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Simple Approach to Micropattern Cells on Common Culture Substrates by Tuning Substrate Wettability

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ABSTRACT

The ability to spatially control cell adhesion and multicellular organization is critical to many biomedical and tissue-engineering applications. This work describes a straightforward method to micropattern cells onto glass, silicone rubber, and polystyrene using commercially available reagents. An elastomeric polydimethylsiloxane stamp is used to contact-transfer extracellular matrix protein onto a surface followed by blocking cell adhesion in the surrounding regions by the physisorption of Pluronic surfactants. Using self-assembled monolayers of alkanethiols on gold as model surfaces to control surface wettability, we found that protein printing was most effective at intermediate to highly wetting surfaces whereas Pluronic adsorption occurred at intermediate to low wetting surfaces. Within a regimen of intermediate wettability both techniques were applied in conjunction to restrict cell adhesion to specified patterns. Adjusting the wettability of common tissue culture substrates to the same intermediate range again allowed the micropatterning of cells, suggesting that this approach is likely to be generally applicable to many types of materials. This technique therefore may allow for wider adoption of cell patterning.

INTRODUCTION

THIS ARTICLE describes a simple and general method to micropattern cells on commonly used tissue culture substrates. Although it is known that the spatial organization of cells and tissues is critical to their function, only more recently have the experimental tools existed to manipulate and study the role of spatial organization in cell behavior.[1,2] These tools have been applied to arrange cells within tissue constructs for maintaining *in vivo* function,[3] and to designate cell localization in cell-based biosensors and transducers.[4] Furthermore, these approaches have also been used to advance cell and tissue-engineering research by delineating the role of cell adhesion, cell shape, and intercellular communication in regulating proliferation, differentiation, contractility, and morphogenesis of cells and tissues.[5–11] However, despite the power of spatial patterning approaches, they have not been widely adopted by the biological and tissue-engineering communities, largely because of the lack of available reagents and specialty surfaces.

Surfaces micropatterned with cellular adhesiveness were first generated by evaporating metal (adhesive to cells) through a stencil mask onto a nonadhesive film.[12] Subsequently, the use of photolithography simplified the technique. The general principle involves projecting patterns of ultraviolet (UV) radiation onto a photosensitive material in order to spatially change its adhesiveness to cells or to expose regions that can be further modified. Various forms of this approach have been developed[7,13–16]; however, in each case it is necessary to repeat the photolithographic step to generate each patterned substrate. As an alternative, Whitesides *et al*. developed the microcontact printing ((CP) technique to pattern cells,[9,11] in which an elastomeric stamp made of polydimethylsiloxane (PDMS) is used to contact-transfer patterns of protein-adhesive alkanethiols directly onto gold-coated surfaces; the remaining regions are coated with

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ethylene glycol-terminated alkanethiols that resist protein adsorption and cell adhesion. Subsequent studies have printed cell-adhesive molecules onto reactive polymer coatings or vice versa.[17,18] The stamps are reusable and many can be cast repeatedly from a single silicon master; thus, once the master containing the desired pattern is generated, patterned substrates can be generated without the specialized equipment and cleanroom environment needed for photolithography. However, ethylene glycol-functionalized alkanethiols and polymer coatings are not commercially available, and high-quality gold-coated surfaces are both expensive and incompatible with some applications.[19]

An alternative approach uses a PDMS membrane containing holes or a stamp containing channels to physically mask a region on a substrate. A solution is flowed into the holes or channels to functionalize the exposed region.[20–24] This approach has the advantage of being compatible with many types of surfaces. However, because the membranes must be sufficiently thick to be handled, or the channels sufficiently high to allow reasonable flow rates, the practical spatial resolution of the patterns appears to be ~5 μm—one order of magnitude larger than is possible by μCP.[25,26] Furthermore, these approaches restrict the complexity, types, and size of the patterns that can be generated; patterns containing thin lines, rough edges, sharp bends, or dead spaces are difficult to create.

Here, we investigated a method that is simple, uses commercially available reagents, is generally applicable to substrates including common tissue culture surfaces, and has submicrometer resolution with no geometric constraints on the pattern. To create regions adhesive to cells, we used μCP to directly print protein onto substrates. This process involves first adsorbing protein onto an elastomeric PDMS stamp containing the pattern of choice and subsequently printing the protein onto the substrate.[27–29] To create nonadhesive regions, we explored the physisorption of Pluronic surfactants, a series of nontoxic triblock polymers (polyethylene glycol–polypropylene glycol–polyethylene glycol) that have been used for several *in vitro* and *in vivo* applications.[30] The hydrophobic polypropylene segment stabilizes Pluronic onto a surface via hydrophobic interactions, while the two polyethylene segments extends into the bulk aqueous medium—protecting the surface from protein adsorption and cell adhesion.[31,32] However, because Pluronic has been successfully adsorbed only to hydrophobic surfaces,[33] it may not be compatible with μCP—a process that requires hydrophilic surfaces.[34] On a series of model surfaces, we verified that indeed the wettability of the surface is a dominant parameter controlling the successful application of both techniques. Importantly, we demonstrate the existence of an overlap in the range of wettability where both techniques are successful. By tuning the wettability of a surface, μCP and adsorption of Pluronic were applied in conjunction to micropattern cells. We demonstrated the general applicability of this approach by micropatterning cells onto surfaces commonly used in tissue culture—glass, silicone rubber, and polystyrene.

MATERIALS AND METHODS

Preparation of the stamps

Multiple stamps were made by replica casting polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning, Midland, MI) against a silicon master made by photolithography as previously described.[35] Briefly, a 2-μm layer of 1813 photoresist (Shipley, Marlborough, MA) was spun onto a 3-in.-diameter silicon wafer (Silicon Sense, Nashua, NH). The wafer was baked at 100°C for 5 min, exposed to UV radiation through a chrome mask (Advance Reproductions, Andover, MA), and developed (351 Developer; Shipley). The silicon master was silanized with fluorosilane [(tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-tricholorosilane; United Chemical Technologies, Bristol, PA) overnight to aid subsequent release of the PDMS by placing the master and a glass slide containing a drop of fluorosilane in a vacuum chamber and evacuated overnight (house vacuum, ~100 torr). Prepolymer of PDMS was poured over the silicon master and cured at 110°C for 15 min. The elastomeric stamp bearing the negative pattern of the master was peeled off, oxidized in an air plasma for 1 min (~200 mtorr) (Plasma Prep II; SPI Supplies, West Chester, PA), and silanized with fluorosilane overnight. The plasma treatment allowed for a more complete silane coupling; however, this step is not necessary for printing on some surfaces.[34] Glass, and UV ozone-treated PDMS and polystyrene, can each be printed on by stamps silanized without prior exposure to plasma treatment. Care was taken after silane treatment to avoid gross distortion of the stamp, which causes microscopic cracks to form on the surface of the stamp. After printing, some stamps were sonicated in ethanol for 5 min, blown dry, and reused.

Preparation of SAMs

Mixed self-assembled monolayers (SAMs) were prepared as previously described.[34] Briefly, silicon wafers (<100>; Silicon Sense) evaporated with gold were immersed in ethanolic 2 mM solutions of the indicated fractions of CH_3-terminated alkanethiol (dodecanethiol; Aldrich, Milwaukee, WI) and OH-terminated alkanethiol (11-mercapto-1-undecanol; Aldrich), or CH_3-terminated alkanethiol and COOH-terminated alkanethiol (11-mercaptoundecanoic acid; Aldrich), for >20 h at 4°C. The substrates were removed from the alkanethiol solution, rinsed with ethanol, and blown dry just before use.

Preparation of common tissue culture substrates

Glass (glass coverslips; Fisher Scientific, Pittsburgh, PA) was cleaned with a solution of 15% H_2O_2 and 15% NH_4OH

in water at 60°C for 30 min. To prepared silanized glass, glass was immersed in 5% dimethyl dichlorosilane (Aldrich) in dichlorobenzene for ~10 s, rinsed with acetone and then water, blown dry, and just before use oxidized by UV-generated ozone (UVO Cleaner; Jelight, Irvine, CA) for 1 min. Polystyrene (Falcon 35-mm bacteriological petri dish; BD Biosciences, Franklin Lakes, NJ) was used as received. PDMS was cured at 60°C overnight in plastic dishes. Oxidized PDMS was prepared by exposing cured PDMS to UV-generated ozone for 7 min and printed within 30 min of UV exposure.

μCP of protein and adsorption of Pluronic

To allow adsorption of proteins, we immersed the stamps for 1 h in an aqueous solution of fluorescently labeled protein (goat-derived antibody [IgG], 10 μg/mL, conjugated to Alexa Fluor 594 [Molecular Probes, Eugene, OR], or fibronectin [50 μg/mL; BD Biosciences]). The stamps were rinsed thoroughly with deionized water, blown dry under nitrogen, and placed in conformal contact with the indicated substrate for >5 s before being peeled off. Subsequently, the substrate was immersed in 0.2% (w/v) Pluronic (fluorescein-labeled F108 [gift from Allvivo, Lake Forest, CA] or F127 [gift from BASF, Mount Olive, NJ]) in phosphate-buffered saline (PBS) for 1 h, and carefully rinsed with water without allowing the surface to dry.

Measurement of contact angles

Contact angles of water under ambient room temperature and humidity were determined with a goniometer (model 100-00; Ramé-Hart, Mountain Lakes, NJ).

Microscopy and image analysis

Images of labeled IgG and Pluronic were acquired with a fluorescence microscope (Nikon, Melville, NY) fitted with a SPOT RT digital camera system (Diagnostic Instruments, Sterling Heights, MI). The fluorescence emission was partially quenched by the gold substrate, and thus longer exposure times were used. Cells adhered onto SAM substrates were fluorescently labeled (CellTracker Green; Molecular Probes) and visualized by fluorescence microscopy. Cells adhered onto common tissue culture substrates were visualized by phase-contrast microscopy.

Cell culture

Bovine pulmonary artery endothelial cells (BPAECs; VEC Technologies, Rensselaer, NY) were cultured in Dulbecco's modified Eagle's medium (DMEM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% calf serum (Invitrogen, Carlsbad, CA). Cells were suspended in trypsin–EDTA, seeded onto the indicated substrates, rinsed 1–2 h after seeding, grown in culture medium for 22–26 h, and fixed in 4% paraformaldehyde. To maintain the patterns of cells for extended periods, care was taken to avoid dewetting of the surface during exchange of the culture medium, which may cause desorption of Pluronic.

RESULTS

Effect of wettability on adsorption of Pluronic

We first characterized the effect of wettability on the adsorption of Pluronic. For this purpose, we examined the adsorption of Pluronic onto two-component mixed self-assembled monolayers (SAMs) that served as well-characterized model surfaces. SAMs were formed by the coadsorption of 2 mM solutions of CH_3-terminated alkanethiol and OH-terminated alkanethiol onto flat gold-coated surfaces. The density of hydroxyl functionalities on the surface determines its wettability and is controlled by the percentage of OH-terminated alkanethiol in the solution used to form the SAMs.[36,37] We refer to the SAMs according to the percentage of OH-terminated alkanethiol in the coating solution; for instance, a "60% OH SAM" refers to a two-component mixed SAM formed from a solution containing 1.2 mM OH-terminated alkanethiol and 0.8 mM CH3-terminated alkanethiol. The SAM surface is fairly homogeneous, as the functionalized alkanethiols distribute uniformly on the SAM surface without aggregating into domains of more than a few tens of angstroms across.[38] Advancing contact angles of the mixed SAMs used in this study were similar to values previously reported.[34,37]

We adsorbed fluorescently labeled Pluronic onto a series of mixed SAM substrates containing increasing densities of hydroxyl functionality, washed several times, and imaged the samples by fluorescence microscopy. The relative fluorescence intensity of each substrate was used to assess semiquantitatively the relative amount of adsorbed Pluronic.[39] Between 0 and 60% OH SAM, the fluorescence intensity was relatively high; despite variations in the intensity of fluorescence, a significant amount of Pluronic was consistently observed on each substrate within this range. Near the regime of 70% OH SAM, the fluorescence intensity started to decrease, and between 80 and 100% OH SAM, the fluorescence intensity was low and similar to background levels (Fig. 1). This trend was also observed for SAMs formed from mixtures of COOH-terminated and CH_3-terminated alkanethiols.

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Micropatterning protein and Pluronic on surfaces with different wettabilities

Whereas Pluronic adsorbed onto hydrophobic surfaces, we have previously shown μCP of protein is successful on hydrophilic surfaces.[34] To examine whether these two processes are mutually exclusive, we printed Alexa Fluor 594-labeled IgG proteins onto a series of mixed SAMs possessing a range of wettabilities, immersed the substrates in a solution of FITC-labeled Pluronic, and ob-

served the localization of protein and Pluronic by fluorescence microscopy. Labeled IgG was chosen for experimental convenience; we have found no differences between printing various proteins including IgG, albumin, streptavidin, fibronectin, and collagen. The surfaces of the stamps were fluorinated to allow printing onto the widest possible range of surfaces.[34]

On the hydrophobic 0% OH SAM, protein did not transfer during μCP, whereas Pluronic adsorbed onto the surface (Fig. 2). On 40% OH SAM, a surface of intermediate wettability, μCP of protein occurred and Pluronic adsorbed onto the surface (Fig. 2); the Pluronic adsorbed preferentially to regions not occupied by the protein, and formed a negative pattern of the printed protein. On the more hydrophilic 70% OH SAM, the pattern of protein transferred during μCP; the fluorescence intensity of Pluronic was higher than the background level but we did not observe a negative pattern (Fig. 2). On each substrate the fluorescence intensity of the printed protein was similar to that of an adsorbed monolayer, and was similar before and after immersion in Pluronic, suggesting it did not cause desorption of the printed protein.

Next, we examined whether the printed protein and the adsorbed Pluronic would, respectively, support and resist cell adhesion to form micropatterns of cells. On similarly prepared SAMs, we printed fibronectin, adsorbed Pluronic, and seeded endothelial cells in 5% serum-containing medium. After 1 day, cells were stained, fixed, and examined by fluorescence microscopy. Cells did not attach to 0% OH SAM (Fig. 2), a surface on which we had observed adsorption of Pluronic but no printing of protein. On 70% OH SAMs, cells initially attached to the regions of the printed protein, but began to migrate into surrounding regions within a few hours, and became randomly oriented by 24 h (Fig. 2). On 40% OH SAM, cells attached and spread exclusively on regions of printed protein and remained in those regions for at least 24 h (Fig. 2).

Micropatterning of cells on common tissue culture substrates

Having demonstrated its applicability on model surfaces of SAMs, we examined this approach on commonly used tissue culture substrates: silicone rubber, polystyrene, and glass. Silicone rubber, the most common type of which is PDMS, has been used to apply mechanical stress to cells and to simulate cyclic loading experienced by cells *in vivo*.[40] Polystyrene is commonly used in two forms. Native, untreated polystyrene is of intermediate wettability and referred to as bacteriological petri plastic, whereas plasma-treated polystyrene is more hydrophilic and often used in tissue culture. Last, glass is often used because of its high optical clarity. Because these surfaces range from hydrophobic (silicone rubber) to hydrophilic (glass) (Table 1) and are chemically distinct, they collectively provide a reasonable test of our approach.

On each surface, we printed Alexa 594-labeled IgG, adsorbed FITC-labeled Pluronic, and observed the transfer of each by fluorescence microscopy. Subsequently, onto the same type of surface, we printed fibronectin, adsorbed Pluronic, seeded cells, and examined the micropatterning of cells after 1 day. On PDMS, μCP resulted in no transfer of protein; however, Pluronic adsorbed onto the surface and subsequently cells did not attach to the surface (Fig. 3). On glass, the pattern of protein transferred during μCP, but there was little or no subsequent adsorption of Pluronic; on this surface, cells attached to the surface, but spread and migrated onto the surrounding regions to cause the pattern to disappear within a few hours (Fig. 3). On untreated polystyrene, both the μCP of protein and the adsorption of Pluronic occurred. The Pluronic adsorbed preferentially to regions not occupied by the pro-

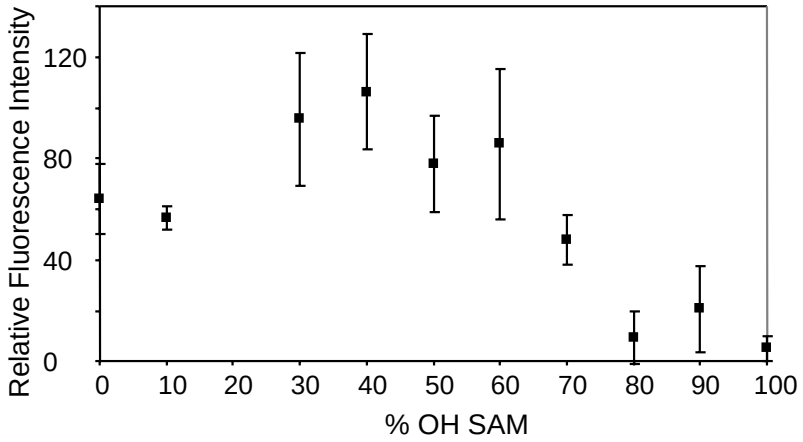

FIG. 1. Adsorption of Pluronic on SAMs. The fluorescence intensity was plotted against substrate composition. Shown are the averages and standard deviations over two experiments. In each experiment, 0 to 100% OH SAMs in 10% increments were made, adsorbed with Pluronic, rinsed, and imaged with equal exposure and illumination conditions. On each substrate, a 700 × 500 μm region was imaged and the pixel intensities were averaged.

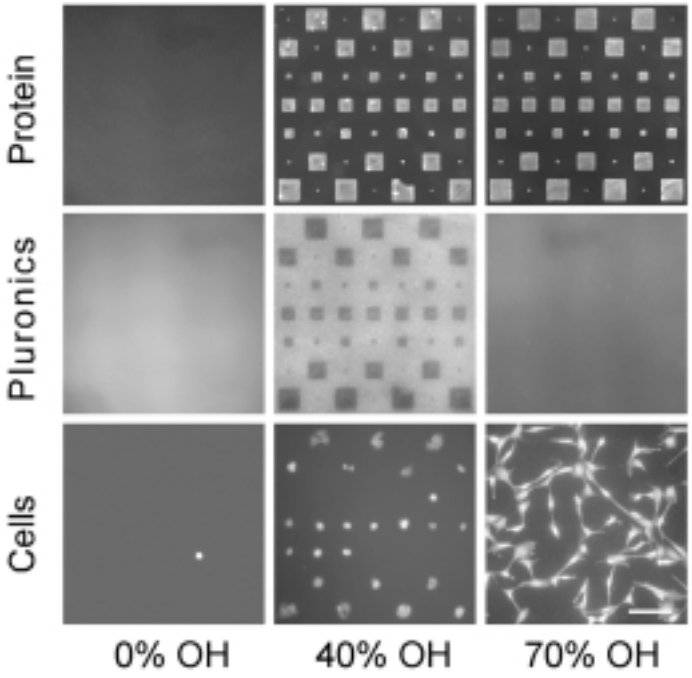

FIG. 2. Micropatterning of protein, Pluronic, and cells on mixed SAMs of different wettabilities. Shown are representative fluorescence micrographs of printed IgG (*top row*) and adsorbed Pluronic (*middle row*) over the same region of the indicated SAM surface, and representative fluorescence micrographs (*bottom row*) of endothelial cells cultured in 5% serum-containing medium on the indicated substrate for 24 h; the substrate was printed with fibronectin and adsorbed with Pluronic. Cells were fluorescently labeled just before being fixed and imaged. Scale bar: 100 μm.

tein, revealing a negative pattern of the printed protein. Cells seeded onto the surface adhered and remained confined to the patterned regions (Fig. 3).

The results on common tissue culture substrates were analogous to findings on the model SAM surfaces. Consistent with results on hydrophobic SAM surfaces, μCP did not occur on the hydrophobic PDMS surface (Table 1), but Pluronic was able to adsorb and block cell adhesion. Conversely, μCP occurred on the hydrophilic glass surface (Table 1); however, without Pluronic in the surrounding regions, adherent cells were able to migrate outside the pattern. On polystyrene, a surface of intermediate wettability (Table 1), both μCP and the adsorption of Pluronic occurred to spatially control the adhesion of cells.

To test whether this approach could be used effectively for PDMS and glass, we moderated the wettability of both surfaces. The wettability of PDMS was increased by oxidizing the surface with UV-generated ozone (Table 1). The

TABLE 1. WETTABILITY OF SURFACES: ADVANCING, SESSILE, AND RECEDING CONTACT ANGLES OF WATER ON VARIOUS SURFACES[a]

Surface	*Advancing (°)*	*Sessile (°)*	*Receding (°)*
0% OH SAM	115	112	101
40% OH SAM	98	90	89
70% OH SAM	63	58	51
PDMS	116	107	96
PDMS, oxidized	118	101	54
Glass	7	4	3
Glass, silanized	101	94	86
Polystyrene	88	80	72

[a]Each value represents the average of four measurements on two independently prepared substrates. Standard deviation $<6°$.

wettability of glass was decreased by exposing the surface to an aliphatic silane (Table 1). On each of the modified surfaces, both μCP and adsorption of Pluronic occurred, and cells were restricted spatially to the micropattern (Fig. 3).

DISCUSSION

We used well-defined model SAM surfaces to systematically vary wettability while keeping other surface parameters such as roughness, orientation, and homogeneity constant. Our results indicate a low degree of wettability is required for the adsorption of Pluronic. Previous studies suggest Pluronic is stabilized on a surface via hydrophobic interactions of its hydrophobic polypropylene oxide segment with the surface,[33] and agree with our observation of the lack of adsorption of Pluronic on hydrophilic surfaces. Increasing the length of the polypropylene oxide segment of Pluronic increases its adsorption and retention on surfaces.[41] In our studies, the use of Pluronic F127 was superior to the shorter, less hydrophobic F108.

We printed protein on SAM surfaces capable of adsorbing Pluronic to examine whether these two processes are mutually exclusive. Our results on model SAM surfaces indicate that the wettability of the surface controls both the microcontact printing of protein and the adsorption of Pluronic. Within a regime of intermediate wettability, both techniques were successfully applied and cells were micropatterned. On such surfaces, the printed protein renders spatially specified regions of the surface more hydrophilic; as a result, Pluronic no longer readily adsorbs onto those regions, but instead occupies the surrounding regions. Thus, the combined use of both techniques allows proteins and Pluronic to spatially separate into regions that are, respectively, adhesive and nonadhesive to cells.

By tuning wettability, we were able to pattern cells on each of the commonly used tissue culture substrates—glass, PDMS, and polystyrene. Results on model and commonly used substrates both indicate that surfaces of inter-mediate wettability can be micropatterned. The wettability, as measured by water contact angles, is dependent on multiple parameters including the chemistry, orientation, roughness, and hardness of the surface.[42] Although it is not yet clear how each of these individual parameters affects μCP of protein and the adsorption of Pluronic, our study provides a guideline for predicting the types of surfaces that can be patterned. Furthermore, it suggests a rational approach for how the wettability of surfaces should be modified, which can be achieved by a variety of direct approaches including UV oxidation, plasma treatment, or silanization as demonstrated in this study.

We have routinely patterned 150-mm-diameter dishes with cells by printing large stamps. Because the stamp readily deforms around dust particles and irregularities on the surface, small local surface defects do not prevent the transfer of the pattern in the surrounding regions, and the pattern can easily be fully transferred over 95% of the printed area.[43] The resolution of pattern that can be printed depends on the resolution of the stamp; nanometer resolution has been achieved.[44] Because of the broad overlap in the range of wettabilities where both μCP and adsorption can occur, the technique is robust and can be adapted on the basis of

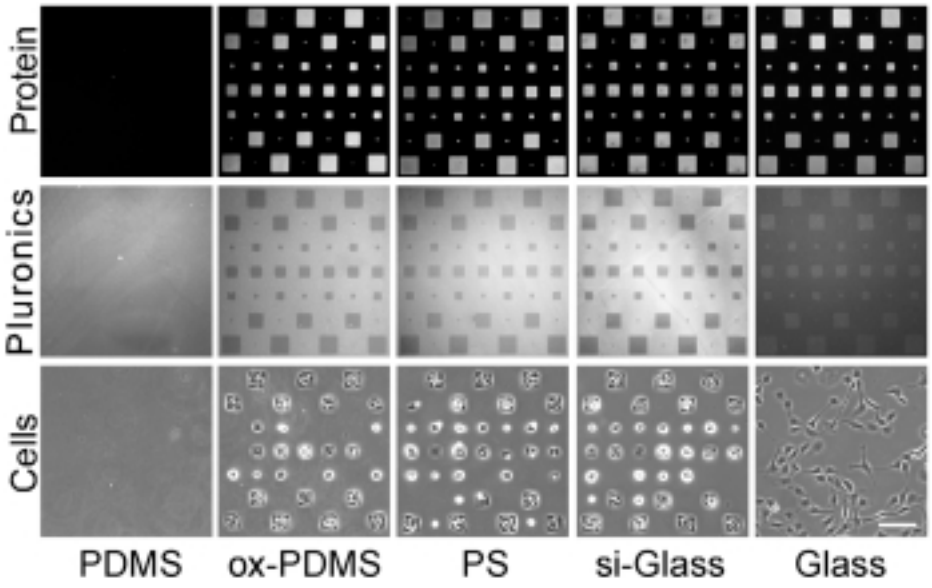

FIG. 3. Micropatterning on common tissue culture substrates. Shown are fluorescence micrographs of printed IgG (*top row*) and adsorbed Pluronic (*middle row*) over the same region of PDMS, oxidized PDMS (ox-PDMS), polystyrene (PS), silanized glass (si-Glass), or glass, and phase-contrast micrographs (*bottom row*) of endothelial cells cultured in 5% serum-containing medium on the indicated substrates for 24 h. Scale bar: 100 μm.

experimental conditions. For example, surfaces including PDMS, polystyrene, and methyl-silanized glass can each be treated with UV-generated ozone for a few minutes without losing the ability of the surface to adsorb Pluronic. On these slightly more hydrophilic surfaces, stamps do not need to be surface treated before being used for microcontact printing.

Past studies have shown that the activity of proteins after μCP is similar to that of the adsorbed protein.[27] We have observed that cells adhered to printed versus adsorbed fibronectin exhibit similar rates of spreading, proliferation, and formation of adhesive structures. Because most cell types rapidly remodel the extracellular matrix proteins after adhering,[45] the printed protein may be quickly remodeled soon after cell adhesion. Nonetheless, to eliminate any possible effects caused by denaturing of the printed protein, antibodies or streptavidin could first be printed onto a surface and subsequently used to capture specific proteins that are more prone to denaturation from solution. We have applied this strategy to capture fibronectin or biotin-labeled proteins from solution for cells to adhere onto (not shown).

We have used this approach to micropattern various cell types including fibroblasts, smooth muscle cells, adipocytes, stem cells, cardiomyocytes, and endothelial cells in serum-containing medium. In each case, the pattern was stable for a period of at least 3 days after seeding, as was shown previously with Pluronic F108.[22,46] In comparison, Pluronic F127 blocked cell adhesion for longer periods and adsorbed onto surfaces of slightly higher wettability. Micropatterns of smooth muscle and endothelial cells have been cultured for at least 4 weeks on F127-blocked substrates.

We have described and demonstrated a general and simple approach to micropattern cells on a variety of surfaces by manipulating the wettability of surfaces in order to successfully apply two straightforward techniques concurrently. All the materials and reagents used were commercially available, and no chemical synthesis was required. The approach requires only the initial photolithographic process to obtain the original silicon master used for casting multiple stamps. The stamps themselves are easy to make, reusable, and require no additional facilities. This straightforward approach may lead to more widespread use of the micropatterning of cells to engineer tissue constructs and study cell biology.

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