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CHAPTER 18

PATTERNING OF CELLS AND THEIR ENVIRONMENT

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INTRODUCTION

Control of the cell culture environment is crucial for understanding cell behavior and for engineering cell function. This chapter describes the use of a set of microfabrication techniques called "soft lithography" for patterning the substrate to which cells attach, the location and shape of the areas to which cells attach, and the fluid environment surrounding the cells, all with micrometer precision. Some examples wherein these techniques have helped to clarify problems in fundamental cell biology are summarized. The methods described are experimentally simple, inexpensive, and well suited for patterning biological materials.

How do tissues assemble *in vivo?* Once an appropriate community of cells has organized, how does it perform its functions? Answering these fundamental biological questions, and using the information thus obtained for engineering tissues, require the ability to study the behavior of cells in controlled environments. Some of the challenges in trying to control the environment experienced by individual cells lie in the size scales of the stimuli that need to be controlled (from the angstrom scale for molecular detail, through the micrometer scale for an individual cell, to the millimeter and centimeter scale for groups of cells) as well as in the number of the types of stimuli that need to be addressed (the composition of the culture media, the topography and chemical composition of the surface to which the cells attach, the nature of neighboring cells, the temperature, etc.).

Microfabrication and micropatterning using stamps or molds fabricated from elastomeric polymers (soft lithography) provide versatile methods for generating 10- to 100-μm-sized patterns of proteins and ligands on surfaces, 50- to 500-μm-sized culture chambers, and 10- to 100-μm-sized laminar flows of culture media in capillaries (Xia and Whitesides, 1998). Soft lithographic methods are relatively simple and inexpensive; the elastomeric polymer most often used in these procedures—poly(dimethylsiloxane), or PDMS—has several characteristics that make it attractive for biological applications. This chapter gives an overview of the application of soft lithography to the patterning of cells, their substrates, and their fluid environment.

SOFT LITHOGRAPHY

As the need of biologists to control and manipulate materials on the micrometer scale has increased, so has the need for new microfabrication techniques. Our laboratory has developed a set of microfabrication techniques that are useful for patterning on the scale of 0.5 μm and larger. We call these techniques "soft lithography" because they use elastomeric (that is, soft) stamps, molds, membranes, or channels (Xia and Whitesides, 1998).

Many other techniques can and have been used to pattern cells and their environment (Hammarback *et al.*, 1985; López *et al.*, 1993a; Park *et al.*, 1998; Vaidya *et al.*, 1998). The most com-

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monly used method has been photolithography. This technique has, of course, been highly developed for the microelectronics industry; it has also been adapted, with varying degrees of success, for biological studies (Hammarback *et al.,* 1985; Kleinfeld *et al.,* 1988; Ravencroft *et al.,* 1998). As useful and powerful as photolithography is (it is capable of mass production at 200-nm resolution of multilevel, registered structures), it is not always the best or only option for biological studies. It is an expensive technology; it is poorly suited for patterning nonplanar surfaces; it provides almost no control over the chemistry of the surface and hence is not very flexible in generating patterns of specific chemical functionalities or proteins on surfaces; it can generate only two-dimensional microstructures; and it is directly applicable to patterning only a limited set of photosensitive materials (e.g., photoresists).

Soft lithographic techniques are inexpensive, are procedurally simple, are applicable to the complex and delicate molecules often required in biochemistry and biology, can be used to pattern a variety of different materials, are applicable to both planar and nonplanar substrates (Jackman *et al.,* 1995), and do not require stringent control (such as a clean room environment) over the laboratory environment beyond that required for routine cell culture (Xia and Whitesides, 1998). Access to photolithographic technology is required only to create a master for casting the elastomeric stamps or membranes, and even then, the requirement for chrome masks—the preparation of which is one of the slowest and most expensive steps in conventional photolithography—can often be bypassed (Deng *et al.,* 1999; Duffy *et al.,* 1998; Grzybowski *et al.,* 1998; Qin *et al.,* 1996). Soft lithography offers special advantages for biological applications, in that the elastomer most often used (PDMS) is optically transparent and permeable to gases, is flexible and seals conformally to a variety of surfaces (including petri dishes), is biocompatible, and can be implanted if desired. The soft lithographic techniques that we will discuss include microcontact printing, patterning with microfluidic channels, and laminar flow patterning.

SELF-ASSEMBLED MONOLAYERS

Because many of the studies involving the patterning of proteins and cells using soft lithography have been carried out on self-assembled monolayers (SAMs) of alkane thiolates on gold, we give a brief discussion of SAMs (Bain and Whitesides, 1988b; Bishop and Nuzzo, 1996; Delamarche and Michel, 1996; Dubois and Nuzzo, 1992; Merritt *et al.,* 1997; Ostuni *et al.,* 1999; Prime and Whitesides, 1993; Ulman, 1996). SAMs are organized organic monolayer films (Fig. 18.1A) that allow control at the molecular level over the chemical properties of the interface by judicious design and fabrication of derivatized alkane thiol(s) adsorbed to the surface of films of gold or silver. The ease of formation of SAMs, and their ability to present a range of chemical functionality at their interface with aqueous solution, make them particularly useful as model surfaces in studies involving biological components. Furthermore, SAMs can be easily patterned by simple methods such as microcontact printing (μCP) with features down to 500 nm in size and smaller (Xia and Whitesides, 1998). These features of SAMs make them the best structurally defined substrates for use in patterning proteins and cells. SAMs on gold are used for the majority of experiments requiring the patterning of proteins and cells, because they are biocompatible, easily handled, and chemically stable [for example, silver oxidizes relatively rapidly, and Ag(I) ions are cytotoxic].

Methods for Preparing Homogeneous SAMs and Mixed SAMs

Single-component (homogeneous) SAMs of alkanethiolates on gold are formed by exposing a surface of gold to a solution containing, or to the vapors of, an alkane thiol (RSH). The surface properties of these SAMs are determined by the nature of the terminal groups (schematically represented by half-circles at the tips of the SAMs in Figs. 18.1 and 18.2). Gold substrates are prepared on glass cover slips or silicon wafers by evaporating a thin layer of titanium (1–5 nm) to promote the adhesion of gold to the support, followed by a thin layer of gold (10–200 nm) (DiMilla *et al.,* 1994). The SAMs formed on these gold substrates are stable to the conditions used for cell culture, but care should be taken to avoid strong light and temperatures above ~70°C, because both can result in degradation of the SAM (Huang and Hemminger, 1993; Ostuni *et al.,* 1999).

Mixed SAMs, or SAMs composed of two or more types of thiols adsorbed to a surface, can be formed directly by coadsorption of two or more alkane thiols from solution (Fig. 18.2A,B) (Bain

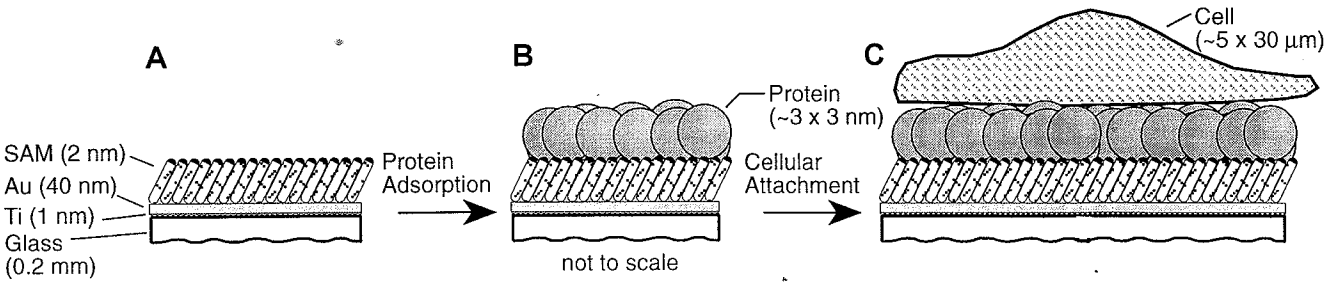

Fig. 18.1. Schematic illustration of the adsorption of proteins and the attachment of a cell to a self-assembled monolayer (SAM). The components of the SAM and the protein molecule are drawn roughly to scale; the cell is much reduced in relative size. See text for details.

and Whitesides, 1988a; Lahiri *et al.*, 1999a; Mrksich *et al.*, 1995), or by the reaction of a nucleophile, such as an amine (H_2NR), with a preformed SAM that presents interchain carboxylic anhydride groups. The anhydride-presenting surface is an exceptionally useful one and is easily prepared by the dehydration of single-component SAMs of $HS(CH_2)_nCO_2H$ (n = 10 or 15) (Fig. 18.2C) (Yan *et al.*, 1998). The anhydride method results in mixed SAMs composed of 1:1 mixtures of terminal amide ($-COHNR$) groups and carboxylic acid ($-CO_2H/-CO_2^-$) groups. The surface properties of mixed SAMs are determined by contributions from the various terminal groups present—the amount of each contribution being determined, qualitatively, by the degree of exposure of that terminal group at the surface (Bain *et al.*, 1989; Bain and Whitesides, 1989). In some cases the contribution of one terminal group will predominate, especially if that terminal group is long or large enough to screen the contribution of the shorter terminal group. For example, the characteristics of mixed SAMs prepared by the interchain anhydride methods are often determined predominantly by nature of the ($-COHNR$) groups, because these are longer and shield the ($-CO_2H/-CO_2^-$) groups (Fig. 18.2C) (Yan *et al.*, 1997). The anhydride method makes it possible to generate mixed SAMs with a range of surface characteristics rapidly and conveniently. This method is experimentally simple, and both $HS(CH_2)_{10}CO_2H$ and a variety of amines are commercially available. It is thus unnecessary to synthesize the alkane thiols required in the older methods of making functionalized SAMs.

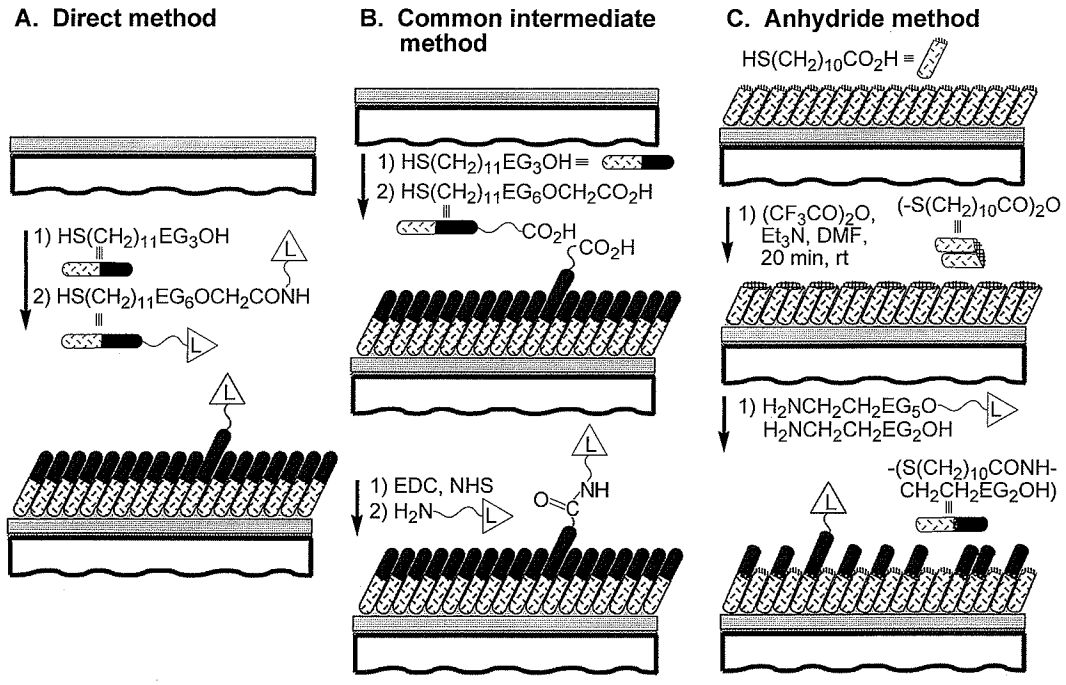

Fig. 18.2. Preparation of mixed SAMs that present a ligand by three methods: direct, common intermediate, and anhydride. These mixed SAMs are useful for the specific adsorption of the protein that binds the incorporated ligand (L). See text for details.

Preventing Protein Adsorption: Inert Surfaces

Proteins play an integral part in the adhesion of cells with surfaces: they are the glue that cements the cells to the surface. Thus, if one can control the interaction of proteins with a surface, one can begin to control the interaction of cells with that surface (Fig. 18.1). Most surfaces adsorb proteins. Thus, the main challenge in controlling the interactions of proteins and cells with surfaces lies in finding surfaces that resist nonspecific adsorption of proteins (surfaces that we call "inert," for brevity). Inert surfaces provide the background necessary for spatially restricting protein adsorption or for preparing surfaces that bind only specific proteins, and are used in patterning proteins and cells (Merritt *et al.,* 1997), as biomaterials (Andrade *et al.,* 1987; Helmus and Hubbell, 1993), and in the construction of biosensors (Mrksich and Whitesides, 1995).

Single-component SAMs terminated in oligo(ethylene glycol) (EG_n), oligomers longer than $n = 3$, resist the adsorption from solution of a variety of proteins that range in size (15–340 kDa) and charge (pI = 6–12) (Prime and Whitesides, 1991, 1993). Mixed SAMs containing as little as 50% of an $HS(CH_2)_{11}EG_6OH$ [mixed with $HS(CH_2)_{11}CH_3$] show good protein resistance, as do mixed SAMs presenting a 1:1 mixture of terminal $-COHN(CH_2CH_2O)_nR$ ($n = 6$, R = H or CH_3) and $-CO_2H/-CO_2^-$ groups. Other work has established that EG_n groups are not unique in their ability to resist adsorption of proteins, and, as an example, SAMs presenting $-CH_2CH_2CH_2S(=O)-$ groups are also effective in resisting protein adsorption (Deng *et al.,* 1996). R. G. Chapman, L. Yan, S. Takayama, R. E. Holmlin, and G. M. Whitesides (unpublished results) have surveyed a large number of functional groups for their ability to resist the nonspecific adsorption of proteins, and found that polar functional groups that do not contain H-bond donors often make good components of inert surfaces. The combination of inert and adsorptive surfaces with soft lithographic techniques enables the facile patterning of proteins and cells.

Controlled Protein Adsorption

Protein-specific mixed SAMs that present ligands for specific adsorption of a protein of interest, while resisting the nonspecific adsorption of other proteins, have been prepared by several techniques: (1) by coadsorption of a thiol that forms inert surfaces with a thiol that presents a ligand specific for a particular protein (Fig. 18.2A) (Mrksich *et al.,* 1995; Sigal *et al.,* 1996); (2) by the common intermediate method, whereby an amine terminated in a ligand is coupled to a mixed SAM presenting $-EG_3OH$ (the inert surface component) and $-EG_6OCH_2COOH$ (the component to which ligands are coupled) (Fig. 18.2B) (Lahiri *et al.,* 1999a); (3) by the anhydride method, whereby a mixture of two amines—one terminated with a ligand and the other with an "inert" functional group (for example, $H_2NCH_2CH_2EG_2OH$)—is allowed to react with a surface that presents interchain anhydride groups (Fig. 18.2C) (R. G. Chapman, L. Yan, and G. M. Whitesides, unpublished results). The common intermediate method and the anhydride method are more convenient than, and preferable to, the direct method, because they require less organic synthesis.

MICROCONTACT PRINTING

Patterning Ligands, Proteins, and Cells Using Microcontact Printing

Microcontact printing is a technique that uses the relief pattern on the surface of an elastomeric PDMS stamp to form patterns on the surfaces of various substrates (Fig. 18.3A) (Xia and Whitesides, 1998). The stamp is "inked" with a solution containing the patterning component, dried, and brought into conformal contact with a surface for intervals ranging from a few seconds to minutes. The patterning component transfers to the substrate in the regions where the stamp contacts the substrate. The kinds of components used as ink for μCP include thiol derivatives that form SAMs on gold and silver; activated silanes that react with the SiOH groups present on the surface of silicon; various ligands (usually amine containing compounds) that react with activated SAMs (generated using the techniques described in the previous section) (Lahiri *et al.,* 1999b; Yan *et al.,* 1998); and robust proteins that can withstand drying and stamping (Bernard *et al.,* 1998; St. John *et al.,* 1998).

Although the use of proteins as the ink is limited to sturdy proteins, even the more delicate ones can be patterned utilizing microcontact printing. This patterning of proteins is accomplished

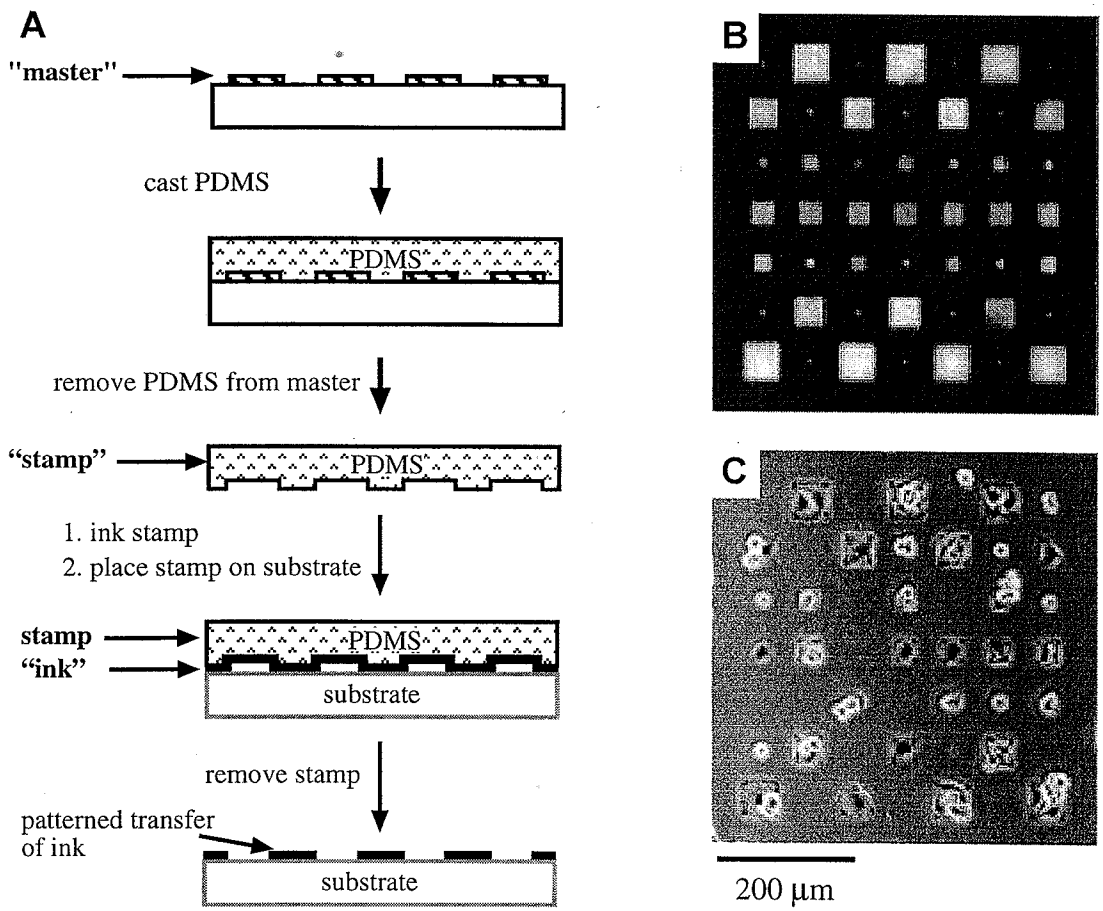

Fig. 18.3. Examples of microcontact printing experiments. (A) Typical procedure. A poly(dimethylsiloxane) (PDMS) stamp is prepared by pouring PDMS prepolymer on a "master," curing the PDMS and removing it from the master. A solution containing the patterning component of interest ("ink") is applied to this stamp and the solution allowed to dry. This inked stamp is placed on a substrate to allow the ink to transfer to the substrate. A patterned substrate remains after removal of the stamp. (B) Selective adsorption of fibronectin onto a surface patterned into protein-adsorptive and non-protein-adsorptive self-assembled monolayers by microcontact printing as visualized by immunostaining. (C) Patterned attachment and spreading of cells on the protein-patterned substrate in B.

by preparing areas of protein-adsorptive SAMs and allowing proteins to adsorb onto those regions from solutions. For example, López *et al.* (1993b) used microcontact printing to pattern gold surfaces into regions terminated in methyl groups, when surrounded by inert oligo(ethylene glycol) groups. Immersion of the patterned SAMs in solutions of proteins such as fibronectin, fibrinogen, pyruvate kinase, streptavidin, and immunoglobulins resulted in adsorption of the proteins on the methyl-terminated regions. The pattern of adsorbed proteins could be characterized by scanning electron microscopy; the layers of adsorbed protein appeared to be homogeneous. Alternatively, proteins can be anchored to ligands patterned onto surfaces by μCP. Lahiri *et al.* (1999b), for example, patterned streptavidin by μCP of its ligand, biotin, onto activated SAMs (using the method illustrated in Fig. 18.2B), and then allowing the protein to bind to the patterned ligand.

All eukarytoic cells, and many prokaryotic cells, are too delicate to be dried or stamped and cannot be patterned directly by μCP. The ability of μCP to create patterns of ligands and proteins, however, allows the patterning of many anchorage-dependent cells (Fig. 18.1), confining them to specific regions of a substrate; these techniques strongly influence the size and shape of the cells (Fig. 18.3C). For example, Mrksich *et al.* (1996) used microcontact printing to pattern gold or silver substrates into regions presenting oligo(ethylene glycol) groups and methyl groups. After coating the substrates with fibronectin, bovine capillary endothelial cells attached only to the methyl-

terminated, fibronectin-coated regions of the patterned SAMs. The cells remained attached in the pattern defined by the underlying SAMs for 5–7 days. μCP has also been used to pattern astroglial cells on silicon substrates (Craighead *et al.*, 1998). Astroglial cells attached selectively to 50-μm-wide bars of *N*-1-[3-(trimethoxysilyl)propyl]diethylenetriamine (DETA) SAMs patterned on a silicon surface. Zhang *et al.* (1999) have synthesized oligopeptides containing a cell adhesion motif at the N terminus connected by an oligo(alanine) linker to a cysteine residue at the C terminus. The thiol group of cysteine allowed the oligopeptides to form monolayers on gold-coated surfaces. A combination of microcontact printing and these self-assembling oligopeptide monolayers was used to pattern gold surfaces into regions presenting cell adhesion motifs and oligo(ethylene glycol) groups that resist protein adsorption. Wheeler *et al.* (1999; Branch *et al.*, 1998) created patterns of covalently bound ligands and proteins on glass cover slips and used these patterns to control nerve cell growth.

Fundamental Studies in Cell Biology Using Microcontact-Printed Substrates

The ability to design SAMs to be either protein adsorptive or nonadsorptive, when combined with the ability of μCP to pattern such SAMs routinely on size scales smaller than that of a single cell (2–50 μm), has led to new studies on the effect of patterned surface environments and cell shape on cell behavior (Table 18.1).

Singhvi *et al.* (1994) used μCP to prepare substrates consisting of square and rectangular islands of laminin surrounded by nonadhesive regions, and studied the behavior of rat hepatocytes on them. The cells conformed to the shape of the laminin patterns, allowing one to control cell shape independently of the extracellular matrix (ECM) ligand density. The investigators compared cells grown on various patterns and observed that cell shape, regardless of ECM ligand density, was the major determinant of cell growth and differentiation. Chen *et al.* (1997, 1999) used μCP to prepare substrates that presented circular cell-adhesive islands of various diameters and interisland spacings. Such patterns allowed them to control the extent of cell spreading without varying the total cell–matrix contact area. They found that the extent of spreading (the projected surface area of the cell) and not the area of the adhesive contact controlled whether the cell divided, remained in stationary phase, or entered apoptosis. Dike *et al.* (1999) used μCP to prepare substrates with cell-adhesive lines of varying widths. They found that bovine capillary endothelial (BCE) cells cultured on 10-μm-wide lines underwent differentiation to capillary tubelike structures containing a central lumen. Cells cultured on wider (30 μm) lines formed cell–cell contacts, but these cells continued to proliferate and did not form tubes. These studies indicate that cell growth, function, and differentiation can be controlled, at least in some cases, by patterning the surface of the substrate to which cells adhere.

In another study, Bailly *et al.* (1998) used micropatterned substrates in studies of the regulation of protrusion shape during chemotactic responses of mammalian carcinoma cells. They plated rat mammary carcinoma cells on gold-coated glass cover slips having 10-μm-wide adhesive lanes. On stimulation with epidermal growth factor (EGF), the cells extended lamellipods laterally, over the nonadhesive part of the substrate. These results showed that lamellipod extension could occur independently of any contact with the substrate. Contact formation was, however, necessary for stabilizing the protrusion.

μCP is an excellent method for patterning surfaces with complex and delicate organic groups, and it is the soft lithographic method that has been most utilized for patterning the substrate.

MICROFLUIDIC PATTERNING

Microfluidic channels can be used to pattern surfaces by restricting the flow of fluids to desired regions of a substrate (Fig. 18.4). The patterning components—such as ligands, proteins, and cells—are deposited from the solution to create a pattern on the substrate.

Delamarche *et al.* (1997, 1998) used microfluidic patterning (μFP) to pattern immunoglobulins with submicron resolution on a variety of substrates, including gold, glass, and polystyrene. Only microliters of reagent were required to cover square millimeter-sized areas. Patel *et al.* (1998) developed a method to generate micron-scale patterns of any biotinylated ligand on the surface of a biodegradable polymer. These investigators prepared biotin-presenting polymer films, and patterned the films by allowing solutions of avidin to flow over them through 50-μm channels fab-

Table 18.1. Examples of patterning cells using microcontact printing

Substrate	Features	Initial component patterned	Proteins patterned	Effect observed (cells patterned)	Refs.
Gold	2- to 80-μm rectangles	Hexadecane thiol, hexa(ethylene glycol)–terminated alkane thiol	Laminin	Primary rat hepatocytes adhere and spread only on laminin-coated islands; cell shape controls cell growth and function	Singhvi *et al.* (1994)
Gold	60-μm lines separated by 120 μm	Hexadecane thiol, tri(propylene sulfoxide)–terminated alkane thiol	Fibronectin	BCE cells were confined to the hexadecane thiol SAM regions for 1–2 days then started to spread into other areas	Deng *et al.* (1996)
Thin gold film (<12 nm) on polyurethane	25- to 50-μm ridges and grooves	Hexadecane thiol, tri(ethylene glycol)–terminated alkane thiol	Fibronectin	Patterned adsorption of fibronectin; cells are confined to tri(ethylene glycol)-terminated alkane thiol SAM regions for at least 5 days	Mrksich *et al.* (1996)
Gold	3- to 40-μm squares and circles	Hexadecane thiol, tri(ethylene glycol)-terminated alkane thiol	Fibronectin, vitronectin, collagen, anti-integrin β_1 antibody, or anti-integrin $\alpha_v\beta_3$	Extent of cell spreading (not the area of adhesive contact) controls apoptosis of bovine capillary endothelial cells	Chen *et al.* (1997, 1999)
Silicon	Features of 1 μm and larger	Octadecyltrichlorosilane, *N*-1-[3-(trimethoxysilyl) propyl]diethylenetriamine, polylysine	—	LRM 55 cells (astroglial cell line) selectively attached to DETA-patterned surfaces in presence of serum-containing media; rat hippocampal neurons attach to poly(lysine)-patterned regions	Craighead *et al.* (1998)
Gold and silicon	Circles and squares of 5 to 80 μm	Hexadecane thiol, tri(ethylene glycol)-terminated alkane thiol	Fibronectin	Cell cycle progression of BCE cells were controlled by cell shape and cytoskeletal tension	Huang *et al.* (1998)
Gold	10-μm lines	Hexadecane thiol, hexa(ethylene glycol)-terminated alkane thiol	Vitronectin	Chemotactic response of MTLn3 metastatic rat mammary adenocarcinoma cells; lamellipod extension is independent of contact with the substratum	Bailly *et al.* (1998)
Gold	Squares and lines of 20 μm and larger	Peptides containing cell adhesion motifs and cysteine, hexa(ethylene glycol)-terminated alkane thiol	—	Human epidermoid carcinoma A431 cells, primary human embryonic kidney 293 cells, bovine aorta endothelial cells, and NIH 3T3 fibroblasts	Zhang *et al.* (1999)
Gold	10- or 30-μm-width lines and 5- or 10-μm squares	Hexadecane thiol, tri(ethylene glycol)-terminated alkane thiol	Fibronectin	BCE cells can be switched between growth, apoptosis, and differentiation by altering the geometry of spreading	Dike *et al.* (1999)
Glass that presents *N*-hydroxysuccinimide esters or aldehydes	10-μm lines	Poly(lysine), bovine serum albumin laminin	BSA, laminin	On a patterned surface, neural somata and dendrites preferred poly(lysine) surfaces whereas axons preferred surfaces presenting laminin/poly(lysine) mixtures	Branch *et al.* (1998) Wheeler *et al.* (1999)

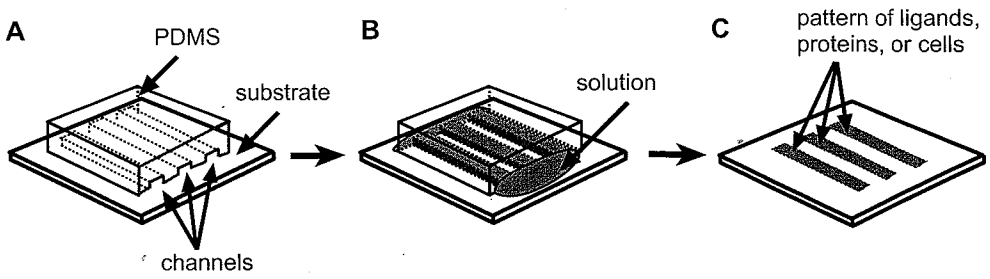

Fig. 18.4. Patterning substrates using microfluidic channels. (A) A poly(dimethylsiloxane) (PDMS) mold is brought into conformal contact with the surface of a substrate to form microfluidic channels. (B) The channels are filled with a solution containing patterning components. (C) The channels are washed clean of the patterning solution and the PDMS mold is removed. This sequence of steps results in generation of patterns of adsorbed proteins on the substrate.

ricated in PDMS. The avidin moieties bound to the biotin groups on the surface, and served as a bridge between the biotinylated polymer and biotinylated ligands. Patterns created with biotinylated ligands containing the RGD or IKVAV oligopeptide sequences determined the adhesion and spreading of bovine aortic endothelial cells and PC12 nerve cells. Folch and Toner (1998) and Folch *et al.* (1999) used μFP to produce patterns of cells on biocompatible substrates. Micropatterns of collagen or fibronectin deposited from fluids in capillaries were used to cause cells to adhere selectively to various biomedical polymers, and to heterogeneous or microtextured substrates. On removing the elastomeric stamp, the bare areas of the substrate could be seeded with more adhesive cell types such as fibroblasts. This procedure produced micropatterned cocultures. By allowing different cell suspensions to flow through different channels, patterns composed of two cell types were also generated.

The mild conditions used with μFP permit the patterning not only of small molecules and proteins, but also of more delicate components such as cells. By filling individual channels with different fluids, multiple components can be patterned at the same time without the need for multiple steps or the accompanying technical concerns of registration.

LAMINAR FLOW PATTERNING

Laminar flow patterning (LFP) is a technique that can pattern surfaces, and the positions of cells on them, in useful ways. It can also pattern fluids (Takayama *et al.*, 1999) themselves. This technique utilizes a phenomenon that occurs in microfluidic systems as a result of their small dimensions—that is, low Reynolds number flow. The Reynolds number (Re) is a nondimensional parameter describing the ratio of inertial to viscous forces in a specific flow configuration. It is a measure of the tendency of a flowing fluid to develop turbulence. The flow of liquids in capillaries often has a low Re and is laminar. Laminar flow allows two or more layers of fluid to flow next to each other without any mixing other than by diffusion of their constituent molecular and particulate components.

Figure 18.5 shows a typical setup for LFP experiments, along with images from some representative work. A network of capillaries is made by bringing a patterned PDMS slab into conformal contact with a petri dish. By flowing different patterning components from the inlets, patterns of parallel stripes are created in the main channel (Fig. 18.5A,B). The positions and environments of cells can be controlled simultaneously in several stripes in the same channel (Fig. 18.5C). Figure 18.5D shows patterning of the substrate with different proteins; Fig. 18.5E shows patterning cell position by deposition from laminar fluid flows; Fig. 18.5F illustrates the use of patterned culture media to deliver chemicals selectively to cells. In this experiment, bovine capillary endothelial cells covered the entire bottom face of the capillary; the fluorescence micrograph visualizes only those cells over which medium containing a fluorescent dye was allowed to flow. Figure 18.5G is another example of patterning the culture media. In this experiment, digestion of fibronectin on the channel surface and sequestering of calcium by ethylenediaminetetraacetic acid (EDTA) cause cells to detach and contract. When a solution of trypsin/EDTA was allowed to flow over only a portion of a cell, the treated part of the cell detached and contracted; the untreated

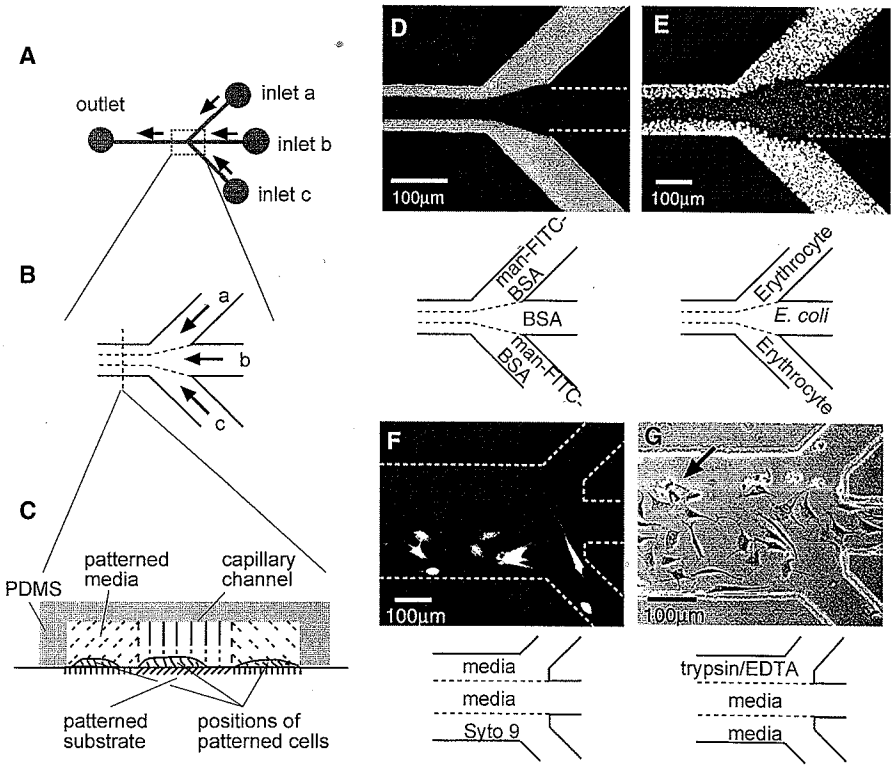

Fig. 18.5 Examples of laminar flow patterning experiments. (A) Top view of the capillary network. A poly(dimethylsiloxane) (PDMS) membrane containing micron-sized channels molded in its surface was placed on the flat surface of a petri dish to form a network of capillaries. (B) A close-up view of the junctions where the inlets converge into a single main channel. Micrographs were obtained for this area of the capillary system. (C) Cross-sectional view of the main channel, looking from the outlet toward the inlet. The patterning of the substrate, the cell positions, and the fluid environment can be controlled simultaneously in the same capillary. (D) Patterning substrate with bovine serum albumin (BSA) and BSA colabeled with mannose and fluorescein (man–FITC–BSA). (E) Patterned cell deposition. Chick erythrocytes and Escherichia coli were deposited selectively in their designated lanes by patterned flow of cell suspensions. Adherent cells were visualized with a fluorescent nucleic acid stain (Syto 9). (F) Using patterned media to selectively stain BCE cells. Syto 9 and media were allowed to flow from the designated inlets. (G) Using patterned media to detach bovine capillary endothelial cells selectively. Trypsin/EDTA and media were allowed to flow from the designated inlets. (D–F) Fluorescence micrographs taken from the top through PDMS. (G) Phase contrast image observed by an inverted microscope looking through the polystyrene petri dish. White dotted lines identify channels not visible with fluorescence microscopy.

part remained spread (for example, see arrow in Fig. 18.5G). Because no physical barriers are required to separate the different liquid streams, different liquids can flow over different portions of a single cell.

LFP has some features that make it complementary to other patterning techniques used for biological applications. It takes advantage of the easily generated multiphase laminar flows to pattern fluids and to deliver components for patterning. The ability to pattern the growth medium is a special feature that cannot be achieved by other processes. This method can pattern over delicate structures, such as a portion of a mammalian cell. This type of patterning is difficult by other techniques. LFP can also give simultaneous control over the surface patterns, cell positioning, and the fluid environment in the same channel.

CONCLUSION AND FUTURE PROSPECTS

Soft lithography brings to microfabrication low-cost, simple procedures, rapid prototyping of custom-designed devices, three-dimensional capability, and biocompatibility. These techniques allow patterning of cells and their environments with great convenience and flexibility at dimensions down to micrometers. We have described three complementary soft lithographic tech-

niques—microcontact printing, patterning using fluids in microfluidic channels, and laminar flow patterning—that are useful in their ability to pattern the cell culture environment.

Microcontact printing is perhaps the simplest method for patterning surfaces and also provides the highest resolution with the greatest flexibility in the shape of the patterns generated. It is most useful when one needs to pattern two types of ligands or proteins, and when the "ink" is able to withstand drying and stamping. Microfluidic channels are well suited for patterning surfaces using delicate objects such as proteins and cells. They are also useful when multiple ligands, proteins, or cells need to be patterned. Laminar flow patterning is similar to patterning with individual microfluidic channels except that the individual flows are kept from mixing with each other by laminar flow, not by physical walls. The ability to pattern the fluid environment is the distinguishing feature of this method, and enables laminar flow to be used to pattern the distribution of different fluids over the surface of a single mammalian cell. This capability allows patterning of portions of a single cell, and remodeling of the cell culture environment, both in the presence of living cells.

The potential applications of soft lithography are just starting to be explored. There are many cell culture environments that we have not discussed in this chapter, but that can potentially be patterned utilizing soft lithographic methods. For example, topographical features created in PDMS affect cell spreading (Flemming *et al.,* 1999). Silicone polymers can sense and affect the mechanical tension within cells (Burton and Taylor, 1997; Sai *et al.,* 1999). With the availability of various three-dimensional fabrication techniques, much more work is expected in three-dimensional patterning of the cell culture substrate (Breen *et al.,* 1999; Jackman *et al.,* 1998; Terfort *et al.,* 1997). The fourth dimension, time, is also an interesting factor. Experiments such as trypsin-mediated remodeling of the exposed surfaces of cells and supports presenting adsorbed proteins using laminar flow demonstrate that real-time temporal changes in the cell culture environment are possible (Takayama *et al.,* 1999). The optical transparency of PDMS makes it possible to pattern the intensity of light in cell cultures (Paul *et al.,* 1999; Xia and Whitesides, 1998). The ability of soft lithography to pattern magnetic materials, and the nonmagnetic character of PDMS, makes it an attractive method for patterning of magnetic fields (Palacin *et al.,* 1996). The gas permeability of PDMS may be useful in patterning the gas of the surrounding cells. PDMS is electrically insulating, and the ability to mold or fabricate electrically conducting wires in it should allow patterning of electric fields (Kenis *et al.,* 1999). Gravitational fields can also be affected: microfluidic culture chambers with adherent cells can be turned upside down without loss of the culture media. Temperature, fluid shear, and other factors may also be accurately patterned.

The potential of a cell is predetermined by its genetics. Realization of that potential depends, inter alia, on whether the cell is exposed to the appropriate environment. Soft lithography provides tools for patterning cells and their environments with a high degree of control. This capability aids efforts to understand fundamental cell biology and advances our ability to engineer cells and tissues. The ease with which electronic components or other nonbiological components can be fabricated with soft lithography also paves the way for the engineering of cells and tissue for use in biosensors and other hybrid systems that combine living and nonliving components.

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References

Andrade, J. D., Nagaoka, S., Cooper, S., Okano, T., and Kim, S. W. (1987). Surfaces and blood compatibility. *Trans. Am. Soc. Artif. Intern. Organs* **33**, 75–84.

Bailly, M., Yan, L., Whitesides, G. M., Condeelis, J. S., and Segall, J. E. (1998). Regulation of protrusion shape and adhesion to the substratum during chemotactic responses of mammalian carcinoma cells. *Exp. Cell Res.* **241**, 285–299.

Bain, C. D., and Whitesides, G. M. (1988a). Formation of two-component surfaces by the spontaneous assembly of monolayers on gold from solutions containing mixtures of organic thiols. *J. Am. Chem. Soc.* **110**, 6560–6561.

Bain, C. D., and Whitesides, G. M. (1988b). Molecular-level control over surface order in self-assembled monolayer films of thiols on gold. *Science* **240**, 62–63.

Bain, C. D., and Whitesides, G. M. (1989). Formation of monolayers by the coadsorption of thiols on gold: Variation in the length of the alkyl chain. *J. Am. Chem. Soc.* **111**, 7164–7175.

Bain, C. D., Evall, J., and Whitesides, G. M. (1989). Formation of monolayers by the coadsorption of thiols on gold: Variation in the head group, tail group, and solvent. *J. Am. Chem. Soc.* **111**, 7155–7164.

Bernard, A., Delamarche, E., Schmid, H., Michel, B., Bosshard, H. R., and Biebuyck, H. (1998). Printing patterns of proteins. *Langmuir* **14**, 2225–2229.

Bishop, A. R., and Nuzzo, R. G. (1996). Self-assembled monolayers: Recent developments and applications. *Curr. Opin. Colloid Interface Sci.* **1**, 127–136.

Branch, D. W., Corey, J. M., Weyhenmeyer, J. A., Brewer, G. J., and Wheeler, B. C. (1998). Micro-stamp patterns of biomolecules for high-resolution neuronal networks. *Med. Biol. Eng. Comput.* **36**, 135–141.

Breen, T. L., Tien, J., Oliver, S. R. J., Hadzic, T., and Whitesides, G. M. (1999). Design and self-assembly of open, 3D lattice mesostructures. *Science* **284**, 948–951.

Burton, K., and Taylor, D. L. (1997). Traction forces of cytokinesis measured with optically modified elastic substrata. *Nature* **385**, 450–454.

Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1997). Geometric control of cell life and death. *Science* **276**, 1425–1428.

Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., and Ingber, D. E. (1999). Micropatterned surfaces for control of cell shape, position, and function. *Biotechnol. Prog.* **14**, 356–363.

Craighead, H. G., Turner, S. W., Davis, R. C., James, C., Perez, A. M., St. John, P. M., Isaacson, M. S., Kam, L., Shain, W., Turner, J. N., and Banker, G. (1998). Chemical and topographical surface modification for control of central nervous system cell adhesion. *J. Biomed. Microdev.* **1**, 49–64.

Delamarche, E., and Michel, B. (1996). Structure and stability of self-assembled monolayers. *Thin Solid Films* **273**, 54–60.

Delamarche, E., Bernard, A., Schmid, H., Michel, B., and Biebuyck, H. (1997). Patterned delivery of immunoglobulins to surfaces using microfluidic networks. *Science* **276**, 779–781.

Delamarche, E., Bernard, A., Schmid, H., Bietsch, A., Michel, B., and Biebuyck, H. (1998). Microfluidic networks for chemical patterning of substrates: Design and application to bioassays. *J. Am. Chem. Soc.* **120**, 500–508.

Deng, L., Mrksich, M., and Whitesides, G. M. (1996). Self-assembled monolayers of alkanethiolates presenting tri(propylenesulfoxide) groups resist the adsorption of protein. *J. Am. Chem. Soc.* **118**, 5136–5137.

Deng, T., Tien, J., Xu, B., and Whitesides, G. M. (1999). Using patterns in microfiche as photomasks in 10-μm scale microfabrication. *Langmuir* **15**, 6675–6581.

Dike, L. E., Chen, C. S., Mrksich, M., Tien, J., Whitesides, G. M., and Ingber, D. E. (1999). Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro Cell. Dev. Biol., Anim.* **35**, 441–448.

DiMilla, P., Folkers, J. P., Biebuyck, H. A., Harter, R., Lopez, G., and Whitesides, G. M. (1994). Wetting and protein adsorption of self-assembled monolayers of alkanethiolates supported on transparent films of gold. *J. Am. Chem. Soc.* **116**, 2225–2226.

Dubois, L. H., and Nuzzo, R. G. (1992). Synthesis, structure, and properties of model organic surfaces. *Annu. Rev. Phys. Chem.* **43**, 437–463.

Duffy, D. C., McDonald, J. C., Schueller, O. J. A., and Whitesides, G. M. (1998). Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). *Anal. Chem.* **70**, 4974–4984.

Flemming, R. G., Murphy, C. J., Abrams, G. A., Goodman, S. L., and Nealey, P. F. (1999). Effects of synthetic micro- and nano-structured surfaces on cell behavior. *Biomaterials* **20**, 573–588.

Folch, A., and Toner, M. (1998). Cellular micropatterns on biocompatible materials. *Biotechnol. Prog.* **14**, 388–392.

Folch, A., Ayon, A., Hurtado, O., Schmidt, M. A., and Toner, M. (1999). Molding of deep polydimethylsiloxane microstructures for microfluidics and biological applications. *J. Biomech. Eng.* **121**, 28–34.

Grzybowski, B. A., Haag, R., Bowden, N., and Whitesides, G. M. (1998). Generation of micrometer-sized patterns for microanalytical applications using a laser direct-write method and microcontact printing. *Anal. Chem.* **70**, 4645–4652.

Hammarback, J. A., Palm, S. L., Furcht, L. T., and Letourneau, P. C. (1985). Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. *J. Neurosci. Res.* **13**, 213–220.

Helmus, M. N., and Hubbell, J. A. (1993). Materials selection. *Cardiovasc. Pathol.* **2**, 53S–71S.

Huang, J. Y., and Hemminger, J. C. (1993). Photooxidation of thiols in self-assembled monolayers on gold. *J. Am. Chem. Soc.* **115**, 3342–3343.

Huang, S., Chen, C. S., and Ingber, D. E. (1998). Control of cyclin D1, p27Kip1, and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol. Biol. Cell* **9**, 3179–3193.

Jackman, R. J., Wilbur, J., and Whitesides, G. M. (1995). Fabrication of submicron features on curved substrates by microcontact printing. *Science* **269**, 664–666.

Jackman, R. J., Brittain, S. T., Adams, A., Prentiss, M. G., and Whitesides, G. M. (1998). Design and fabrication of topologically complex, three-dimensional microstructures. *Science* **280**, 2089–2091.

Kenis, P. J. A., Ismagilov, R. F., and Whitesides, G. M. (1999). Microfabrication inside capillaries using multiphase laminar flow patterning. *Science* **285**, 83–85.

Kleinfeld, D., Kahler, K. H., and Hockberger, P. E. (1988). Controlled outgrowth of dissociated neurons on patterned substrates. *J. Neurosci.* **8**, 4098–4120.

Lahiri, J., Isaacs, L., Tien, J., and Whitesides, G. M. (1999a). A strategy for the generation of surfaces presenting ligands for studies of binding based on an active ester as a common reactive intermediate: A surface plasmon resonance study. *Anal. Chem.* **71**, 777–790.

Lahiri, J., Ostuni, E., and Whitesides, G. M. (1999b). Patterning ligands on reactive SAMs by microcontact printing. *Langmuir* **15,** 2055–2060.

López, G. P., Albers, M. W., Schreiber, S. L., Carroll, R. W., Peralta, E., and Whitesides, G. M. (1993a). Convenient methods for patterning the adhesion of mammalian cells to surfaces using self-assembled monolayers of alkanethiolates on gold. *J. Am. Chem. Soc.* **115,** 5877–5878.

López, G. P., Biebuyck, H. A., Härter, R., Kumar, A., and Whitesides, G. M. (1993b). Fabrication and imaging of two-dimensional patterns of proteins adsorbed on self-assembled monolayers by scanning electron microscopy. *J. Am. Chem. Soc.* **115,** 10774–10781.

Merritt, M. V., Mrksich, M., and Whitesides, G. M. (1997). Using self-assembled monolayers to study the interactions of man-made materials with proteins. *In* "Principles of Tissue Engineering" (R. P. Lanza, W. L. Chick, and R. Langer, eds.), pp. 211–223. R. G. Landes, Austin, TX.

Mrksich, M., and Whitesides, G. M. (1995). Patterning self-assembled monolayers using microcontact printing: A new technology for biosensors? *Trends Biotechnol.* **13,** 228–235.

Mrksich, M., Grunwell, J. R., and Whitesides, G. M. (1995). Bio-specific adsorption of carbonic anhydrase to self-assembled monolayers of alkanethiolates that present benzenesulfonamide groups on gold. *J. Am. Chem. Soc.* **117,** 12009–12010.

Mrksich, M., Chen, C. S., Xia, Y., Dike, L. E., Ingber, D. e., and Whitesides, G. M. (1996). Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 10775–10778.

Ostuni, E., Yan, L., and Whitesides, G. M. (1999). The interaction of proteins and cells with self-assembled monolayers of alkanethiolates on gold and silver. *Colloids Surf. B: Biointerfaces* **15,** 3–30.

Palacin, S., Hidber, P. C., Bourgoin, J.-P., Miramond, C., Fermon, C., and Whitesides, G. M. (1996). Patterning with magnetic materials at the micron scale. *Chem. Mater.* **8,** 1316–1325.

Park, A., Wu, B., and Griffith, L. (1998). Integration of surface modification and 3D fabrication techniques to prepare patterned poly(⅗-lactide) substrates allowing regionally selective cell adhesion. *J. Biomater. Sci., Polym. Ed.* **9,** 89–110.

Patel, N., Padera, R., Sanders, G. H. W., Cannizzaro, S. M., Davies, M. C., Langer, R., Roberts, C. J., Tendler, S. J. B., Williams, P. M., and Shakesheff, K. M. (1998). Spatially controlled cell engineering on biodegradable polymer surfaces. *FASEB J.* **12,** 1447–1454.

Paul, K. E., Breen, T. L., Aizenberg, J., and Whitesides, G. M. (1999). Maskless lithography: Embossed photoresist as its own optical element. *Appl. Phys. Lett.* **73,** 2893–2895.

Prime, K. L., and Whitesides, G. M. (1991). Self-assembled organic monolayers: Model systems for studying adsorption of proteins at surfaces. *Science* **252,** 1164–1167.

Prime, K. L., and Whitesides, G. M. (1993). Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): A model system using self-assembled monolayers. *J. Am. Chem. Soc.* **115,** 10714–10721.

Qin, D., Xia, Y., and Whitesides, G. M. (1996). Rapid prototyping of complex structures with feature sizes larger than 20 μm. *Adv. Mater.* **8,** 917–919.

Ravencroft, M. S., Bateman, K. E., Shaffer, K. M., Schessler, H. M., Jung, D. R., Schneider, T. W., Montgomery, C. B., Custer, T. L., Schaffner, A. E., Liu, Q. Y., Li, Y. X., Barker, J. L., and Hickman, J. J. (1998). Developmental neurobiology implications from fabrication and analysis of hippocampal neuronal networks on patterned silane-modified surfaces. *J. Am. Chem. Soc.* **120,** 12169–12177.

Sai, X., Naruse, K., and Sokabe, M. (1999). Activation of pp60src is critical for stretch-induced orienting response in fibroblasts. *J. Cell Sci.* **112,** 1365–1373.

Sigal, G. B., Bamdad, C., Barberis, A., Strominger, J., and Whitesides, G. M. (1996). A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance. *Anal. Chem.* **68,** 490–497.

Singhvi, R., Kumar, A., Lopez, G. P., Stephanopolous, G. N., Wang, D. I. C., Whitesides, G. M., and Ingber, D. E. (1994). Engineering cell shape and function. *Science* **264,** 696–698.

St. John, P. M., Davis, R., Cady, N., Czajka, J., Batt, C. A., and Craighead, H. G. (1998). Diffraction-based cell detection using a microcontact printed antibody grating. *Anal. Chem.* **70,** 1108–1111.

Takayama, S., McDonald, J. C., Ostuni, E., Liang, M. N., Kenis, P. J. A., Ismagilov, R. F., and Whitesides, G. M. (1999). Patterning cells and their environments using multiple laminar fluid flows in capillary networks. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 5545–5548.

Terfort, A., Bowden, N., and Whitesides, G. M. (1997). Three-dimensional self-assembly of millimetre-scale components. *Nature (London)* **386,** 162–164.

Ulman, A. (1996). Formation and structure of self-assembled monolayers. *Chem. Rev.* **96,** 1533–1554.

Vaidya, R., Tendler, L. M., Bradley, G., O'Brien, M. J., Cone, M., and Lopez, G. P. (1998). Computer-controlled laser ablation: A convenient and versatile tool for micropatterning bifunctional synthetic surfaces for applications in biosensing and tissue engineering. *Biotechnol. Prog.* **14,** 371–377.

Wheeler, B. C., Corey, J. M., Brewer, G. J., and Branch, D. W. (1999). Microcontact printing for precise control of nerve cell growth in culture. *J. Biomech. Eng.* **121,** 73–78.

Xia, Y., and Whitesides, G. M. (1998). Soft lithography. *Angew. Chem., Int. Ed. Engl.* **37,** 550–575.

Yan, L., Marzolin, C., Terfort, A., and Whitesides, G. M. (1997). Formation and reaction of interchain carboxylic anhydride groups on self-assembled monolayers on gold. *Langmuir* **13,** 6704–6712.

Yan, L., Zhao, X.-M., and Whitesides, G. M. (1998). Patterning a preformed, reactive SAM using microcontact printing. *J. Am. Chem. Soc.* **120,** 6179–6180.

Zhang, S., Yan, L., Altman, M., Lässle, M., Nugent, H., Frankel, F., Lauffenburger, D. A., Whitesides, G. M., and Rich, A. (1999). Biological surface engineering: A simple system for cell pattern formation. *Biomaterials* **20,** 1213–1220.