Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10775–10778, October 1996
Cell Biology

Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold

(biocompatibility/cell culture/microfabrication/tissue engineering)

MILAN MRKSICH*, CHRISTOPHER S. CHEN†, YOUNAN XIA*, LAURA E. DIKE†, DONALD E. INGBER†, AND GEORGE M. WHITESIDES*

*Department of Chemistry, Harvard University, Cambridge, MA 02138; and †Departments of Surgery and Pathology, Children's Hospital and Harvard Medical School, Enders 1007, 300 Longwood Avenue, Boston, MA 02115

Contributed by George M. Whitesides, May 28, 1996

ABSTRACT **This paper describes a method based on experimentally simple techniques–microcontact printing and micromolding in capillaries–to prepare tissue culture substrates in which both the topology and molecular structure of the interface can be controlled. The method combines optically transparent contoured surfaces with self-assembled monolayers (SAMs) of alkanethiolates on gold to control interfacial characteristics; these tailored interfaces, in turn, control the adsorption of proteins and the attachment of cells. The technique uses replica molding in poly(dimethylsiloxane) molds having micrometer-scale relief patterns on their surfaces to form a contoured film of polyurethane supported on a glass slide. Evaporation of a thin (<12 nm) film of gold on this surface-contoured polyurethane provides an optically transparent substrate, on which SAMs of terminally functionalized alkanethiolates can be formed. In one procedure, a flat poly(dimethylsiloxane) stamp was used to form a SAM of hexadecanethiolate on the raised plateaus of the contoured surface by contact printing hexadecanethiol [$HS(CH_2)_{15}CH_3$]; a SAM terminated in tri(ethylene glycol) groups was subsequently formed on the bare gold remaining in the grooves by immersing the substrate in a solution of a second alkanethiol [$HS(CH_2)_{11}(OCH_2CH_2)_3OH$]. When this patterned substrate was immersed in a solution of fibronectin, the protein adsorbed only on the methyl-terminated plateau regions of the substrate [the tri(ethylene glycol)-terminated regions resisted the adsorption of protein]; bovine capillary endothelial cells attached only on the regions that adsorbed fibronectin. A complementary procedure confined protein adsorption and cell attachment to the grooves in this substrate.**

This report describes a simple and general method to fabricate optically transparent surfaces contoured into grooves of defined size and shape and to use self-assembled monolayers (SAMs) of alkanethiolates on gold to control cell attachment to these substrates. We have used SAMs extensively to control the adsorption of proteins and the attachment of mammalian cells to *planar* surfaces (refs. 1–5; for pioneering work by other groups, see refs. 6–10). By patterning the formation of SAMs using microcontact printing (11, 12)–an experimentally simple and nonphotolithographic technique–into regions that promote or resist the adsorption of protein, the attachment of cells to surfaces could be confined to rows 10–100 μm wide (M.M., L.E.D., J. Tien, D.E.I., and G.M.W., unpublished results), or to islands, for the attachment of single cells (14). The present work extends this methodology to include control over the *topography* of surfaces used for cell culture; the method employs an elastomeric stamp having micrometer-scale patterns of relief to mold a thin film of polyurethane and SAMs to control the properties of these contoured surfaces.

A number of groups have used contoured surfaces to study the effects of topography on cell alignment, migration, and metabolism (15–20); this work has demonstrated the importance of substrate topography in controlling the behavior of cells. The procedures used to fabricate the substrates used in these studies have three limitations: (*i*) The molecular properties of the surfaces are not well-controlled (nor can these properties be tailored easily). (*ii*) The substrates (silicon) are optically opaque, and attached cells cannot be visualized using conventional light microscopy. (*iii*) The preparation of the substrates requires photolithographic techniques that are not routinely available in biological laboratories. The methodology described in this report uses more flexible and convenient techniques for microfabrication–microcontact printing (11, 12) and micromolding in capillary channels (21)–to create substrates contoured into grooves and plateaus. The methodology is general in that it allows surfaces having a variety of topologies to be fabricated easily, and it permits control at the molecular scale over the interfacial properties of the substrates.

SAMs of alkanethiolates on gold are prepared by immersing a substrate coated with a thin film of gold in an ethanolic solution of a long-chain alkanethiol [$HS(CH_2)_nX$, $10 < n < 25$]. The sulfur atoms coordinate to the gold, and the trans-extended alkyl chains pack tightly: the terminal group, X, is confined to the interface between the SAM and the aqueous phase; the properties of the interface are dominated by the identity of this group (22–24).

For studies involving the attachment of cells, we have used glass slides coated with thin optically transparent films of gold (10–12 nm) (4). SAMs terminated in methyl groups are hydrophobic and adsorb protein quickly and irreversibly from solution. SAMs terminated in short oligomers of the ethylene glycol group [$-S(CH_2)_{11}(OCH_2CH_2)_nOH$, n = 2 to 7] resist essentially completely the nonspecific adsorption of proteins; *in situ*, these SAMs resist even the adsorption of "sticky" proteins such as fibrinogen (3). For the same reason, SAMs terminated in oligo(ethylene glycol) groups resist the attachment of cells–and the spreading of attached cells–over periods of several days in culture (M.M., L.E.D., J. Tien, D.E.I., and G.M.W., unpublished results; 14).

MATERIALS AND METHODS

Materials Used in Fabrication. Poly(dimethylsiloxane) (PDMS) was purchased from Dow Corning (Sylgard 184). PDMS stamps were prepared from photolithographically produced masters as described (11); flat stamps were prepared by casting the prepolymer against a clean silicon wafer (25). Silicon wafers were purchased from Silicon Sense (3-inch <111> orientation; 1 inch = 2.54 cm). Prepolyurethane

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SAM, self-assembled monolayer; PDMS, poly(dimethylsiloxane); BCE, bovine capillary endothelial.

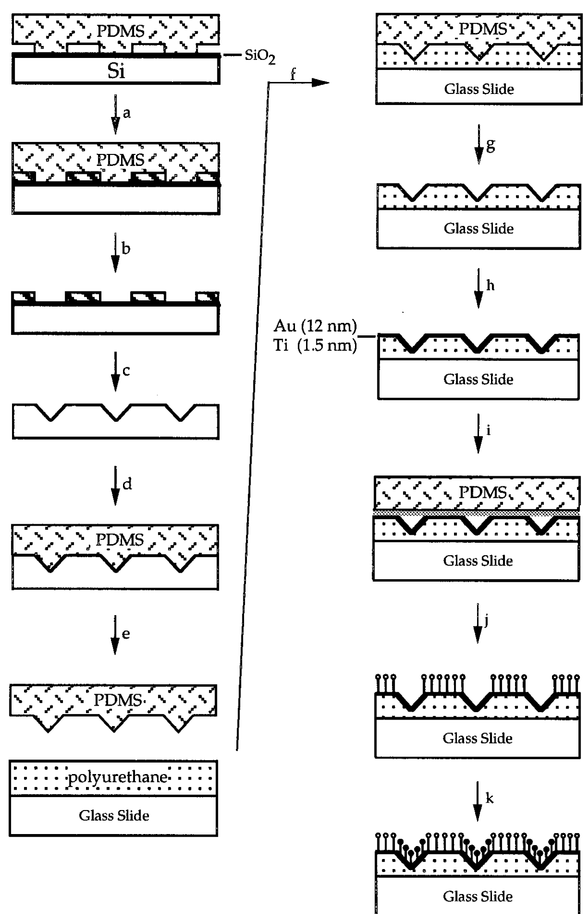

FIG. 1. Procedure for preparing contoured substrates. The method is explained in the text.

(Norland Optical Adhesive 68) was purchased from Norland Products (New Brunswick, NJ). Hexadecanethiol was purchased from Aldrich and purified by silica gel chromatography using 19:1 (vol/vol) hexanes/ethyl acetate as the eluent. The tri(ethylene glycol)-terminated alkanethiol was synthesized as described (26). All other chemicals and solvents were purchased from Aldrich and used as received.

Attachment of Cells to Substrates. The contoured substrates were placed in Petri dishes containing phosphate-buffered saline (PBS; 20 ml; 10 mM sodium phosphate/100 mM sodium chloride, pH = 7.4). A solution of fibronectin (Organon Teknika–Cappel) in PBS (400 μl; 2.5 mg/ml) was added. After 2 hr, the solution was diluted by the addition of PBS ($\approx$200 ml) and the substrates were removed from solution under a stream of buffer and transferred immediately to Petri dishes containing defined medium [low-glucose Dulbecco's modified Eagle's medium (DMEM)/10 mM Hepes/1% bovine serum albumin (BSA)/high density lipoprotein (10 μg/ml)/transferrin (10 μg/ml)/fibroblast growth factor (5 μg/ml)]. Bovine capillary endothelial (BCE) cells were plated on these substrates and maintained in culture for several days (37°C, 10% CO_2/90% air) (27); the medium was initially exchanged 2 hr after inoculation with cells and then daily thereafter. After 3 days, the cells were fixed with paraformaldehyde and either stained for filamentous actin by using rhodaminated-phalloidin (Sigma) or sputtered with gold and observed by scanning electron microscopy.

RESULTS AND DISCUSSION

Fabrication of Substrates. Our method for fabrication of contoured substrates involved four steps (Fig. 1): (*i*) Preparation of a master pattern in silicon by micromolding in capillaries using an elastomeric stamp, followed by anisotropic chemical etching of the silicon (other procedures would also work). (*ii*) Transfer of the topographical pattern into a film of polyurethane on a glass coverslip. (*iii*) Evaporation of a thin optically transparent film of gold on the polyurethane. (*iv*) Formation of patterns of SAMs of alkanethiolates on the gold.

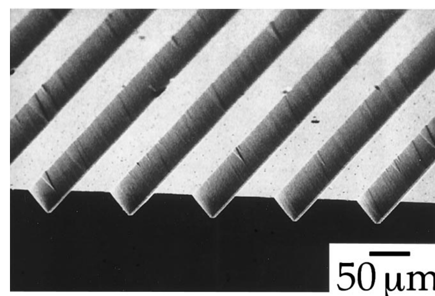

FIG. 2. Scanning electron micrograph of a contoured film of polyurethane supported on a glass slide (as in Fig. 1, part g). The contoured substrate was frozen in nitrogen, fractured along a plane perpendicular to the array of lines, and sputtered with gold (20 nm) prior to the electron microscopy.

To accomplish the first step, we prepared a PDMS stamp using the procedure described for microcontact printing (11). The stamp was placed on a silicon <111> wafer having a layer of silicon dioxide; the recessed features of the stamp formed a network of channels (Fig. 1). When a drop of prepolyurethane was placed on the wafer and in contact with the stamp, capillary action caused the liquid to fill the channels completely (arrow a). The prepolymer was cured with UV light and the stamp was removed from the surface to leave a pattern of the polymer at the surface (arrow b). This polymer protected the underlying SiO_2 from dissolution in an aqueous solution of HF (1%); the exposed regions of silicon were then etched anisotropically in an aqueous solution of KOH (4 M, 15% isopropanol, 60°C) to give V-shaped grooves (arrow c) (28). A PDMS stamp was cast from this substrate (arrow d), peeled away (arrow e), and gently pressed onto a drop of liquid prepolyurethane on a glass coverslip (arrow f). The structure was cured under UV light with the stamp in place, and the stamp was then peeled away to give the contoured substrate (arrow g). Fig. 2 shows a scanning electron micrograph of this fabricated substrate. This same PDMS stamp could be used to fabricate multiple substrates.

Evaporation of a thin layer of titanium (1.5 nm; to promote adhesion of the gold to the polyurethane) and a thin layer of gold (12 nm) provided a contoured substrate (arrow h) on which SAMs could be assembled. In one example, the plateaus of the substrate were derivatized selectively with a SAM of hexadecanethiolate by contact printing with a flat stamp (25) (arrow i); this procedure left the gold surface of the grooves unmodified (arrow j). A SAM terminated in tri(ethylene glycol) groups was formed in the grooves by immersing the substrate in a solution of the second alkanethiol ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$) (arrow k). Substrates having a reversed pattern of SAM were prepared by first printing the tri(ethylene glycol)-terminated alkanethiol onto the plateaus and then immersing in a solution of hexadecanethiol.‡

Directed Attachment of Cells. We examined the attachment of BCE cells on two fibronectin-coated contoured surfaces: one having ridges 25 μm wide and separated by V-shaped

‡Ellipsometric measurements showed that microcontact printing of the tri(ethylene glycol)-terminated alkanethiol resulted in < 50% formation of SAM. We determined empirically that it was necessary to repeat the microcontact printing three times before immersing the substrate in a solution of hexadecanethiol to passivate the ridges of the contoured substrates.

trenches of equal width and the second having ridges and grooves 50 μm wide. For all substrates, the attachment of cells depended strictly on the properties of the SAM and not on the topology of the substrate; SAMs presenting tri(ethylene glycol) groups resisted the adsorption of fibronectin and the subsequent attachment of BCE cells; fibronectin adsorbed to methyl-terminated SAMs and allowed efficient attachment of cells in these areas. Substrates modified uniformly with a SAM of hexadecanethiolate presented fibronectin at all regions and allowed efficient attachment of the BCE cells on both the plateaus and grooves, with little preference for either region (Fig. 3*A*). For substrates presenting fibronectin only on their plateaus, cells attached exclusively to the plateaus; no cells attached to the grooves presenting a SAM of tri(ethylene glycol) groups (Fig. 3*C*). For substrates whose grooves were coated with fibronectin, cells attached only to the sides of the grooves: many cells stretched across both sides of the grooves without contacting the bottom edge (Fig. 3*B*).

These contoured substrates have many characteristics that make them useful for experimental manipulation of cultured cells. Because the substrates are optically transparent, attached cells can be observed in culture using standard light microscopy. Fig. 4 shows optical micrographs of cells that were stained with Coomassie blue; cells were also visible by phase contrast without staining.

The gold-coated substrates are compatible with fluorescence microscopy. Fig. 4*C* shows a fluorescent micrograph of the filamentous actin network of cells confined to ridges after staining with rhodaminated-phalloidin. These substrates also have the stability required for use in cell culture. After 5 days, the BCE cells remained attached to the contoured substrates and continued to divide; the cells also did not invade regions that were modified with a SAM terminated in tri(ethylene glycol) groups.

SAMs of alkanethiolates in this methodology provide many opportunities for tailoring the molecular structures of the surfaces to control their interfacial characteristics. For example, the properties of SAMs that present electroactive groups can be switched by applying a potential to the gold substrate (29, 30); the thin optically transparent films of gold used here still have the electrical conductivity of bulk gold (31). SAMs that present ligands of low molecular weight have been prepared for fundamental studies of bio-specific adsorption of proteins at interfaces (32). SAMs presenting chelates of Ni(II) are useful for immobilizing His-tagged proteins from cell extracts (33). A variety of analytical techniques–surface plasmon resonance (SPR) spectroscopy (3, 32, 33), ellipsometry (1, 2), scanning electron microscopy (34), and quartz crystal microbalance (35)–can be used to study the interactions of proteins with SAMs on gold. SPR is especially useful because it is a noninvasive technique that can detect ≈2% of a

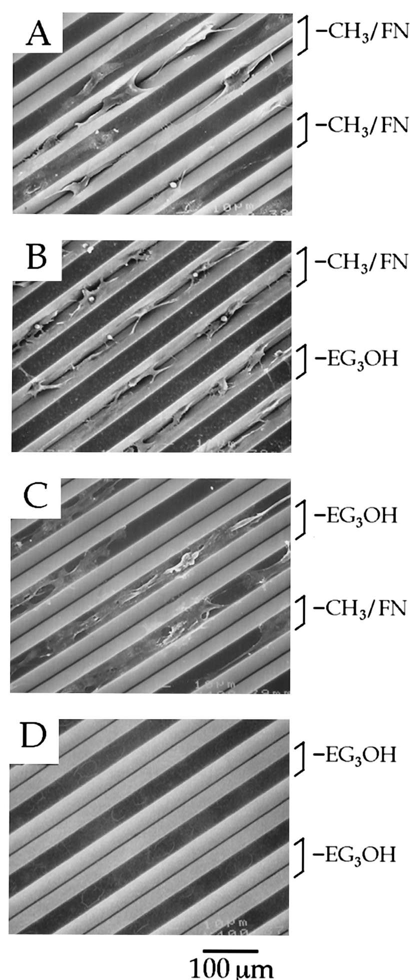

FIG. 3. Scanning electron microscopy images of endothelial cells cultured on contoured substrates having ridges and grooves 25 μm wide. The substrates were tailored with SAMs presenting either methyl or tri(ethylene glycol)(EG_3OH) groups; fibronectin (FN) adsorbed only on the methyl-terminated regions; cells attached only to these regions presenting fibronectin. (*A*) The entire substrate presents fibronectin (FN). (*B*) Only the grooves present fibronectin. (*C*) Only the ridges present fibronectin. (*D*) None of the areas present fibronectin. After 2 days in culture, cells were fixed in Karnovsky's fixative, critical-point-dried, and sputtered with 20 nm of gold. The scale bar applies to all images.

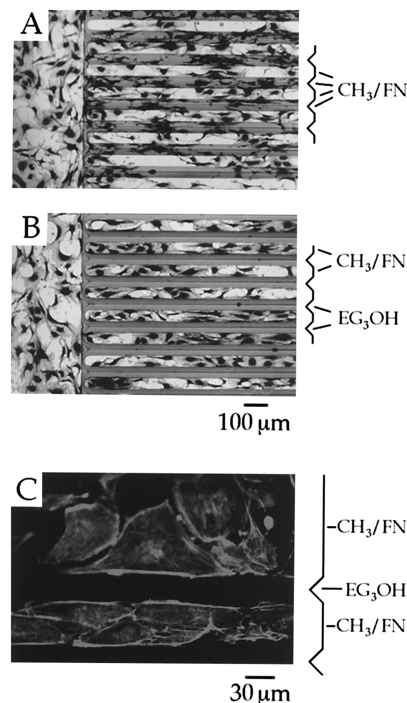

FIG. 4. Optical micrographs of endothelial cells cultured on contoured substrates having ridges and grooves 50 μm wide. (*A*) The entire substrate was tailored with a SAM presenting methyl groups and fibronectin (FN). (*B*) Only the plateau regions were tailored with a SAM presenting methyl groups and fibronectin. Both photographs are at the same magnification and include an unpatterned planar region to the left. Cells were fixed in 3.7% paraformaldehyde and stained with 1:1 Giemsa/Coomassie. (*C*) Optical micrograph of endothelial cells attached on a contoured surface after fluorescence staining of the filamentous actin microfilaments with rhodaminated-phalloidin. The upper region shows cells on a planar nonpatterned region and the lower region shows cells confined to a plateau 50 μm wide. Cells were fixed in 3.7% paraformaldehyde prior to staining with the phalloidin.

monolayer of protein, and it provides both kinetic and thermodynamic parameters.

In summary, this report describes a flexible methodology to prepare optically transparent contoured surfaces appropriate for fundamental studies of the relationships between the molecular structure and topology of a surface and the behavior of cells attached to the surface. This experimental system may also find use in applied cell culture, including the development of supports for the immobilization of cells in bioreactors and substrates for tissue engineering. The range of geometries of features that can be formed is limited only by the availability of appropriate master templates; these templates are often created using techniques common in microfabrication but are also available from other sources (e.g., diffraction gratings). This methodology can be used to prepare contoured substrates having features with dimensions down to the submicrometer range without requiring access to the special facilities and instrumentation used currently in microfabrication (13).

This work was supported by the National Institutes of Health (Grants GM 30367 to G.M.W. and CA 55833 to D.E.I.), the Office of Naval Research, and the Advanced Research Projects Agency. D.E.I. is a recipient of a Faculty Research Award from the American Cancer Society. M.M. is grateful to the American Cancer Society for a postdoctoral fellowship.

1. Prime, K. L. & Whitesides, G. M. (1993) *J. Am. Chem. Soc.* **115,** 10714–10721.
2. Prime, K. L. & Whitesides, G. M. (1991) *Science* **252,** 1164–1167.
3. Mrksich, M., Sigal, G. B. & Whitesides, G. M. (1995) *Langmuir* **11,** 4383–4385.
4. DiMilla, P. A., Folkers, J. P., Biebuyck, H. A., Harter, R., Lopez, G. P. & Whitesides, G. M. (1994) *J. Am. Chem. Soc.* **116,** 2225–2226.
5. Lopez, G. P., Albers, M. W., Schreiber, S. L., Carroll, R., Peralta, E. & Whitesides, G. M. (1993) *J. Am. Chem. Soc.* **115,** 5877–5878.
6. Kleinfeld, D., Kahler, K. H. & Hockberger, P. E. (1988) *J. Neurosci.* **8,** 4098–4120.
7. O'Neill, C., Jordan, P. & Riddle, P. (1990) *J. Cell Sci.* **95,** 577–586.
8. Britland, S., Clark, P., Connolly, P. & Moores, G. (1992) *Exp. Cell Res.* **198,** 124–129.
9. Stenger, D. A., Georger, J. H., Dulcey, C. S., Hickman, J. J., Rudolph, A. S., *et al.* (1992) *J. Am. Chem. Soc.* **114,** 8435–8442.
10. Spargo, B. J., Testoff, M. A., Nielsen, T. B., Stenger, D. A., Hickman, J. J. & Rudolph, A. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11070–11074.
11. Kumar, A., Biebuyck, H. A. & Whitesides, G. M. (1994) *Langmuir* **10,** 1498–1511.
12. Mrksich, M. & Whitesides, G. M. (1995) *Trends Biotechnol.* **13,** 228–236.
13. Wilbur, J. L., Kim, E., Xia, Y. & Whitesides, G. M. (1995) *Adv. Mater.* **7,** 649–652.
14. Singhvi, R. Kumar, A., Lopez, G. P., Stephanopoulos, G. N., Wang, D. I. C., Whitesides, G. M. & Ingber, D. E. (1994) *Science* **264,** 696–698.
15. Chou, L., Firth, J. D., Uitto, V.-J. & Brunette, D. M. (1995) *J. Cell Sci.* **108,** 1563–1573.
16. Clark, P., Connolly, P., Curtis, A. S. G., Dow, J. A. T. & Wilkinson, C. D. W. (1991) *J. Cell Sci.* **99,** 73–77.
17. Meyle, J., Gultig, K., Brich, M., Hammerle, H. & Nisch, W. (1994) *J. Mater. Sci. Mater. Med.* **5,** 463–466.
18. Hoch, H. C., Staples, R. C., Whitehead, B., Comeau, J. & Wolf, E. D. (1987) *Science* **235,** 1659–1662.
19. Green, A. M., Jansen, J. A., van der Waerden, J. P. & von Recum, A. F. (1994) *J. Biomed. Mater. Res.* **28,** 647–653.
20. Schmidt, J. A. & von Recum, A. F. (1992) *Biomaterials* **13,** 1059–1069.
21. Kim, E., Xia, Y. & Whitesides, G. M. (1995) *Nature (London)* **376,** 581–584.
22. Whitesides, G. M. & Gorman, C. B. (1995) in *Handbook of Surface Imaging and Visualization,* ed. Hubbard, A. T. (CRC, Boca Raton, FL), pp. 713–732.
23. Dubois, L. H. & Nuzzo, R. G. (1992) *Annu. Rev. Phys. Chem.* **43,** 437–463.
24. Mrksich, M. & Whitesides, G. M. (1996) *Annu. Rev. Biophys. Biophys. Chem.*, in press.
25. Jeon, N. L., Nuzzo, R. G., Xia, Y., Mrksich, M. & Whitesides, G. M. (1995) *Langmuir* **11,** 3024–3026.
26. Pale-Grosdemange, C., Simon, E. S., Prime, K. L. & Whitesides, G. M. (1991) *J. Am. Chem. Soc.* **113,** 12–20.
27. Ingber, D. E. & Folkman, J. (1989) *J. Cell Biol.* **109,** 317–330.
28. Kim, E., Kumar, A. & Whitesides, G. M. (1995) *J. Electrochem. Soc.* **142,** 628–633.
29. Abbott, N. & Whitesides, G. M. (1994) *Langmuir* **10,** 1493–1497.
30. Wong, J. Y., Langer, R. & Ingber, D. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3201–3204.
31. Gorman, C. B., Biebuyck, H. A. & Whitesides, G. M. (1995) *Langmuir* **11,** 2242–2246.
32. Mrksich, M., Grunwell, J. R. & Whitesides, G. M. (1995) *J. Am. Chem. Soc.* **117,** 12009–12010.
33. Sigal, G. B., Bamdad, C., Barberis, A., Strominger, J. & Whitesides, G. M. (1996) *Anal. Chem.* **68,** 490–497.
34. Lopez, G. P., Biebuyck, H. A., Harter, R., Kumar, A. & Whitesides, G. M. (1993) *J. Am. Chem. Soc.* **115,** 10774–10781.
35. Ward, M. D. & Buttry, D. A. (1990) *Science* **249,** 1000–1007.