

DOI: 10.1002/sml.200600431

Massively Parallel Dip-Pen Nanolithography of Heterogeneous Supported Phospholipid Multilayer Patterns**Steven Lenhart,* Peng Sun, Yuhuang Wang,
Harald Fuchs, and Chad A. Mirkin*

Phospholipids are an essential component of biological membranes, as their lyotropic liquid-crystalline nature enables them to self-assemble into two-dimensional bilayer sheets under physiological conditions.^[1] Micro- and nanoscopic heterogeneities, such as lipid rafts^[2,3] and focal adhesions,^[4,5] are vital to the biological function of lipid bilayer membranes. Lithographically patterned phospholipid membranes can be used as cell-surface models^[6] and have been used in several applications, including biochemical sensors,^[7] drug screening and delivery,^[8,9] the analysis of cell-cell interactions,^[3,10] and to address fundamental biological questions in membrane trafficking.^[11] However, in order to create model systems that are capable of mimicking the structural complexity of biological membranes, a method is necessary that allows both high-resolution patterning and parallel deposition of different phospholipid materials over large areas. Here, we demonstrate that a new, noncovalent modality of dip-pen nanolithography (DPN) is a suitable approach for the rapid fabrication and integration of large-scale phospholipid nanostructure libraries on a variety of substrates. This method provides a lateral resolution down to 100 nm and an areal throughput of 5 cm² min⁻¹.

Previously, micropatterned lipid bilayers on solid supports have been prepared using several approaches. A microarrayer that deposits nanoliter droplets of phospholipid solutions can be used to create arrays of supported lipid bi-

layers which are physiologically fluid and can thus be applied to mimic the behavior of natural biomembranes.^[9] Microcontact printing and related soft lithographic techniques have also been used to create arrays of supported lipid bilayers.^[12] This technique was used to investigate the binding of a protein (annexin V) and an anti-inflammatory drug (nimesulide) with supported lipid bilayers.^[8] Diffusion barriers within a single bilayer can be created by patterning the substrate underlying a supported lipid bilayer using photolithography.^[13] When this technique is combined with the high lateral resolution of scanning-probe lithography, two lipids of different composition can be integrated by a nano-grafting method to achieve pixel sizes of 1 μm, although only in a serial process.^[14]

Microdomains play important roles in the *in vivo* function of biological membranes. For instance, protein clustering on a length scale of 5–200 nm takes place within the focal adhesions that govern cell-surface interactions and motility.^[5] The exact size of lipid domains in biological membranes, collectively referred to as lipid rafts, is still a matter of debate, although most agree that they are sub-micrometer in size.^[2] A method that allows both high-resolution patterning and parallel integration of different phospholipid materials is lacking, yet necessary for the fabrication of model systems that are capable of mimicking these complex biological membrane patterns.

DPN makes use of an atomic force microscope tip to directly deposit molecular inks onto a surface, reproducibly allowing line widths below 20 nm in the case of alkanethiols on gold.^[15,16] The ability for DPN to operate under ambient conditions makes it particularly well suited to the fabrication of biomolecular arrays. This unique capability has been demonstrated by the fabrication of DNA and protein arrays with sub-100-nm lateral resolution.^[17,18] The fabrication of small spot sizes locally concentrates the analyte, which enables the detection of very low bulk analyte concentrations.^[19] Importantly, DPN has an advantage over other types of lithography in that many different chemical functionalities can be integrated onto a single surface^[16] without the risk of feature cross-contamination, as in the previously used indirect methods.^[14] Since the line widths and spot sizes in DPN are independent of the contact force of the microscope tip on the substrate, the technique can be readily carried out in parallel with arrays of cantilevers over centimeter length scales.^[20,21]

We found that the phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, $T_m = -16.5^\circ\text{C}$) is a suitable ink for DPN under humidity-controlled conditions.^[22] The phase behavior of this compound is well characterized as it is a standard model system for unsaturated lipid bilayer membranes.^[23] At room temperature and the humidity range used in this work (40–95%), the compound is in the lamellar liquid-crystalline (L_α) phase.^[24,25] The head-to-head spacing for a single bilayer of DOPC in the L_α phase has been measured to be 3.53 nm by X-ray diffraction,^[26] and temperature-controlled atomic force microscopy (AFM) of phospholipid bilayers in this phase shows 3.4–3.6-nm steps.^[27] Consistent with those measurements, we observe step heights of (3.4 ± 0.2) nm in AFM topographical images of

[*] Dr. S. Lenhart, Prof. H. Fuchs
Institut für NanoTechnologie
Forschungszentrum Karlsruhe GmbH
76021 Karlsruhe (Germany)
Fax: (+49)7247-826368
E-mail: lenhart@int.fzk.de

Dr. S. Lenhart, Prof. H. Fuchs
Physikalisches Institut, Universität Münster
48149 Münster (Germany)

P. Sun, Dr. Y. Wang, Prof. C. A. Mirkin
Department of Chemistry and
International Institute for Nanotechnology
Northwestern University, Evanston, IL 60208 (USA)
Fax: (+1) 847-467-5123
E-mail: chadnano@northwestern.edu

[**] S.L. is grateful to A. Ulrich and A. Parikh for the helpful discussions and to M. Kohl for the evaporated metal films. C.A.M. acknowledges the NSF-NSEC program, a NIH Director's Pioneer Award, and the AFOSR for supporting this work.

Supporting information for this article is available on the WWW under <http://www.small-journal.com> or from the author.

the DPN patterns (see Supporting Information, Figure S1). The humidity is the most critical parameter in both the coating of the tips and the DPN writing of DOPC. An atomic force microscope tip can be reproducibly coated with DOPC at room temperature by placing the tip in contact with the pure material while it is sufficiently hydrated. Under ambient humidity (30–50%), the ink does not flow onto the tip. However, at a higher humidity (>70%), the phospholipid ink becomes sufficiently fluid and readily coats the tip.

While most ink–substrate combinations used for DPN make use of covalent binding between the ink and the substrate, this approach is undesirable for the patterning of biomimetic phospholipid membranes, because covalent links between the lipids and the surface prevent lateral mobility within the lipid bilayers.^[6,28] However, owing to the amphiphilic nature of the phospholipids, it is possible to use physisorption as a driving force for the ink transport from the tip to the surface. We have been able to use this technique to reproducibly generate DOPC patterns on silicon wafers, glass slides (either untreated or made hydrophilic by treatment with oxygen plasma), evaporated metal films, and hydrophobic polystyrene cut from petri dishes.

Rather than spreading laterally over the surface to form a monolayer like covalent DPN inks, the phospholipid ink tends to stack three dimensionally on the surface. However, when left at high humidity for extended periods of time (e.g., 90% for 1 hour), the multilayer stacks will slowly spread on hydrophilic or hydrophobic surfaces to form a thin, homogeneous layer (data not shown). We therefore attribute this stacking effect to the slow spreading kinetics of the phospholipids in air. The heights of the DOPC features measured by AFM exhibit a strong correlation with the tip velocity and the relative humidity (Figure 1). A reflection-mode micrograph of DOPC films deposited from an atomic force microscope tip onto plasma-oxidized silicon by scanning 5- μm areas at various speeds exhibits different colors for each deposited square, a consequence of optical interference due to different film thicknesses (Figure 1A). AFM measurements show a

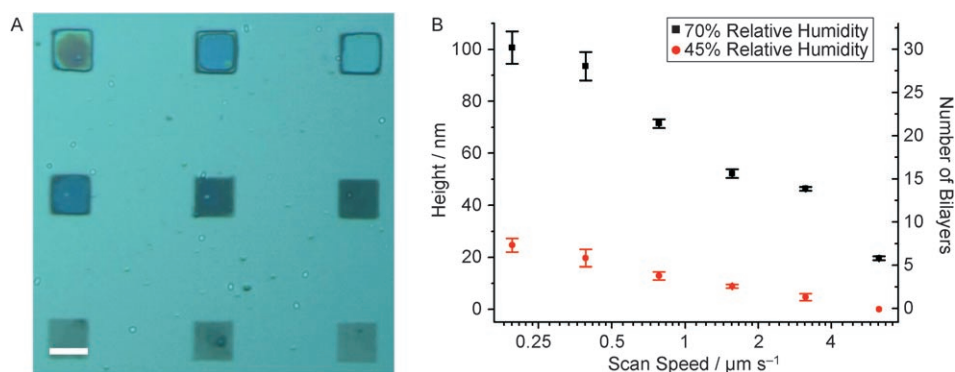


Figure 1. Control of multi-bilayer stacking. A) Reflection-mode optical micrograph of phospholipid squares patterned on plasma-oxidized silicon at various speeds; scale bar: 5 μm . B) The height of phospholipid multilayers (and the corresponding number of bilayer stacks) measured by AFM is plotted as a function of scan speed (on a logarithmic scale) at two different relative humidities. Error bars represent the standard deviation between the heights of eight different cross sections of a single square.

linear dependence of the film thickness on the logarithm of the tip velocity (Figure 1B). This trend is analogous to the lateral diffusion observed in conventional covalent DPN.^[29] The relative humidity plays an important role, both in the inking of the tip and in the multilayer stacking during writing. It is therefore likely that both the hydration-dependent phase behavior of the phospholipid ink^[24] and the water meniscus condensed between the microscope tip and the substrate^[30–32] govern transport of the phospholipid ink.

By careful optimization of the scan speed and relative humidity, we were able to control the thickness of the material deposited to that of a single bilayer (Figure 1A) with line widths down to (93 ± 18) nm, as determined by AFM (see Supporting Information, Figure S2). A fluorescence micrograph of a serially patterned array of 25 features of DOPC doped with 1 mol% of a rhodamine-labeled lipid shows that one can routinely prepare features consisting of contiguous lines (Figure 2). Structures with two different dye layers (the green pattern being DOPC doped with 1 mol% of a 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) labeled lipid) on the same substrate were also fabricated by serial DPN (Figure 2C). Since the physisorbed patterns are swept

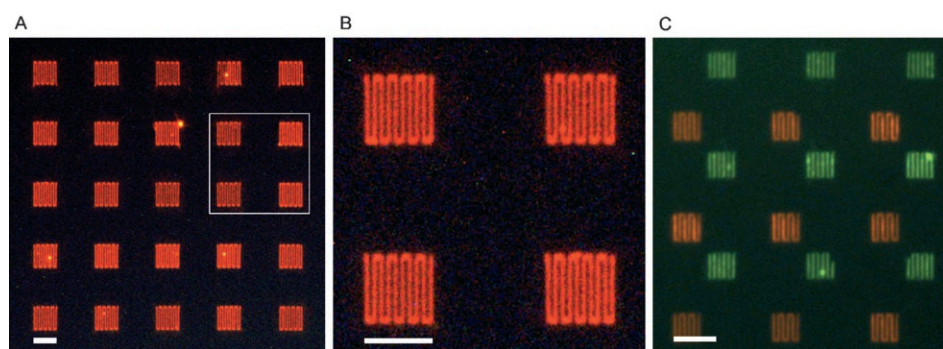


Figure 2. Fluorophore-doped phospholipid patterns deposited onto a polystyrene petri dish. A) An array of 25 contiguous line features. B) A higher magnification of the region highlighted by the white square in (A). Lines corresponding to the path of the atomic force microscope tip can be seen within each square; the line widths are 250 nm. C) Two-component pattern created serially by using two different tips inked with phospholipid doped with two different fluorescent dyes. Scale bars: 5 μm .

away when imaged in contact mode, nanoscale alignment marks using the same tip for DPN writing and imaging could not be used. Therefore, optical alignment marks were used to align the different tips, which resulted in a misalignment of about 2 μm between the two layers.

A major advantage of DPN over other lithographic techniques is that different inks can be simultaneously deposited from different atomic force microscope tips in a single array, provided that the two inks can write under the same environmental conditions (if this is not the case, then active pen arrays become necessary to deposit the different materials in series^[33,34]). This method is used to test the fluidity of the DOPC patterns (Figure 3). In this experiment,

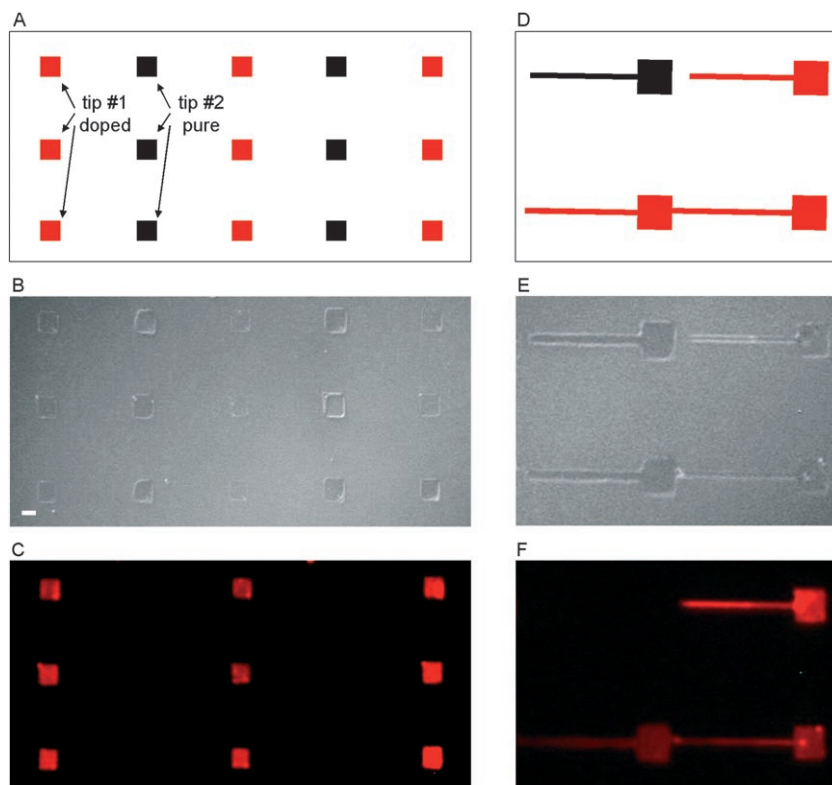


Figure 3. Parallel writing of two different inks (fluorophore-doped and pure DOPC) as a test of membrane fluidity. A) Schematic drawing of the heterogeneous ink pattern programmed to be fabricated in parallel from a one-dimensional array of atomic force microscope tips. B) Bright-field micrograph of the phospholipid squares (doped and pure) that were fabricated simultaneously. C) Fluorescence micrograph of the same area in (B) showing that only the doped squares fluoresce. D) Schematic drawing of the pattern fabricated to test the fluidity by connecting fluorophore-doped DOPC reservoirs with their neighbors of pure DOPC. E) Bright-field micrograph of the connected squares (bottom) and an unconnected control (top). F) Fluorescence micrograph showing that the fluorophore-doped lipid has diffused into the previously pure lipid square upon connection, which demonstrates the fluidity of the membranes. Scale bar: 5 μm .

inkwells are used to coat every second tip in a one-dimensional array with fluorophore-doped DOPC, while the other tips are coated with pure DOPC. Figure 3A shows a simple pattern where each tip was programmed to deposit three squares. Fluorescence is only observed from the patterns created by every second tip, thus eliminating the possibility of cross-contamination between neighboring tips (Figure 3B). Each square was then connected to the neighbor-

ing squares of different composition by drawing a fine line of the same ink. Upon connecting the squares, the fluorescent dye diffuses into the pure lipid squares, which indicates that the phospholipid patterns retain their fluidity at humidity levels >40% (Figure 3D–F).

DPN is well suited to large-scale parallel deposition of lipid membranes, because the pattern dimensions are independent of the contact force of the microscope tip on the substrate. To illustrate this, a two-dimensional cantilever array consisting of 55 000 tips was used to simultaneously pattern over a surface area of 1 cm^2 (Figure 4). Each tip wrote “INT” three times at 70% relative humidity and the total writing time required was only 12 seconds. This surface

was later immersed in pure water, and the fluorescent patterns were still visible in the water after overnight storage (see Supporting Information, Figure S3). We attribute the stability of the multilayer patterns in water to the partial wetting of phospholipids on the substrate. For this situation to occur, the interfacial tensions involved (solid/water, solid/lipid, water/lipid) must be such that spreading is thermodynamically unfavorable, thus resulting in a nonzero contact angle.^[35]

Upon exposure to water, dehydrated phospholipids are known to spontaneously spread to form lipid membranes on hydrophilic surfaces.^[36,37] We found the dehydrated multilayer patterns formed by DPN to be suitable precursors for the formation of such membranes. Multilayer squares patterned on hydrophilic glass (oxidized by an oxygen plasma) spread to form circles of homogeneous fluorescence intensity, which eventually fused with their neighbors. Fluorescence recovery after photobleaching (FRAP) measurements on mem-

branes connected to their neighbors showed complete recovery, which indicated the fluidity and connectivity of the patterns (see Supporting Information, Figure S4).

In conclusion, the hydrated phospholipid DOPC can be used as a universal ink for noncovalent patterning on a variety of surfaces, ranging from hydrophilic silicon to hydrophobic polystyrene. The lateral resolution spans the 100-nm to several micrometer length scales, and the patterning can

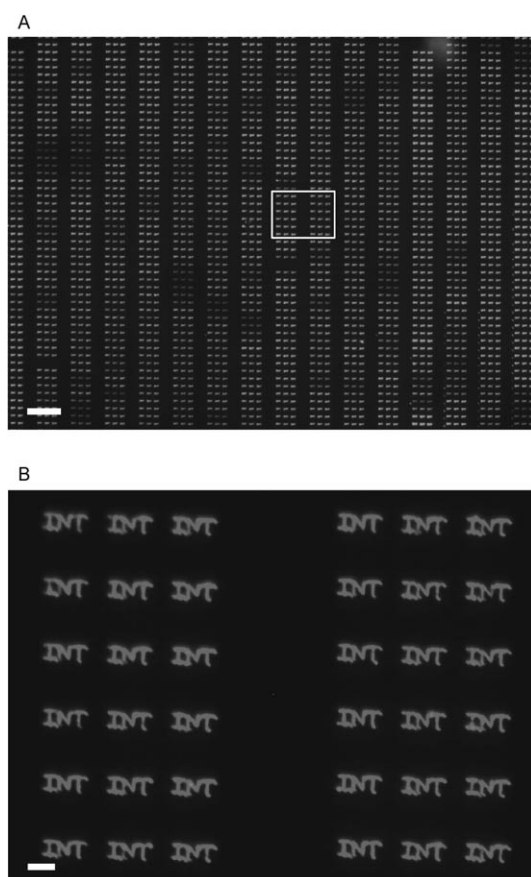


Figure 4. Fluorescence micrographs of phospholipid patterns fabricated in parallel using a two-dimensional cantilever array consisting of 55 000 tips covering 1 cm². A) Overview showing the patterning of a large area; scale bar: 100 μ m. B) A higher magnification of the area outlined by the white square in (A); scale bar: 10 μ m. The substrate here was a glass coverslip used as purchased, without further cleaning or treatment.

be carried out in parallel using cantilever arrays on the centimeter length scale. Simultaneous writing of different inks enables the precise alignment and integration of chemically distinct phospholipid patterns. The resulting multilayer patterns are fluid and stable on certain surfaces upon immersion in water, while on other surfaces they can spread to form supported lipid bilayer membranes. Furthermore, the ability of DPN to fabricate and integrate arbitrary phospholipid patterns of submicrometer line widths opens many new possibilities in the design of membrane chemical heterogeneity (e.g., lipid raft arrays^[2,3]) and membrane structural constructs (e.g., lipid nanotube-vesicle networks^[38]), where it is desirable to create complex physical and chemical membrane networks.

Experimental Section

The phospholipid DOPC and fluorophore-doped lipids 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) (18:1 NBD/PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (18:1

lissamine rhodamine/PE) were purchased from Avanti Polar Lipids, Alabaster, AL, USA. Chloroform and dichloromethane solvents were HPLC grade, from Sigma. Nanopure water with a resistivity of 18.2 M Ω cm was used.

DPN writing and tip coating was carried out using a commercial DPN writer (Nscriptor™) and the following accessories: single tips of the A type (S1), one-dimensional tip arrays of the D type (A26), and inkwells of type W4 (NanoInk Inc., Chicago, IL, USA). The inkwells were filled with a chloroform or dichloromethane solution of the phospholipid ink (1 μ l, 10 mM, doped with 1 mol% of the dye in the case of doped inks). The solvent was allowed to evaporate for at least 2 h before coating the tips. Tips were inked by placing them in contact with the inkwell and increasing the humidity to \approx 95% for at least 30 min.

The two-dimensional array was fabricated and aligned as described.^[21] The tips were inked using an ink pad prepared by spin-coating the ink solution (0.5 mL) onto a glass slide at 1000 rpm. The array was aligned and placed in contact with an ink pad. To facilitate the movement of ink onto the tips, a drop (10 μ l) of water was placed on the corner of the ink pad and allowed to spread through the phospholipid coating on the ink pad.

Keywords:

atomic force microscopy • dip-pen nanolithography • multilayers • patterning • phospholipids

- [1] P. Yeagle, *The Structure of Biological Membranes*, 2nd ed., CRC Press, Boca Raton, FL, **2005**.
- [2] L. Rajendran, K. Simons, *J. Cell Sci.* **2005**, *118*, 1099.
- [3] K. D. Mossman, G. Campi, J. T. Groves, M. L. Dustin, *Science* **2005**, *310*, 1191.
- [4] T. P. Lele, C. K. Thodeti, D. E. Ingber, *J. Cell. Biochem.* **2006**, *97*, 1175.
- [5] M. Arnold, E. A. Cavalcanti-Adam, R. Glass, J. Blummel, W. Eck, M. Kantlehner, H. Kessler, J. P. Spatz, *ChemPhysChem* **2004**, *5*, 383.
- [6] M. Tanaka, E. Sackmann, *Nature* **2005**, *437*, 656.
- [7] D. Anrather, M. Smetazko, M. Saba, Y. Alguel, T. Schalkhammer, *J. Nanosci. Nanotechnol.* **2004**, *4*, 1.
- [8] S. Majd, M. Mayer, *Angew. Chem.* **2005**, *117*, 6855; *Angew. Chem. Int. Ed.* **2005**, *44*, 6697.
- [9] V. Yamazaki, O. Sirenko, R. J. Schafer, L. Nguyen, T. Gutschmann, L. Brade, J. T. Groves, *BMC Biotechnol.* **2005**, *5*, 18.
- [10] M. Wu, D. Holowka, H. G. Craighead, B. Baird, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13798.
- [11] A. S. Cans, N. Wittenberg, R. Karlsson, L. Sombers, M. Karlsson, O. Orwar, A. Ewing, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 400.
- [12] J. S. Hovis, S. G. Boxer, *Langmuir* **2001**, *17*, 3400.
- [13] J. T. Groves, N. Ulman, S. G. Boxer, *Science* **1997**, *275*, 651.
- [14] B. L. Jackson, J. T. Groves, *J. Am. Chem. Soc.* **2004**, *126*, 13878.
- [15] D. S. Ginger, H. Zhang, C. A. Mirkin, *Angew. Chem.* **2004**, *116*, 30; *Angew. Chem. Int. Ed.* **2004**, *43*, 30.
- [16] R. D. Piner, J. Zhu, F. Xu, S. H. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661.
- [17] L. M. Demers, D. S. Ginger, S. J. Park, Z. Li, S. W. Chung, C. A. Mirkin, *Science* **2002**, *296*, 1836.
- [18] K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702.

- [19] K. B. Lee, E. Y. Kim, C. A. Mirkin, S. M. Wolinsky, *Nano Lett.* **2004**, *4*, 1869.
- [20] K. Salaita, S. W. Lee, X. F. Wang, L. Huang, T. M. Dellinger, C. Liu, C. A. Mirkin, *Small* **2005**, *1*, 940.
- [21] K. Salaita, Y. Wang, J. Fragala, R. A. Vega, C. Liu, C. A. Mirkin, *Angew. Chem.* **2006**, *118*, 7257; *Angew. Chem. Int. Ed* **2006**, *45*, 7220
- [22] This technique is also compatible with other phospholipids; for instance, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, $T_m = -2.6^\circ\text{C}$) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, $T_m = 23.5^\circ\text{C}$) have been successfully patterned. Pure POPC and POPC mixed with 20% cholesterol were readily patterned under the same conditions as DOPC. DMPC could be patterned at 35°C and 75% humidity, but not at lower temperatures. This finding suggests that the phospholipid ink must be at least 10°C above its gel-liquid crystal transition temperature.
- [23] A. S. Ulrich, M. Sami, A. Watts, *Biochim. Biophys. Acta* **1994**, *1191*, 225.
- [24] H. Binder, *Appl. Spectrosc. Rev.* **2003**, *38*, 15.
- [25] E. Strandberg, T. Sparrman, G. Lindblom, *Adv. Colloid Interface Sci.* **2001**, *89*, 239.
- [26] S. Tristram-Nagle, H. I. Petrache, J. F. Nagle, *Biophys. J.* **1998**, *75*, 917.
- [27] S. Schuy, A. Janshoff, *ChemPhysChem* **2006**, *7*, 1207.
- [28] Y. Fang, A. G. Frutos, J. Lahiri, *ChemBioChem* **2002**, *3*, 987.
- [29] J. Haaheim, R. Eby, M. Nelson, J. Fragala, B. Rosner, H. Zhang, G. Athas, *Ultramicroscopy* **2005**, *103*, 117.
- [30] B. L. Weeks, J. J. DeYoreo, *J. Phys. Chem. B* **2006**, *110*, 10231.
- [31] B. L. Weeks, M. W. Vaughn, J. J. DeYoreo, *Langmuir* **2005**, *21*, 8096.
- [32] S. Rozhok, P. Sun, R. Piner, M. Lieberman, C. A. Mirkin, *J. Phys. Chem. B* **2004**, *108*, 7814.
- [33] D. Bullen, C. Liu, *Sens. Actuators A* **2006**, *125*, 504.
- [34] D. Bullen, S. W. Chung, X. F. Wang, J. Zou, C. A. Mirkin, C. Liu, *Appl. Phys. Lett.* **2004**, *84*, 789.
- [35] F. Brochard-Wyart, in *Soft Matter Physics* (Ed.: C. E. W. M. Daoud), Springer, Berlin, **1995**, p. 1.
- [36] J. Nissen, K. Jacobs, J. O. Radler, *Phys. Rev. Lett.* **2001**, *86*, 1904.
- [37] M. C. Howland, A. R. Sapuri-Butti, S. S. Dixit, A. M. Dattelbaum, A. P. Shreve, A. N. Parikh, *J. Am. Chem. Soc.* **2005**, *127*, 6752.
- [38] M. Karlsson, K. Sott, M. Davidson, A. S. Cans, P. Linderholm, D. Chiu, O. Orwar, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11573.

Received: August 18, 2006
Published online on December 13, 2006