

Compatibility of Mammalian Cells on Surfaces of Poly(dimethylsiloxane)

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This paper describes the influence of the composition of poly(dimethylsiloxane) (PDMS) on the attachment and growth of several different types of mammalian cells: primary human umbilical artery endothelial cells (HUAECs), transformed 3T3 fibroblasts (3T3s), transformed osteoblast-like MC3T3-E1 cells, and HeLa (transformed epithelial) cells. Cells grew on PDMS having different ratios of base to curing agent: 10:1 (normal PDMS, PDMS_N), 10:3 (PDMS_{CA}), and 10:0.5 (PDMS_B). They were also grown on “extracted PDMS” (normal PDMS that has reduced quantities of low molecular-weight oligomers, PDMS_{N,EX}) and normal PDMS that had been extracted and then oxidized (PDMS_{N,EX,OX}); all surfaces were exposed to a solution of fibronectin prior to cell attachment. Generally, fibronectin-coated PDMS is a suitable substrate for culturing mammalian cells. Compatibility of cells on some surfaces, however, was dependent on the cell type: PDMS_{N,EX,OX} caused cell detachment of 3T3 fibroblasts and MC3T3-E1 cells, and PDMS_{CA} caused detachment of HUAECs and HeLa cells. Growth of cells on PDMS_N, PDMS_{N,EX}, and PDMS_B was comparable to growth on tissue culture-treated polystyrene for most of the cell types. All cells grew at similar rates on PDMS substrates regardless of the stiffness of the substrate, for substrates having Young’s moduli ranging from $E = 0.60 \pm 0.04$ to 2.6 ± 0.2 MPa (for PDMS_B and PDMS_{N,EX}, respectively).

Introduction

This paper examines the influence of the composition of poly(dimethylsiloxane) (PDMS, in the form of flat slabs) on the attachment and growth of four different types of mammalian cells: primary human umbilical artery endothelial cells (HUAECs), transformed 3T3 fibroblasts (3T3s), transformed osteoblast-like MC3T3-E1 cells, and HeLa (transformed epithelial) cells. We grew cells on normal PDMS, PDMS having either excess curing agent or excess base, PDMS that had been extracted with solvent that removed low molecular-weight components,¹ and hydrophilic (oxidized) PDMS; we exposed all substrates to a solution of fibronectin (an extracellular matrix (ECM) protein) before cell attachment. We observed a preference for cells to attach to PDMS synthesized using a normal (10:1) or lower than normal (10:0.5) ratio of base to curing agent, and to surfaces that were not oxidized.

The physical and chemical properties of a substrate affect the attachment and growth of cells on it.^{2–5} For example, many studies have demonstrated that different topographical features on a surface affect cell attachment;^{6–10} the role of the surface chemistry of materials

used for growing cells has been less examined. We characterized the compatibility of PDMS for culturing different cell types. PDMS is a material that has been used extensively in medical implants and biomedical devices because of its biocompatibility,^{11–14} low toxicity,^{12,15,16} and high oxidative and thermal stability.^{17,18} PDMS is elastic, optically transparent, has low permeability to water, and has low electrical conductivity.^{19,20} These properties, in addition to the ease with which it can be fabricated into microstructures using soft-lithography,²¹ have made this material attractive for use in studies of cell biology, including those of contact guidance, chemotaxis, and mechanotaxis.^{22–27}

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Table 1. Advancing Contact Angle of Water on Different Surfaces Used for Cell Culture

	surface						
	PDMS _{CA}	PDMS _B	PDMS _{N,EX}	PDMS _{N,EX,OX}	PDMS _N	glass ^a	PS ^b
$\theta_a^{\text{H}_2\text{O}}$ (deg)	105 ± 2	95 ± 3	99 ± 4	<15	100 ± 4	61 ± 2	36 ± 3

^a Borosilicate glass; surfaces of glass were untreated and used as received. ^b Tissue culture-treated non-pyrogenic polystyrene.

The most widely used form of PDMS for soft lithography derives from a two-part polymer: a base and a curing agent whose components undergo a hydrosilylation reaction upon cross-linking.²⁸ In this paper, we use Sylgard 184 (produced by Dow Corning). This PDMS is made up of a base that consists of dimethylsiloxane oligomers with vinyl-terminated end groups, platinum catalyst, and silica filler (dimethylvinylated and trimethylated silica), and a curing agent that contains a cross-linking agent (dimethyl methylhydrogen siloxane), and an inhibitor (tetramethyl tetravinyl cyclo-tetrasiloxane).²⁹ Cross-linking occurs when the vinyl and silicon hydride groups undergo a hydrosilylation reaction to form a Si–C bond.^{28,30,31} The suggested proportion of base to curing agent for PDMS is 10:1 (which we call “normal” PDMS in this paper); the proportion of cross-linked to un-cross-linked groups generated in curing depends on the processing conditions (for example, the temperature and time allowed for curing) in addition to the ratio of components. As these conditions are likely to vary for different users of this material, the amount of un-cross-linked, low molecular-weight oligomers from either the base or the curing agent may also differ for each elastomer. The processing conditions also affect the stiffness of the polymer.

To probe the effect of these low molecular-weight species on the biocompatibility of cultured mammalian cells, we fabricated PDMS substrates having three different ratios of base to curing agent (w/w): 10:1 (normal PDMS, which we call PDMS_N), 10:3 (PDMS having excess curing agent, PDMS_{CA}), and 10:0.5 (PDMS having excess base, PDMS_B). We also used normal PDMS that had been extracted to remove un-cross-linked materials (PDMS_{N,EX}), or extracted and then oxidized (PDMS_{N,EX,OX}). The 10:1 ratio of normal PDMS gives optimal cross-linking density between the two components when cured, although we have reported that as much as 5% of the total weight of the polymer comprise un-cross-linked oligomers included in the elastomer.¹ An increase in the ratio of curing agent to base (i.e., 10:3) increases the number of un-cross-linked dimethyl methylhydrogen siloxane groups, and dimethylsiloxane monomers having silicon hydride end-groups, and the amount of excess inhibitor. The fabrication of PDMS having less than optimal quantities of curing agent (i.e., 10:0.5) leaves more of the vinyl-terminated dimethylsiloxane units un-cross-linked and increases the amount of platinum catalyst and silica filler (dimethylvinylated and trimethylated silica) in the elastomer. The composition of the PDMS affects the surface properties of the elastomer, because the composition determines the types of un-cross-linked (and thus mobile) components that may reside on (or migrate to) the surface of the polymer.^{1,32,33} We observed

that these differences in composition of PDMS also affect the attachment and growth of cells.

Results and Discussion

Fabrication and Analyses of Surfaces. *Contact Angle of Surfaces.* Because the main components of PDMS are the repeating dimethylsiloxane units, $-\text{OSi}(\text{CH}_3)_2-$, the surface of normal PDMS is hydrophobic; the contact angle of water on this surface is $\theta_a^{\text{H}_2\text{O}}(\text{PDMS}_N) = 100^\circ \pm 4^\circ$ (Table 1). PDMS samples containing excess base or curing agent also have surfaces that are hydrophobic ($\theta_a^{\text{H}_2\text{O}}(\text{PDMS}_{CA}) = 105^\circ \pm 2^\circ$; $\theta_a^{\text{H}_2\text{O}}(\text{PDMS}_B) = 95^\circ \pm 3^\circ$). Exposing normal PDMS to an air or oxygen plasma introduces silanol (Si–OH) groups in the place of methyl groups (Si–CH₃) and makes the surface hydrophilic $\theta_a^{\text{H}_2\text{O}}(\text{PDMS}_{N,OX}) < 15^\circ$.^{21,34,35} PDMS that has been treated with plasma can be kept hydrophilic by keeping the surfaces in contact with water (or polar organic solvents).¹⁹ If the surface is left in contact with air, however, surface rearrangement occurs and new hydrophobic groups migrate to the surface, lowering the surface free energy.^{19,20} The high mobility of the dimethylsiloxane chains in PDMS results from the low glass transition temperature ($T_g = -127^\circ\text{C}$) of this material.^{36,37}

Previously, we reported that extracting PDMS with a solvent that swells the PDMS removes many of the un-cross-linked oligomers from the bulk of the elastomer and decreases the rate of regeneration of the hydrophobic surface after plasma oxidation.¹ We followed this extraction procedure using pentane, a highly swelling solvent, to make pieces of “extracted PDMS” (PDMS_{N,EX}). The surfaces of these extracted pieces were then oxidized in an air plasma to make “extracted and oxidized PDMS” (PDMS_{N,EX,OX}) that were wetted completely by water. PDMS_{N,EX,OX} surfaces that were left in contact with air had $\theta_a^{\text{H}_2\text{O}} < 15^\circ$ for 4 days, but surfaces that were placed into contact with water within a few minutes after oxidation were wetted by water indefinitely (i.e., they remained hydrophilic); we followed the latter procedure for treating the PDMS_{N,EX,OX} used for cell culture.

Studies of protein adsorption on surfaces of self-assembled monolayers (SAMs)^{38,39} have revealed that, in general, more proteins adsorbed on hydrophobic surfaces (–CH₃) than on hydrophilic surfaces (–OH). Some pro-

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(37) The glass transition temperature, T_g , is the temperature below which flexibility is so reduced that the material becomes glassy. The T_g of PDMS ($T_g = -127^\circ\text{C}$) is the lowest reported for any polymer (see ref above). The low T_g of PDMS means that the dimethylsiloxane chains are highly flexible and mobile within the elastomer, making rearrangements feasible.

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teins, especially large ones with MW > 200 kD, adsorbed to some extent on both types of surfaces (albeit more proteins adsorbed on the hydrophobic ones).⁴⁰ We infer from these studies that more fibronectin, a large protein (MW = 440 kD), will adsorb on PDMS surfaces having $-\text{CH}_3$ termini than those presenting $-\text{OH}$ termini. It is difficult to conclude, however, whether a high surface coverage of fibronectin will better mediate cell adhesion than a low surface coverage of fibronectin, because the conformation of the protein on a surface also plays an important role in cell adhesion.^{5,41–44} The conformation of fibronectin on a surface, and the surface density and conformation of polypeptide sequences exposed on the surface, parameters important for recognition by cells, are difficult to determine experimentally.^{45,46} In addition, because the surfaces of polymers such as PDMS are not molecularly well-ordered, we are limited in our ability to correlate the surface chemistry of PDMS and the adhesion and growth of cells. In this paper, therefore, we use the cell density to measure the suitability of each composition of PDMS for cell culture.

Stiffness of Substrates. The stiffness of a substrate influences the attachment and growth of cells.^{7,47–49} We determined the Young's modulus (E), a measure of the stiffness (or elasticity) of a material, of different compositions of PDMS that were cured for different amounts of time and at different temperatures (Figure 1). The Young's modulus can be calculated using the formula: $E = (F/A)(L/\Delta L)$, where F is the applied force, A is the unstressed cross-sectional area that is perpendicular to the force, L is the unstressed length, and ΔL is the change in length due to the effect of the applied force.

The Young's modulus measures the amount of elastic deformation in a polymer when an applied force causes the polymers in the cross-linked network to stretch.⁵⁰ Generally, a high degree of cross-linking in a polymer makes the polymer stiffer (higher E), and, thus, more difficult to stretch, than a polymer that is less cross-linked, because it is more difficult for parts of a polymer to move away from their initial position when they are more highly cross-linked to neighboring parts. The degree of cross-linking in PDMS and, therefore, the stiffness of the polymer vary depending on the conditions of curing and the ratio of base to curing agent. High cross-linking in PDMS was achieved by curing the polymer at a higher temperature (190 °C), or for a longer period of time (24 h), than the suggested curing conditions (100 °C for 1 h or 60 °C for 4 h)²⁹ and, generally, by using a 10:1 ratio of base to curing agent (Figure 1). For example, samples of PDMS that were cured at 70 °C for 2 h, followed by 190 °C for 2 h, were stiffer than the respective samples cured at 70 °C for 2 h, for all compositions of PDMS.

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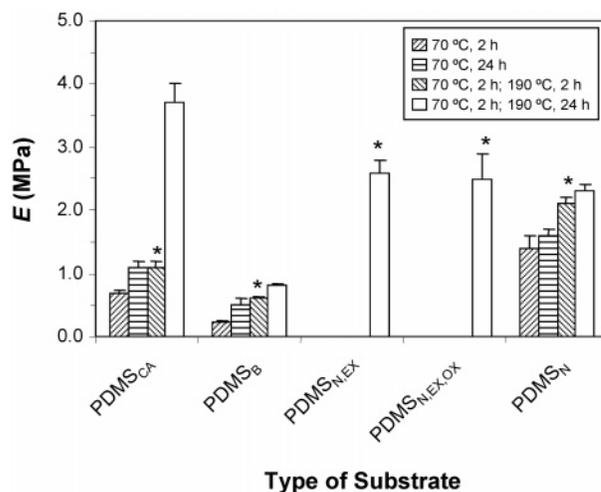


Figure 1. Young's modulus (E , MPa) of PDMS surfaces that have different ratios of base to curing agent (w/w) and that were cured under different conditions. PDMS_{CA} and PDMS_B contained ratios of 10:3 and 10:0.5 base to curing agent, respectively. Extracted (PDMS_{NEX}), extracted and oxidized (PDMS_{NEX,OX}), and normal PDMS (PDMS_N) were made with ratios of 10:1 base to curing agent. Substrates that were used in the cell experiments are marked with “*”. At least 16 measurements were taken for each datum, using pieces of PDMS with dimensions $\sim 50 \text{ mm} \times 5.0 \text{ mm} \times 1.1 \text{ mm}$, *lwh*. Error bars represent one standard deviation from the mean.

PDMS that was fabricated with excess curing agent (10:3, PDMS_{CA}) was less stiff than PDMS_N when cured at a low temperature (70 °C), or for a small amount of time at a high temperature (2 h, 190 °C). These conditions were insufficient to fully cross-link the excess curing agent with the base. When PDMS_{CA} was cured at 190 °C for 24 h, however, the polymer was stiffer ($E = 3.7 \pm 0.3 \text{ MPa}$) than PDMS_N that had been cured under similar conditions ($E = 2.3 \pm 0.1 \text{ MPa}$); the excess curing agent in PDMS_{CA} promoted further cross-linking in the polymer at this high temperature. PDMS that was fabricated with excess base (10:0.5, PDMS_B) was less stiff than PDMS_N for all conditions of curing, because not enough curing agent was present to cross-link efficiently many of the dimethylsiloxane oligomers.

PDMS that was extracted (PDMS_{NEX}) and PDMS that was both extracted and oxidized (PDMS_{NEX,OX}) had values of stiffness of 2.6 ± 0.2 and $2.5 \pm 0.4 \text{ MPa}$, respectively. We measured the stiffness of PDMS_{NEX} and PDMS_{NEX,OX} only after curing the samples in an oven at 190 °C for 24 h to remove excess solvent (during extraction) from the elastomer. The values of stiffness of these elastomers were comparable to those of PDMS_N that were cured under similar conditions.

To test the influence of stiffness on the attachment and growth of cells, we grew cells on substrates that had values of stiffness in the order: PDMS_{NEX} \approx PDMS_{NEX,OX} > PDMS_N > PDMS_{CA} > PDMS_B. PDMS_N, PDMS_{CA}, and PDMS_B were cured at 70 °C, 2 h and then 190 °C, 2 h; PDMS_{NEX} and PDMS_{NEX,OX} were cured at 70 °C, 2 h and then 190 °C, 24 h. These substrates are marked in Figure 1 with “*”.

Growth of Cells on PDMS. To test whether cells grow preferentially on a particular type of PDMS surface, we grew human umbilical artery endothelial cells (HUAECs), 3T3 fibroblasts, osteoblast-like MC3T3-E1 cells, and HeLa (epithelial) cells on five different types of PDMS substrates: PDMS_{CA}, PDMS_B, PDMS_{NEX}, PDMS_{NEX,OX}, and PDMS_N, as well as on borosilicate glass slides (G) and tissue culture-treated polystyrene (PS), which were used

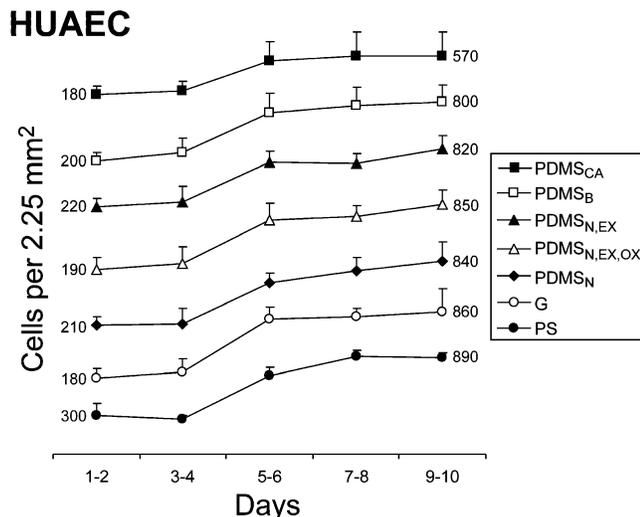


Figure 2. Growth of human umbilical artery endothelial cells (HUAEC) on different surfaces of PDMS. PDMS_{CA} and PDMS_B contained ratios of 10:3 and 10:0.5 base to curing agent, respectively. PDMS that was extracted (PDMS_{N,EX}), extracted and oxidized (PDMS_{N,EX,OX}), and normal PDMS (PDMS_N) were made with ratios of 10:1 base to curing agent. Glass (G) and polystyrene (PS) substrates were used as controls. The y-axis is linear, but the curves float on the y-axis and the absolute positions of the curves are meaningless. Instead, the average numbers of cells per 2.25 mm² on days 1–2 and days 9–10 are presented beside each of the curves. Error bars for each datum represent one standard deviation from the mean and correspond to >40 measurements.

as controls. All surfaces were exposed to a solution of fibronectin (5 μg/mL in PBS) for 1 h at 37 °C, prior to the attachment of cells.

We seeded cells at a density of 2.5 × 10⁴ per well, and the cells were allowed to grow for 1–10 days in each of the wells. Each day, the cells on each type of substrate were stained with a nucleic acid stain, fixed using paraformaldehyde, and imaged using fluorescent microscopy. Cell growth was determined by the cell density per 2.25 mm², the surface area viewed by a 5× objective on a Hamamatsu video camera (which corresponds to one image). At least five images were taken for each substrate, and at least four substrates were tested for each type of surface. The numbers of cells counted on each type of surface for a particular day were, therefore, averaged over at least 20 images. Each datum in Figures 1–4 corresponds to the average number of cells on two consecutive days (i.e., the numbers of cells counted on the first and second days were averaged and corresponded to one datum).⁵¹

HUAECs. Fewer cells attached onto PDMS and glass on days 1–2 (~200 ± 90 cells per 2.25 mm² on PDMS and glass) than onto PS (300 ± 120 cells per 2.25 mm², Figure 2). On all surfaces, HUAECs showed little or no growth from days 1–4, but began to grow significantly between days 4–5. HUAECs reached confluence at a density of ~800 cells per 2.25 mm² by days 5–6 on all types of surfaces except for PDMS_{CA}; cells on surfaces of PDMS_{CA} reached confluence only on some substrates by days 8 and 9. Cells began to detach, however, on half of the substrates of each type beginning on day 6, while cells remained confluent on the others. Cells detached in certain areas

(51) The data for two consecutive days were averaged because there was some variability in cell growth for cells on the same type of substrate; for example, some cells reached confluence on day 5, while others were confluent on day 6. Because we wanted to observe a general trend for cells on each type of substrate, averaging data for consecutive days made the graphs clearer and easier to make statistical comparisons between the different types of substrates.

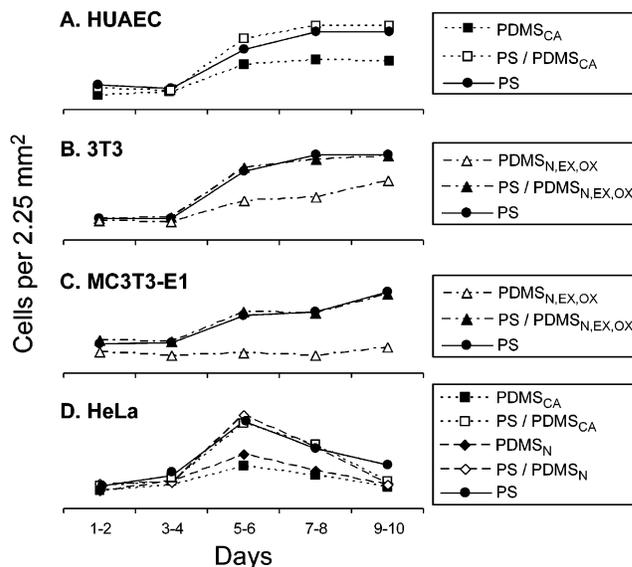


Figure 3. Numbers of cells that grew on polystyrene that was either in physical contact with PDMS (denoted “PS/PDMS_X”, where X is the composition of PDMS that exhibited detachment or poor attachment of cells for a particular cell type) or not in physical contact with PDMS (denoted “PS”, the control). Plots of PDMS_X are also shown for comparison. Error bars were removed for clarity. (Note: The curves float on the y-axis, and the curves for each cell type, A–D, are not on the same linear scale.) (A) HUAECs detached from surfaces of PDMS_{CA}. Attachment and growth of cells on PS/PDMS_{CA} was similar to that on PS. (B) 3T3s detached from surfaces of PDMS_{N,EX,OX}. Attachment and growth of cells on PS/PDMS_{N,EX,OX} was similar to that on PS. (C) Some MC3T3-E1 cells attached to surfaces of PDMS_{N,EX,OX}, but cells did not proliferate on this surface. Attachment and growth of cells on PS/PDMS_{N,EX,OX} was similar to that on PS. (D) HeLa cells detached from surfaces of PDMS_{CA} and PDMS_N. The attachment and growth of cells on all surfaces of PS were similar.

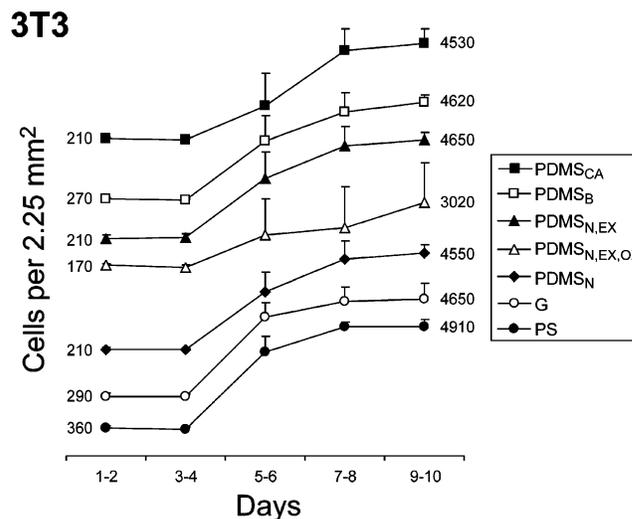


Figure 4. Growth of 3T3 fibroblasts on different surfaces of PDMS. PDMS_{CA} and PDMS_B contained ratios of 10:3 and 10:0.5 base to curing agent, respectively. PDMS that was extracted (PDMS_{N,EX}), extracted and oxidized (PDMS_{N,EX,OX}), and normal PDMS (PDMS_N) were made with ratios of 10:1 base to curing agent. Glass (G) and polystyrene (PS) substrates were used as controls. (Refer to Figure 2 caption.)

throughout the confluent sheet of cells, leaving behind empty gaps on the surfaces. This detachment was noticeable for all types of surfaces on day 7.

On glass, HUAECs detached and left behind small aggregates of cells covering the whole surface of the

substrate on day 7. These remaining cells continued to grow to confluence from days 8–10, although some cells on glass began to detach on day 10. We observed similar behavior on surfaces of PDMS_N and PS: cells reached confluence from days 8–10 after detaching from the surfaces during the previous days. On day 10, cells began to detach on some surfaces of PDMS_N and PS. Cells remaining on substrates of PDMS_{CA}, PDMS_B, PDMS_{N,EX}, and PDMS_{N,EX,OX} did not grow to confluence on days 8–10, but aggregated into small clumps throughout each of the surfaces.

Numbers of cells were similar throughout the 10-day period on all PDMS substrates except for on PDMS_{CA}. On some of the PDMS_{CA} substrates, cells grew to high densities by days 5–6 and reached confluence on day 9, but on other substrates, cells detached throughout the 10-day period. The complete detachment of cells from surfaces (as opposed to detachment of cells from surfaces during migration or cell division) may indicate an unfavorable interaction between the cell and its local environment. Here, the local environment consists of the interactions between the cell and solution, cell and substrate, and interactions between cells. Because HUAECs on PDMS_{CA} detached even before reaching confluence, detachment probably did not reflect cell–cell interactions (i.e., contact inhibition). HUAECs detached more frequently on PDMS_{CA} rather than the other compositions of PDMS, indicating that the components in the curing agent, dimethyl methylhydrogen siloxane or the inhibitor (tetramethyl tetravinyl cyclotetrasiloxane), may have played a role in detachment.⁵² Unfortunately, it is difficult to isolate either of these components from PDMS to test which one is responsible for cell detachment, without affecting the physical properties of the polymer.

To determine whether detachment of cells from substrates was surface- or solution-mediated, we counted the cells that grew on PS that resided in the same well as the substrates that exhibited detachment. Figure 3A shows the numbers of endothelial cells on PS that were in the same well as PDMS_{CA}, denoted PS/PDMS_{CA}. The numbers of cells on this surface were similar to cells grown on PS in the absence of PDMS, within experimental error. These data indicate that detachment was not mediated through the solution, either by the curing agent, which may have leached into the solution, or by cell-derived soluble molecules that can induce detachment, synthesized because of changes in cell physiology in response to the chemistry of the surface. Also, contamination of the solution could not have been responsible for detachment of cells on PDMS_{CA}. Detachment was probably mediated by direct contact of the cells with the surface and, therefore, the components of the curing agent on the surface (via a layer of ECM protein).

Cell detachment is a (or perhaps, several) complicated process(es); it may occur through different mechanisms, many of which are not well-understood at the molecular level.^{53–56} For example, detachment can occur by the dissociation of receptor–ligand bonds,^{45,57,58} unfavorable

conformation of ECM proteins for cell receptor binding,⁴¹ or the transmission of growth-inhibitory signals.⁵³ The mechanism of detachment of HUAECs on PDMS_{CA} is beyond the scope of this paper.

In summary, HUAECs grew on all types of substrates tested, but detached more frequently and in higher numbers on PDMS_{CA} than on the other substrates. PDMS_{CA} was, therefore, the poorest substrate for HUAECs. Although the rates of growth of HUAECs were comparable on PDMS_B, PDMS_{N,EX}, PDMS_{N,EX,OX}, PDMS_N, G, and PS, behavior of the cells on PDMS_N, in terms of detachment and regrowth, was more similar to that on G and PS than the other types of PDMS; therefore, we recommend culturing HUAECs on PDMS_N.

3T3 Fibroblasts. Cells attached to PDMS in lower numbers than on PS (days 1–2: 170 ± 60 to 270 ± 90 cells per 2.25 mm^2 on PDMS; 360 ± 100 cells on PS, Figure 4). This difference in initial attachment may have allowed the cells on PS to grow to higher numbers by days 9–10, as compared to the other surfaces tested. 3T3s showed little or no growth from days 1–4 on all surfaces, but began to grow significantly between days 4 and 5. For PDMS_{CA}, PDMS_B, PDMS_{N,EX}, and PDMS_N, a large error was obtained for days 5–6 because the numbers of cells varied significantly on these days between different experiments. In other words, cells on these surfaces grew fastest either between days 4 and 5, or between days 5 and 6.

The numbers of cells that were counted on PDMS_{N,EX,OX} varied throughout the 10-day period. Fewer cells attached initially to PDMS_{N,EX,OX} (170 ± 60 cells per 2.25 mm^2) than the other surfaces, and cells detached on various days between days 5–10. On the substrates from which cells detached, the remaining cells on the surface appeared rounded. These observations are consistent with previous reports of detachment of fibroblasts from oxidized PDMS due to weak attachment.⁵⁹ Low amounts of fibronectin on hydrophilic surfaces may lead to low ligand-mediated cell spreading, absence of maturation, and eventually cell death through a series of signals from the nonengaged adhesion receptors to the mechanism responsible for apoptosis.⁴⁶

The detachment of cells on PDMS_{N,EX,OX} was limited to this surface, as cells grown on PS/PDMS_{N,EX,OX} proliferated at similar rates as those on PS (Figure 3B). For those PDMS_{N,EX,OX} surfaces from which cells did not detach, cells were able to grow. For example, on some surfaces, cells reached 3000 per 2.25 mm^2 by day 6, consistent with growth on most of the other types of surfaces (PDMS_B, PDMS_{N,EX}, PDMS_N, G, PS), and some cells reached confluence by day 9.⁶⁰

Generally, PDMS that is coated with fibronectin is a good substrate for culturing 3T3 fibroblasts. 3T3s became confluent on all types of surfaces tested at a density of ~ 4500 cells per 2.25 mm^2 by days 7–8, except for on PDMS_{N,EX,OX}. The densities of 3T3s on surfaces of PDMS_{CA}, PDMS_B, PDMS_{N,EX}, and PDMS_N were generally comparable to the controls during the 10 days. These compositions of PDMS, therefore, can be used for culturing 3T3

(52) Detachment of HUAECs on some of the PDMS_{CA} substrates, and not on others, may be caused by variations in the compositions of components inside different batches of PDMS prepolymer and curing agent. For instance, the percentages of components in the prepolymer and curing agent are stated as ranges, instead of exact numbers, by the manufacturer.

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(60) Detachment of 3T3 fibroblasts on some of the PDMS_{N,EX,OX} substrates, and not on others, may be due to the inhomogeneity of the oxidized substrates. Oxidation of PDMS using a plasma cleaner does not give uniform oxidation of the surface, as shown by the large errors in contact angle measurements in a previous report (see ref 1).

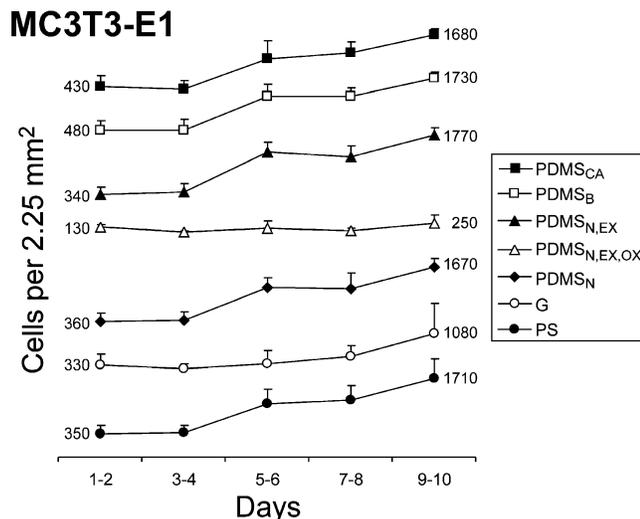


Figure 5. Growth of osteoblast-like MC3T3-E1 cells on different surfaces of PDMS. PDMS_{CA} and PDMS_B contained ratios of 10:3 and 10:0.5 base to curing agent, respectively. PDMS that was extracted (PDMS_{N,EX}), extracted and oxidized (PDMS_{N,EX,OX}), and normal PDMS (PDMS_N) were made with ratios of 10:1 base to curing agent. Glass (G) and polystyrene (PS) substrates were used as controls. (Refer to Figure 2 caption.)

fibroblasts, whereas oxidized surfaces of PDMS are not recommended.

MC3T3-E1 Cells. On days 1–2, similar numbers of MC3T3-E1 cells attached on PDMS_{N,EX} and PDMS_N, G, and PS ($\sim 340 \pm 240$ cells per 2.25 mm², Figure 5); higher numbers were observed for cells on PDMS_{CA} and PDMS_B ($\sim 450 \pm 260$ cells per 2.25 mm²). Attachment was poor on PDMS_{N,EX,OX} (130 ± 70 cells per 2.25 mm²) on days 1–2, and generally poor throughout the 10-day period. Rates of growth of MC3T3-E1 cells on surfaces of PDMS_{CA}, PDMS_B, PDMS_{N,EX}, PDMS_{N,EX,OX}, PDMS_N, and PS were similar: there was little or no growth from days 1–4, but cells began to grow significantly between days 4–5. Between days 5–8, there was little growth, but cells grew again from days 8–9. Cells reached confluence at a density of ~ 1700 cells per 2.25 mm² on days 9–10 on all surfaces except for PDMS_{N,EX,OX}; the poor attachment of MC3T3-E1 cells on PDMS_{N,EX,OX} was specific to this surface (Figure 3C).

Growth of MC3T3-E1 cells was inconsistent on glass: cells seemed to grow well on some glass substrates but not well on others, even on the same day. For example, cells were able to reach confluence on some surfaces of glass on days 9 and 10, but detached on others. The variability of growth on glass is probably associated with the ability of the cells to attach to this substrate. Weak attachment and poor spreading of MC3T3-E1 cells on untreated glass slides has been reported, as cells are unable to form focal adhesions on these surfaces.^{61,62} Treating glass with ECM proteins such as fibronectin (10 μ g/mL, incubated at 37 °C for 1 h) or 55% fetal bovine serum enhanced the strength of attachment and spreading of cells.⁶³ Even though the glass we used was treated with fibronectin (5 μ g/mL, incubated at 37 °C for 1 h), we observed weak attachment and, as a result, detachment of cells on some substrates.

In summary, PDMS is a good substrate for growing osteoblast-like MC3T3-E1 cells. Rates of growth of MC3T3-

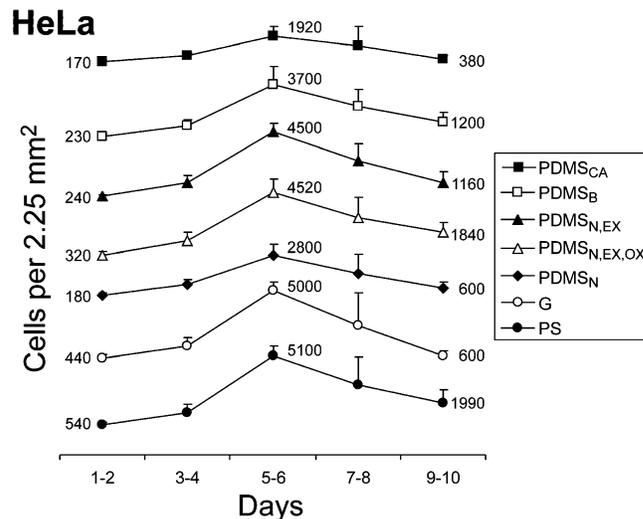


Figure 6. Growth of HeLa epithelial cells on different surfaces of PDMS. PDMS_{CA} and PDMS_B contained ratios of 10:3 and 10:0.5 base to curing agent, respectively. PDMS that was extracted (PDMS_{N,EX}), extracted and oxidized (PDMS_{N,EX,OX}), and normal PDMS (PDMS_N) were made with ratios of 10:1 base to curing agent. Glass (G) and polystyrene (PS) substrates were used as controls. The average numbers of cells per 2.25 mm² on days 1–2 and days 9–10 are presented beside each of the curves; average numbers for days 5–6 are listed in the center. (Refer to Figure 2 caption.)

E1 cells were similar on all types of PDMS, except for PDMS_{N,EX,OX}, and growth on these surfaces was comparable to growth on PS. Cells did not attach well on PDMS_{N,EX,OX}, and attachment was inconsistent on borosilicate glass; thus, growth of MC3T3-E1 cells is not recommended on these substrates.

HeLa Cells. On days 1–2, a higher number of HeLa cells attached onto PS and glass (540 ± 150 and 440 ± 250 cells per 2.25 mm²) than any of the PDMS substrates (Figure 6). Of the PDMS substrates, cells attached best onto PDMS_{N,EX,OX} (320 ± 210 cells), followed by PDMS_{N,EX} (240 ± 120 cells) and PDMS_B (230 ± 130 cells); the fewest cells attached onto PDMS_N (180 ± 100 cells) and PDMS_{CA} (170 ± 120 cells). On all types of surfaces, HeLa cells grew in number between days 2 and 3, but began to grow significantly between days 4–5. HeLa cells reached confluence at a density of ~ 4500 cells per 2.25 mm² between days 5–6 on PDMS_{N,EX} and PDMS_{N,EX,OX}, as well as on G and PS. Cells also reached confluence on PDMS_B, but confluence occurred between days 5–7 on different substrates. Cells did not reach confluence on PDMS_{CA} or PDMS_N within the 10 days.

After reaching confluence, cells began to detach on each type of PDMS substrate, beginning on day 7. On G and PS, cells did not begin to detach until day 8, and, thus, a large error was associated for days 7–8. The rates of detachment on all surfaces were similar.

Overall, the best substrates for growing HeLa cells to confluence are PDMS_{N,EX,OX} and PDMS_{N,EX}, PS, and glass; PDMS_B is also recommended, but the rate of growth is more variable on this type of surface. HeLa cells do not reach confluence on PDMS_{CA} and PDMS_N; therefore, we consider these poor substrates for culturing this cell type. Because detachment of cells occurred on all types of surfaces after 2 days of reaching confluence, this behavior is probably independent of the surface chemistry of the substrate.

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Effect of Stiffness of Substrates on the Growth of Cells. HUAEC, 3T3, MC3T3-E1, and HeLa cells grew at similar rates on substrates regardless of the stiffness of the substrate. For example, cells attached and grew to similar numbers on the softest ($E = 0.60 \pm 0.04$ MPa, PDMS_B) and hardest ($E = 2.6 \pm 0.2$ MPa, PDMS_{N,EX}) PDMS substrates that we tested, for each of the cell types (Figures 2, 4–6). Although the range of stiffness of the substrates we fabricated was narrow, others have observed that 3T3 cells do not grow differently on PDMS substrates having a broader range of stiffness: $E = 12 \pm 1$ kPa to 2.5 ± 0.2 MPa.⁷ We also did not observe differences between cells grown on PDMS and the controls; silicate glass⁶⁴ and polystyrene⁶⁵ have typical Young's moduli of 70 and 3 GPa, respectively. Furthermore, Wang et al. have shown that substrate flexibility regulates growth and apoptosis of normal, but not transformed 3T3 cells, possibly because transformed cells lose their capability to probe the stiffness of substrates.⁴⁹ This characteristic may explain why we did not observe differences in the rate of growth of cells on substrates of different stiffness, as all of the cells that we cultured were transformed, except for the primary HUAECs.

Summary of Observations. The attachment and growth characteristics of cells differ for different cell types cultured in similar environments.⁶⁶ Here, we tested four types of cells, HUAECs, 3T3 fibroblasts, osteoblast-like MC3T3-E1 cells, and HeLa (epithelial) cells, a set representative of the common types of cells used in cell culture, on surfaces of PDMS. These cells included primary cells (HUAECs) and transformed cells (3T3 fibroblasts, MC3T3-E1, and HeLa cells). We observed that, in general, the rates of growth of cells on surfaces of normal PDMS are comparable to rates of growth on PS for all cell types. The different compositions of PDMS, however, affect cell attachment and growth for certain cell types, and the ability of these surfaces to proliferate cells is, therefore, cell-type dependent. For all cell types, variations in the composition of normal PDMS by excess base (PDMS_B) did not significantly affect cell growth, and cells were able to reach confluence on these surfaces. We expect that most types of cells will be biocompatible with PDMS_N, PDMS_{N,EX}, and PDMS_B. PDMS having excess curing agent (PDMS_{CA}) caused variability in cell growth for HUAEC and HeLa cells, but did not affect the growth of 3T3 fibroblasts and MC3T3-E1 cells. 3T3 fibroblasts and MC3T3-E1 cells attached poorly, however, on PDMS_{N,EX,OX} (and glass for MC3T3-E1 cells). PDMS_{CA} and PDMS_{N,EX,OX} may cause variability in cell attachment and growth for other types of cells as well. We also observed that the stiffness of the substrate did not influence attachment and proliferation of cells for all cell types.

Conclusions

PDMS is a useful material for cell biology because it can be easily manipulated to have different sizes, shapes, and dimensions by simple soft-lithographic techniques.^{67–69} Variations in the processing conditions (i.e., time and

temperature of curing, the ratio of base to curing agent), and the manipulation of PDMS during assembly of PDMS-based devices (i.e., oxidation of surfaces), may result in changes in the physical properties of PDMS, such as the surface chemistry and stiffness of the substrate. These properties may adversely influence the attachment and growth of certain types of cells. For instance, fabrication of devices made in PDMS used to culture and analyze cells often requires oxidizing the surfaces to seal the device irreversibly. These hydrophilic surfaces may result in poor initial attachment, and detachment of certain cell types such as 3T3 fibroblasts and osteoblast-like MC3T3-E1 cells. Another example of a processing condition that may negatively affect cell attachment is a sealing technique that involves fabricating pieces of PDMS having different ratios of base to curing agent. This process requires fabricating one piece of PDMS having excess curing agent, fabricating another having excess base, partially curing each of these pieces, bringing together the two pieces, and then curing that assembly.^{70,71} If cells are cultured in devices made by this method, excess curing agent in the polymer may cause detachment of certain types of cells (e.g., HUAEC and HeLa cells) and may inhibit cells from reaching confluence (e.g., HeLa cells). These obstacles can be avoided, however, by choosing the processing conditions of PDMS, and the techniques used in assembling devices, that favor the attachment and growth of a particular cell type. Overall, PDMS is a suitable substrate for culturing various types of mammalian cells, and there are many advantages of fabricating devices made in this material (i.e., low cost, ease of fabrication, transparency, permeability to gas), rather than in conventional materials such as glass, silicon, and polystyrene.

Materials and Methods

Materials. Sylgard 184 Silicone, a two-part poly(dimethylsiloxane) (PDMS) elastomer, was purchased from Essex Brownell (Edison, NJ). Pentane and acetone were obtained from Sigma-Aldrich Co. (St. Louis, MO) and used as received.

Fabrication of Surfaces. PDMS substrates were fabricated having three different ratios of base to curing agent (by weight): 10:1 (normal PDMS, PDMS_N), 10:3 (PDMS_{CA}), and 10:0.5 (PDMS_B). The base and curing agent were mixed by hand for 5 min and then degassed under vacuum until all of the air bubbles were removed (typically, ~30 min). The prepolymer was poured onto flat polystyrene Petri dishes to achieve a thickness of ~1 mm, and then cured at 70 °C for 2 h. The cured PDMS was removed from the polystyrene dish, placed onto a Pyrex Petri dish, and then placed in a 190 °C oven for 2 h. The PDMS was cooled to room temperature (25 °C), and then cut manually into ~4 × 7 mm pieces.

Extracted PDMS was fabricated by a procedure reported elsewhere.¹ Briefly, pieces of normal PDMS were immersed into a stirred solution of ~140 mL of pentane (a "high-swelling" solvent¹) for 24 h, while changing the solvent once after ~12 h. The pieces were removed from this solvent, and then immersed into a stirred solution of ~140 mL of acetone (a "low-swelling" solvent¹) for 24 h, also changing the solvent once after ~12 h. The pieces were removed from acetone, dried with nitrogen, and then dried at 190 °C for ~24 h. Oxidized PDMS was fabricated by oxidizing the extracted PDMS pieces for 60 s in a SPI Plasma Prep II plasma cleaner (~2 mTorr, 60 s) (SPI Supplies, West Chester, PA). The oxidized pieces of PDMS were placed in a 7:3 (v/v) ethanol/water solution within a few minutes after plasma treatment.

We used glass cover slips (VWR Corp.) as the glass substrates. Both PDMS and glass substrates were cleaned before use in cell

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culture by immersing them in a 7:3 (v/v) ethanol/water solution for 24 h, followed by rinsing with Dulbecco's phosphate buffered saline (PBS, JRH Biosciences, Kansas City, MO).

Contact Angle Measurements. Advancing contact angles were measured on static drops of water using a Ramé-Hart goniometer with a Matrix Technologies Electrapipet to control the advancement of the drop. Typically, >30 measurements were taken for each type of surface presented in Table 1. We measured the advancing contact angles of water on flat pieces of PDMS (1.5 cm × 1.5 cm × 0.2 cm, *lwh*) that were fabricated by the methods described above. Contact angle measurements on extracted and oxidized PDMS were performed 10 min after the surface was oxidized.

Stiffness Measurements. The Young's modulus was measured for different compositions of PDMS (PDMS_{CA}, PDMS_B, PDMS_{N,EX}, PDMS_{N,EX,OX}, and PDMS_N) that had been cured for different amounts of time (2, 24 h) and at different temperatures (70, 190 °C). Flat slabs of PDMS were fabricated by molding PDMS prepolymer between flat pieces of polystyrene and glass slides, separated by 1.1 mm thick glass slides. The PDMS was cut manually to pieces having the dimensions 50 mm × 5.0 mm × 1.1 mm, *lwh*, using a razor blade (VWR Corp.). For measurements of Young's modulus, the pieces of PDMS were clamped at both ends using binder clips; one clip was attached to a clamp on a ring stand, leaving the PDMS hanging by gravity. Different weights (20–500 g) were hung to the binder clip on the other end of the PDMS. Images of the elongation of PDMS were recorded using a digital camera; the changes in length of the PDMS were measured directly on the digital image. At least four measurements were taken for each sample, and four samples of each composition (for a particular curing condition) were measured. Each datum in Figure 1 was, therefore, an average of at least 16 measurements. The Young's modulus was averaged over these measurements in the linear range of elongation.

Cell Culture and Reagents. PDMS and glass substrates were placed into the wells of a 24-well plate. The PDMS substrates were placed into conformal contact with the bottom of the well; otherwise, air bubbles were present underneath the substrate after the medium was added. (When the substrates were incubated in the medium, these air bubbles resulted in detachment of the substrates from the bottoms of the wells. These substrates tended to float at the air/liquid interface due to the high surface tension of the medium. Cells on floating substrates were not included in the analyses.) At least two samples of each type of surface were prepared for each day, over a series of 10 days, for each type of cell. Each day, the cells on two samples of each type of surface were stained, fixed, and visualized. These experiments were repeated at least twice for each cell type.

To prepare the surfaces for seeding the cells, a solution of fibronectin (5 μg/mL in PBS) was added to each well. We allowed the solution of fibronectin to be in contact with the substrates for 60 min at 37 °C (without agitation) to allow a uniform layer of fibronectin to adsorb onto the substrates to facilitate cell attachment. After the solution of fibronectin was removed from the wells, the substrates were rinsed with medium.

3T3 fibroblasts, MC3T3-E1, and HeLa cells were grown in Dulbecco's Modification of Eagle's Medium (JRH Biosciences)

containing 10% fetal bovine serum (Hyclone Laboratories, Pittsburgh, PA) and glutamine-penicillin-streptomycin antibiotics (295 μg/mL, 100 units/mL, and 100 μg/mL respectively, Irvine Scientific, Santa Ana, CA). 3T3, MC3T3-E1, and HeLa cell cultures were maintained at 37 °C in a humidified 10% CO₂ incubator.

Human umbilical artery endothelial cells (HUAEC) were grown in EGM-2MV-Microvascular Endothelial Cell Medium-2 (Cambrex Corp., East Rutherford, NJ); these cultures were maintained at 37 °C in a humidified 5% CO₂ incubator.

Confluent cells were treated with trypsin/EDTA (JRH Biosciences) and then resuspended in the appropriate medium at a density of 2.5 × 10⁴ cells/mL. A 1 mL suspension was added to each well containing the fibronectin-coated substrates, and an additional 0.5 mL of medium was added to each well. The cells were allowed to attach and grow for up to 10 days in the appropriate incubator, and the medium in each well was changed every 3 days.

Visualization and Imaging of Cells. The nuclei of the cells were fluorescently dyed with Hoechst nucleic acid stain (Molecular Probes, Eugene, OR), 10 min before imaging. A 5 μL aliquot of the 10 mg/mL solution of dye was added to the medium in each well. The cells were incubated for 10 min at 37 °C, the medium was removed, and the substrates were rinsed carefully with PBS. The cells were fixed by adding a 4% paraformaldehyde solution (in PBS) to the wells, incubating the substrates for 5–10 min, and then rinsing the substrates with PBS.

Images of fluorescently labelled cells were acquired by a Hamamatsu video camera (ORCA-ER) using Metamorph software (Universal Imaging Inc., Downingtown, PA). For each substrate, 5–10 fluorescent images were taken using a 5× Fluotar lens. Each image covered an area of 2.25 mm², and thus at least 11.25 mm² of each surface was imaged. Cells were counted on each image using an image analysis routine using ImageJ software (<http://rsb.info.nih.gov/ij/>). The data reported in Figure 2 represent the average number of cells that were counted in each image, on at least four different substrates. The numbers of cells counted on each type of surface for a particular day were, therefore, averaged over at least 20 images. Each datum in Figures 1–4 corresponds to the average number of cells on two consecutive days, and the error bars represent the standard deviation from the mean.

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