{1}{6}}$$

where $d_{d,v}$ is the droplet diameter for varicose break-up and c is a constant, which is approximately 2. Substituting the first into the second equations yields:

$$d_{d,\,v} = \left(\frac{16\rho\varepsilon_0 Q^3}{\gamma K}\right)^{\frac{1}{6}}$$

These scaling laws are used for design and operational purposes. EHDA can be used in very widely different applications, as the injection of liquid in a mass spectrometer, spraying liquid on surfaces to produce a homogeneous thin coating layer, the production of a particle mist for administration of medicines to the bronchial tract of patients or even the dispensing of living cells [8, 12]. In this work we assume that we spray in the cone-jet mode, although we did not visualize the spray itself, since our aim was to demonstrate the applicability of EHDA to deposit nanovolumes in a controlled way.

2 Materials and methods

Experimental methods used for controlled dispensing of sub-microliter droplets of viscous liquids using EHDA are described. In EHDA, an electric field is used to overcome the viscous forces. The switching variant of EHDA is employed to dispense viscous fluids without the prior need to heat the liquid, which is particularly advantageous in situations where the liquid to be dispensed is thermally unstable as in the case of concentrated protein solutions. This method allows controlled dispensing of viscous liquids, which is very difficult to achieve without heating in other dispensing methods.

The generic EHDA experiment consists of a conducting nozzle that contains the liquid (stream) to be dispensed and the target surface, which is kept at a high potential difference with respect to the nozzle. In Fig. 2, the resulting mist consisting of minute droplets that form a continuous spray is visualized by the scattered/reflected light. The formed conical-shaped liquid body hanging at the bottom of the metallic cylindrical tube emits from a jet a fine mist that fans out on its way to the target surface.

In this work we investigated the possibility of modulating the produced liquid flow to achieve controlled deposition of liquids. To realize this, different approaches can be used that modulate either the electric field strength that is generated between the liquid supply nozzle and the target surface and/or the liquid flow rate.

As the electric field depends on the applied voltage and the distance between the spraying cone and the target surface, two variables could be used to modulate the spraying process: changing the applied voltage and varing the distance. We investigated the effect of a changing high voltage to start and stop the spraying of liquid. Previously, the use of a variable distance between a surface and the nozzle has been successfully exploited to modulate the deposition of very small volumes in nanoliter cavities produced in a silicon wafer [7, 13].

In Fig. 3, we show the basic schematic design of a switching EHDA system that uses a modulation of the applied voltage to generate a controlled liquid stream. The variable voltage in our experiments was generated using three different methods.

The simplest method consisted of a high voltage relay that could be opened and closed by a controlling voltage. Using a voltage divider circuit it is possible to have a constant high voltage present to which a high voltage pulse is added to generate the voltage difference that makes the setup spray liquid in the cone-jet mode. In this case a constant high voltage power supply was used that could be controlled by a variable current. A constant voltage is used to maintain a situation close to a non spraying cone,

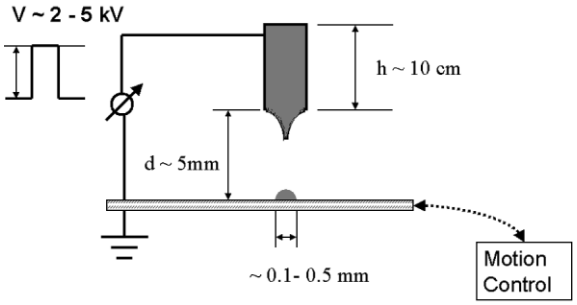

Figure 3. Schematic drawing of the relevant controlled variables in the EHDA system. Basic EHDA system parameters are: V, the high voltage; d, nozzle target distance; and h, the hydrostatic pressure used. The length of the liquid supply line was about 1 m, the other values typically used are indicated.

as the formation of the cone is a relatively slow process. Switching the high voltage relay generated the pulsed voltage to make the cone spray. The use of a high voltage relay resulted in a frequent breakdown of the relay and a reduced flexibility to vary the constant bias voltage and cannot be recommended for this type of experiment.

A more flexible variable high voltage was realized using a voltage-controlled high voltage power supply. To have full flexibility in the amplitude of the constant bias voltage and the pulsed voltage, a summation amplifier was used to combine the manually adjustable constant voltage and the adjustable variable voltage. The switching of the variable voltage was done using an analog switch. In this realization, the low voltage waveform was used to generate the driving electric field using a voltage-controlled high voltage power supply (MP5, Start Spellman Ltd., Pulborough, UK).

A third method used a programmable digital to analog voltage generator to achieve an arbitrary waveform that was subsequently used to generate the high voltage as described in the previous method. In our experience the use of the summation amplifier and the analog switch was sufficiently flexible to generate a reliable switching EHDA spray at relatively low cost. All quantitative results shown here were obtained using the third method.

In our experiments we used hydrostatic pressure to influence the flow of liquid, and we did not rely on (very expensive) pumps that can transport a few nanoliter per minute. The balance between the capillary forces and a hydrostatic pressure in the range of 10 cm water pressure was exploited to maintain a filled capillary. In all cases the total dispensed volume was negligible to the amount of liquid in the liquid system to maintain the pressure.

As the aim of the experiments was not only to switch the spraying of the electrospray, which could always be achieved by the electronics described above, but also the controlled deposition of liquids, the position of the spray with respect to the target surface must be adjustable. Two basic scenarios can be envisioned: the surface can be moved with respect to the cone or *visa versa*, in combination with a variation of the effective field strength. In this study, we moved the target surface, kept the nozzle at a fixed position and applied a variable high voltage to induce the spraying. From a safety point of view, we preferred to move the earthed target surface, while keeping the nozzle and the liquid supply at a high voltage. A schematic drawing of the experimental design is given in Fig. 3.

A reliable system that could combine both the switching of the field and the positioning was achieved only after shielding the generated electric fields from the controlling computer system. The use of optocouplers alone was not sufficient to prevent premature crashing of the software in the PC that was used to control the experiment due to electromagnetic interference. Design and construction of a well-shielded electronic system allowed us to combine motion and distribution. The dispensation was started after the motion had stopped.

3 Results and quantification

3.1 Dispensed volumes

Two methods to vary the total dispensed volume were explored: the duration of the applied field was used to con-

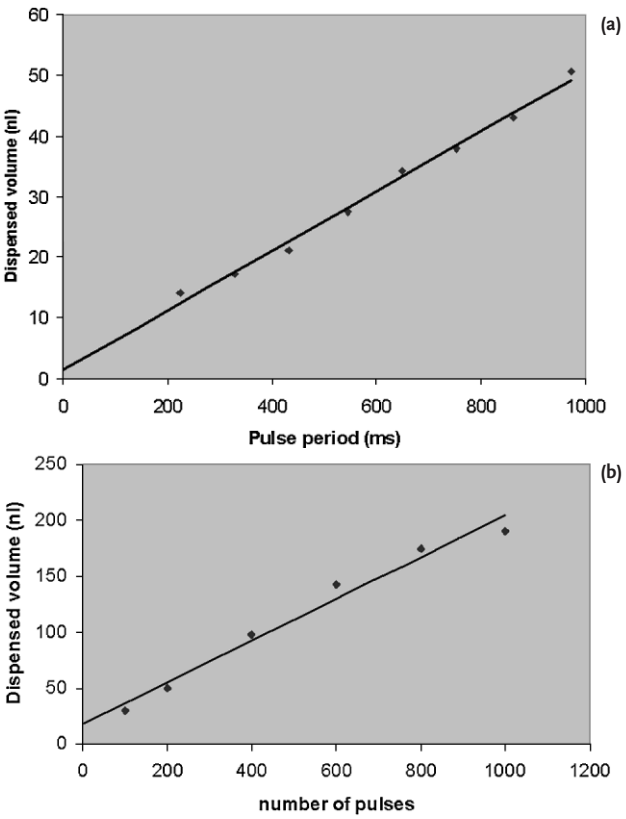

Figure 4. (a) Dispensed volume as a function of the dispensing period. (b) Dispensed volume as a function of the number of pulses.

trol the spraying time or the number of pulses used to create a liquid deposition (LD) was varied.

The total amount of dispensed material was determined by weighing the target, a standard microscope slide, before and after the experiment. The experiments were performed by dispensing pure PEG400. As PEG400 does not evaporate very fast the dispensed patterns were very stable after deposition. The dispensed pattern could be seen by the naked eye several months after dispensation. We assume that evaporation or adsorption of water could be neglected on the time scale of our experiments. The results of this experiment are shown in Fig. 4a, and a linear increase of the dispensed volume as a function of the pulse period was observed. In each case 500 spraying periods, *i.e.*, LDs, using a 4-kV high voltage pulse were performed allowing us to weigh the amount deposited directly after the experiment (total duration 540 s). It should be noted that, at the shortest dispensing time of 224 ms, the spray just started to spray, while at shorter dispensing periods no detectable mass was sprayed. A minimum number of 100 LDs resulted in a mass increase of at least 3.3 mg, which could be readily measured using a standard analytic balance. This amount corresponds to a volume of 30 nL per shot calculated using the number of LDs and the density of PEG400 (1120 kg/m^3). In our experiments we used weighing, and assumed that there is little variation in the LD amount produced in these experiments as we determine the average mass of hundreds of LDs.

To determine the mass of individual LDs two other methods were considered: determining the perimeter of the deposited LD and calculating the LD volume using the shape, which is determined by interfacial forces, or, alternatively, using a quartz microbalance to weigh single LDs. We have not yet explored these methods extensively. One of the problems of the quartz microbalance method is that it suffers from charge built-up as the deposited LD is charged and the balance itself is part of the high voltage circuit.

After we established a stable spraying condition, *e.g.*, using a pulse duration of 540 ms, we investigated the variation of the LD mass as a function of the number of pulses. The average mass deposited was used to calculate the volume of the individual LDs. We observed a linear dependence on the number of pulses that can be used to control the dispensed amount of liquid (Fig. 4b), allowing us to dispense amount between 20 and 200 nL in a controlled way.

3.2 Visualization of the deposition

In Fig. 5a the result of a computer-controlled dispensation of an array of PEG200 LDs is shown. The line A points at one of the corners of the dispensed array, while line B points at the nozzle that is brought to a high voltage of 4 kV at the start of each dispense. The liquid is dispensed on a standard microscope slide (76×26 mm) and a metal clip is used to earth the slide and microscope stage. Line C has a length of approximately 26 mm. The slide holder is part of a Leica DMLA microscope (Leica Microsystems, Rijswijk, The Netherlands), which is mounted on a linear X-Y translation stage (8MT175-100, Altechna Co. Ltd., Vilnius, Lithuania). In Fig. 5b, details of the deposited array are shown after transfer of the slide to another (stereo)-microscope, with variable magnification.

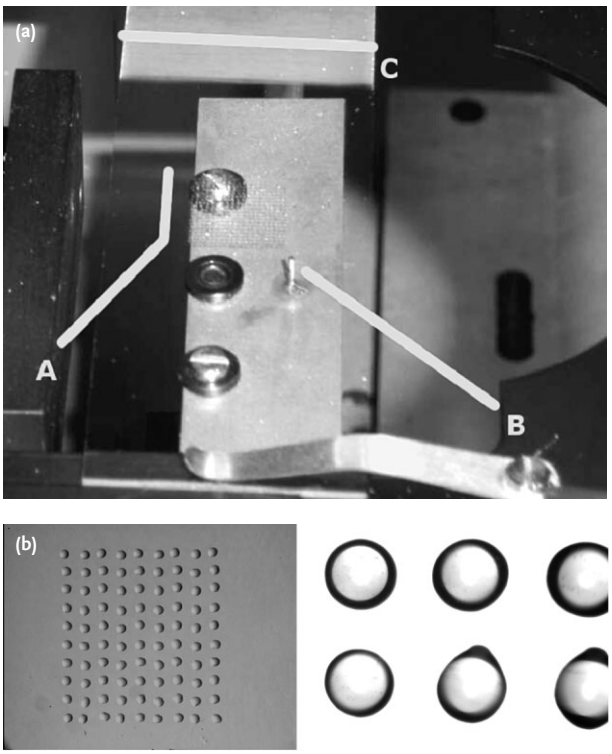

Figure 5. (a) Prototype of an inverted geometry EHDA system. (A) Deposited array of PEG200 LDs with a 0.6-mm spacing between the depositions. (B) Stainless steel needle used to spray the liquid (part 5125CH-B, EDF Inc, East Providence, RI, USA). (C) Length scale (width of the microscope slide is 26 mm). (b) Dispensed array viewed using transmitted light. The droplets were dispensed with the prototype upright spraying system shown in (a), and visualized in a bright-field microscope with variable magnification. The droplets stick to the glass due to the surface forces. Array (9×10) with PEG200 LDs, deposit radius approximately 0.2 mm, droplet spacing 0.6 mm.

After the feasibility of upright spraying was demonstrated, a construction was designed to incorporate the dispensing system in the Leica DMLA microscope. The whole system is depicted in Fig. 6a, where the position of the original condensor is now taken by the dispensing system. In Fig. 6b, the dispensing system can be seen with the bottom part of the dispensing needle (nozzle) visible (red plastic, top middle part of the picture, A). The cable B carries the high voltage and the cables labeled C are the earth connections. The transparent tube in the middle of the picture is the liquid supply to the needle/nozzle (D). The nozzle mounting is attached to the transport

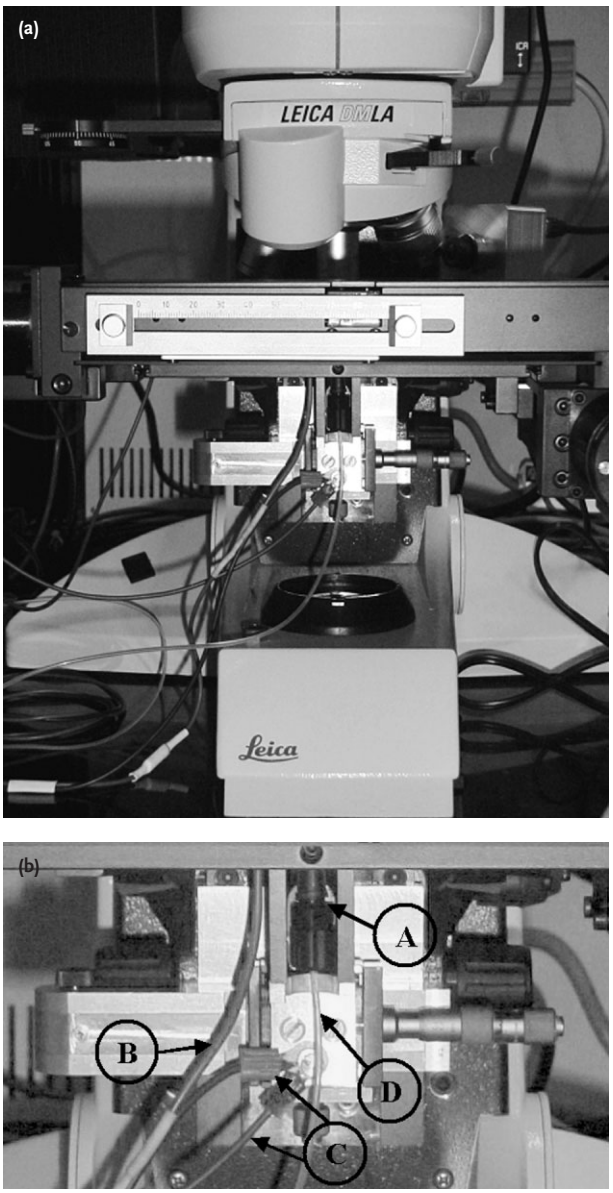

Figure 6. (a) Nozzle mount placed at the position of the condensor lens system in a Leica DMLA microscope. Using a 10_ magnifying long-working-distance objective either the deposit is visualized or the rim of the nozzle. (b) Detailed view of the implementation in a upright microscope system (Leica DMLA). Nozzle mounting with bottom part of the dispense needle visible (top middle part of the picture, panel A). Positive high voltage cable (B) and earth connections (C), the transparent tube in the middle of the picture is the liquid supply to the needle (D). The nozzle mounting is attached to the transport mechanism of the (removed) condenser lens system of the microscope. The visualization of the dispensing process is done from the top with epi-illumination and captured with a CCD camera. A schematic overview of the nozzle assembly is given in Fig. 7.

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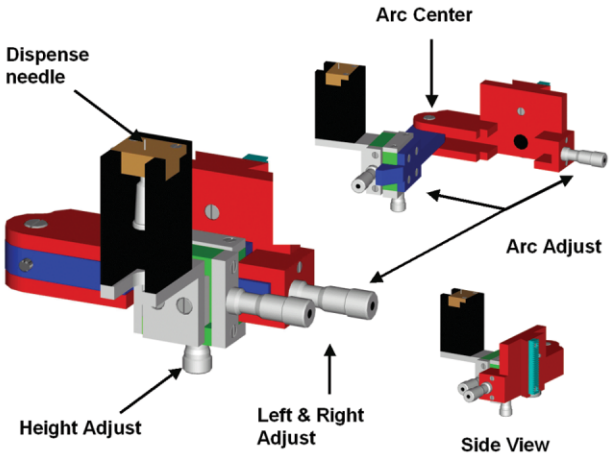

Figure 7. Degrees of freedom of the nozzle assembly. The nozzle assembly can be swung out of the dispense position under the microscope to allow for inspection. Three micrometers can be used to adjust the position of the nozzle with respect to the optical axis of the microscope. The horizontal micrometer is used to adjust the distance between the nozzle and the target surface (height adjust).

CCD camera at video rate (768×576 pixels at 25 Hz). This configuration allows for the real-time registration of the dispensing process of a nanoliter LD or, by changing the focus, the study of the liquid surface at the tip of the nozzle. The dispensation can take place at another location by moving the motorized X-Y translation stage of the microscope, is this way one can dispense any LD pattern.

The design drawing of the electrospray unit is shown in Fig. 7. The arm that carries the dispensing nozzle can be swung away from the microscope for adjustment or cleaning. When the nozzle is in position in the microscope, a micrometer can be used to adjust the height of the nozzle with respect to the earthed target surface. The center of the nozzle can be centered in the view of the microscope using the combined action of the two other micrometers. Using different objectives the amount of detail captured can be varied.

4 Discussion

It is shown that EHDA is a suitable method for dispensing viscous liquids, such as PEG400, starting at a minimal LD volume of about 30 nL. In view of the equipment used to detect and measure the LD volume, this is close to the limit that is possible to dispense reliably. Further progress could be expected using a voltage higher than the voltage of 5 kV used. This did allow us to use larger distances between the nozzle and the target surface, but the use of higher voltages made it more difficult to achieve fast switching. In the reported experiments, the switching time was largely determined by the rise and fall time of the high voltage circuit used. The described setup can also be used for generic dispensing of other low-conducting liquids and even deter-

gents, some of which were tested in this work (PEG200, PEG400, PEG2000 and PEG4000 50% in water, ethylene glycol, 2-propanol, dioxane, Triton X-100).

The construction of a nozzle assembly that can be mounted at the position of the condensor of a microscope opens the opportunity to visualize the dispensing process with high resolution. By moving the motorized stage of the microscope, we were able to 'write' a liquid path using an EHDA spray. The simultaneous observation and control allows for the design of a feedback loop where the pulse duration and/or the number of pulses can be used to dispense a pre-determined amount of material.

For every liquid one has to determine the appropriate dispensation parameters and the distance between the surface and the nozzle tip. This procedure can be quite laborious and was not attempted in this work, although this will be necessary for the construction of a calibrated system that can be used routinely. Although we were able to dispense many different liquids, we were not able to dispense aqueous solutions due to their high surface tension. Fortunately, those solutions are often not very viscous and can be dispensed using other methods.

A practical application of switching EHDA is in protein nano-crystallization where an array of small containers (100–200 nL) is filled with different mixtures. These mixtures are then observed for a prolonged period using microscopic techniques to monitor the formation of protein crystals, which are in turn used in 3-D structure determination using X-ray diffraction. All solutions contain a protein concentration of about 10–30 g/L and some of these mixtures contain a high concentration of polyethylene glycol with a high molar mass (up to 10%, M_r=20 000). In practice, half of the volume in the container comprises the protein solution and the other half is a mixture with variable composition, which is called the precipitant. The precipitant is varied to influence the solubility of the protein and to induce nucleation of protein crystals. It is shown here that EHDA can be used successfully to dispense some of the components that can not be dispensed by piezoelectric or bubble jet methods, such a polyethylene glycols.

A reliable EHDA system can be designed at relatively low cost that can dispense low-conductive viscous solutions in the nanoliter range. With a suitable design of the control electronics, it is possible to dispense nanoliter amounts in a controlled way. The use of direct observation of the spraying jet can also provide knowledge about the importance of the different operational parameters on the dispensing process. One important factor is the changing geometry due to the accumulation of liquid on the target, which influences the electric field and thus the deposition process. Here we assumed that this in negligible. This aspect deserves future attention especially when larger amounts of liquid are to be deposited.

Using a sufficiently fast high switching voltage, much smaller LDs could be produced at a higher production rate, which will enable EHDA to be used for other applications as a sub-nanoliter dispensing method. It would be of interest to investigate whether the bias voltage applied to pre-form the Taylor cone does indeed contribute to a faster switching of the electrospray. Although, much improvement to the system shown here can still be made, EHDA is at present, as far as we know, the only method capable of dispensing nanovolumes of highly viscous thermally unstable liquids.

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