Micropallet arrays with poly(ethylene glycol) walls†‡

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Arrays of releasable micropallets with surrounding walls of poly(ethylene glycol) (PEG) were fabricated for the patterning and sorting of adherent cells. PEG walls were fabricated between the SU-8 pallets using a simple, mask-free strategy. By utilizing the difference in UV-transmittance of glass and SU-8, PEG monomer was selectively photopolymerized in the space surrounding the pallets. Since the PEG walls are composed of a cross-linked structure, the stability of the walls is independent of the pallet array geometry and the properties of the overlying solution. Even though surrounded with PEG walls, the individual pallets were detached from the array by the mechanical force generated by a focused laser pulse, with a release threshold of 6 μJ. Since the PEG hydrogels are repellent to protein adsorption and cell attachment, the walls localized cell growth to the pallet top surface. Cells grown in the microwells formed by the PEG walls were released by detaching the underlying pallet. The released cells/pallets were collected, cultured and clonally expanded. The micropallet arrays with PEG walls provide a platform for performing single cell analysis and sorting on chip.

Introduction

Research and development for the separation of cells while they remain adherent has increased dramatically over the past few years.[1–5] The task of separating adherent cells is important as most cells naturally grow in an adherent manner and when in their native state can be analyzed for additional attributes (*e.g.* morphology, growth rate). One approach has been to use laser microdissection (LM) to isolate cells from a microfabricated culture vessel. LM has been used for over a decade to collect individual or small groups of cells from fixed tissue sections.[6] Toner's group incorporated LM with a microwell array to sort lymphocytes.[7] Poly(ethylene glycol) (PEG) photolithography was used to create microwells composed of PEG hydrogel surrounding a glass surface. Revzin's group followed this work by combining a LM technique for cell collection with a microarray of co-cultured hepatocytes and fibroblasts.[8] Small groups of hepatocytes were removed using a form of LM called laser catapulting.[9] A collagen film with overlying cells were cut out with a focused laser, and then expelled from the array with a laser-generated shock wave produced by a single laser pulse positioned beneath the cell–collagen matrix. The mechanical force imparted by the laser propelled the cells into a collection vessel for later genomic analysis. Both of these studies used fixed cells, although live cell sorting by these traditional LM approaches has been described.[10,11]

Recently, our group has worked to develop an alternate approach for sorting adherent cells. This cell sorting strategy uses arrays of releasable, microfabricated elements, termed pallets, formed from the biocompatible photoresist SU-8.[12] The SU-8 is photolithographically defined on a standard microscope slide to create the pallet array. The pallet surfaces can be modified with proteins or gels to enhance cell attachment and growth.[12,13] To culture cells on these arrays, cells are initially placed in suspension, but are allowed to settle and grow on individual pallets prior to analysis. In past work, placement of cells only on the pallets has been accomplished by generating a continuous region of air between the pallets referred to as a virtual air wall.[12,14] Subsequent to analysis by microscopy, individual pallets containing the desired cells are released from the array using a pulsed laser and are then collected.[13,15] Recent studies of the selection and expansion of single cells have demonstrated a high rate of viability after laser-based release.[13,16]

The ability to exclude cells from the region between the pallets is necessary to establish a cell-based array using pallets.[12] The virtual air wall works well in this regard, but has limitations. The air wall is generated by coating the surface of the array's glass substrate with a perfluoroalkylsilane layer to render the glass hydrophobic.[12] When cell media or buffer is added to an array treated in this manner, a continuous bubble of air is trapped between the pallets by Cassie-Baxter wetting.[14] The stability of Cassie-Baxter wetting on the array depends on a number of variables. The properties of the wetting solutions (*e.g.* surface tension) influence the stability of Cassie-Baxter

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wetting. The virtual air walls are not stable in low-surface tension solutions such as alcohol–water mixtures or highly concentrated protein solutions. The properties of the array itself which can be quantified as a roughness factor determine the stability of the Cassie-Baxter wetting. The roughness factor depends on the pallet size, height and spacing between pallets. The stability of Cassie-Baxter wetting is diminished by larger pallets, greater inter-pallet spacings, and shorter pallet heights. These factors provide constraints on the geometries available for array design. For example, highly motile cells (some fibroblasts, neutrophils) can cross over to adjacent pallets when the pallets are separated by a small distance (<50 μm). For these cell types, it is desirable to have widely spaced pallets; however, the virtual walls have limited stability at large inter-pallet gaps. To overcome these limits, a solid barrier that serves the same function as the air wall would be of great value.

PEG has been widely used to produce surfaces and structures in order to create micro-environments for applications in tissue engineering and cell-based assays.[17–19] In these applications, PEG's resistance to protein adsorption and cell attachment are its chief strengths.[20] PEG can also be easily photopolymerized into micro-scale gel forms.[21–23] In previous reports, photomasks have been used to selectively polymerize PEG hydrogels on flat silicon oxide surfaces.[24,25] In a similar manner, high density arrays of microwells created by photopolymerized PEG hydrogel walls have been fabricated and used to guide cell attachment on glass, as was described above.[7,26,27]

In the current report, PEG was selectively polymerized on the micropallet array within the inter-pallet space. By utilizing the difference in UV-transmittance between glass and SU-8, a simple one-step and mask-free process was used to create PEG hydrogel walls surrounding each pallet in place of the virtual air walls. The micro-scale structures created by this approach were wells possessing a base which could be selectively removed using a single, focused laser pulse. With this laser-based technique, single adherent cells cultured on the array could be released and collected in a viable manner. Since the formation of the hydrogel is independent of the inter-pallet spacing and pallet length, a wider range of array geometries can be created with the PEG walls compared to that with the virtual air walls. Pallet arrays with PEG walls may have a wider range of applications than that of virtual walls. The mask-free method for patterning the PEG hydrogel may find utility in the fabrication of PEG-based structures.

Materials and methods

Materials

SU-8 photoresist (formulation 10) and SU-8 developer were purchased from MicroChem Corp. (Newton, MA, USA). Poly(ethylene glycol) diacrylate (PEG-DA; MW 575), 2-hydroxy-2-methylpropiophenone and L-glutamine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Collagen I from rat tail tendon was purchased from BD Biosciences (San Jose, CA, USA). 3-(*N*-allylamino)propyltrimethoxysilane and (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane were purchased from Gelest Inc. (Morrisville, PA, USA). Silicone O-rings (24 mm outer diameter) were purchased from McMaster-Carr (Los Angeles, CA, USA). Before use, the silicon O-rings were washed in distilled water for 24 h, rinsed with ethanol, and then dried in a 50 °C oven. The Sylgard 184 silicone elastomer kit was purchased from Dow Corning (Midland MI, USA). All other reagents were from Fisher Scientific (Pittsburgh, PA, USA).

Fabrication of micropallet arrays

Pallets composed of SU-8 were fabricated on a glass slide in a manner similar to that described previously.[12,15] Glass slides were cleaned by immersing them in freshly prepared piranha solution (3 : 1 concentrated H_2SO_4/30% H_2O_2 by volume) for 30 min. *Caution: piranha solution is highly corrosive. Extreme care should be taken when handling it.* The slides were then rinsed with deionized water and dried with a nitrogen stream. The slides were dehydrated on a 200 °C hot plate for at least 5 min before use. SU-8 films of 30 μm thickness were obtained by spin-coating SU-8 photoresist on the glass slides following the protocol provided by MicroChem Corp.[28] Briefly, approximately 2–3 mL of SU-8 was dispensed to the center of glass slides, and then the resist was spin-coated at 500 rpm for 10 s followed by 1000 rpm for 30 s on a WS-200-4NPP spin coater (Laurell Technologies Corp.). The coated slides were baked on a hot plate at 65 °C for 3 min followed by a second bake at 95 °C for 7 min to remove organic solvent. To prepare SU-8 pallets, the SU-8 film was exposed to UV light through a photomask with the designed features for 35 s using an Oriel collimated UV source (6.8 mW cm^{-2}). The post-exposure baking was performed on a hot plate at 65 °C for 1 min followed by a second bake at 95 °C for 3 min. The SU-8 samples were then developed in SU-8 developer for 4 min, rinsed with 2-propanol, and dried by a nitrogen stream.

Silane-based modification of the pallet array

After fabrication of SU-8 pallets on a glass substrate, the pallet array was baked for 10 min on a hot plate at 95 °C to remove any solvent trapped on the surface. Then the pallet array was treated with oxygen plasma (Technics 500-II plasma system) for 5 min at the power of 200 W and a pressure of 0.2 Torr. The arrays were then coated with a layer of 3-(*N*-allylamino)propyltrimethoxysilane. The array and a small plastic Petri dish containing 150 μL of 3-(*N*-allylamino)propyltrimethoxysilane were placed inside a 100 mm internal diameter Wheaton dry-seal desiccator. The desiccator was then attached to an oil-free diaphragm vacuum pump (Vacubrand, Fisher Scientific) for 1 min (7 Torr). The desiccator was detached from the pump and maintained under vacuum for 16 h at room temperature. Afterward, the array was placed under a high vacuum (2×10^{-3} Torr) for 2 h to remove any unreacted silane molecules using a standard oil vacuum pump (Fisherbrand, Fisher Scientific). The array was rinsed with ethanol, purge dried with a nitrogen stream, and baked on a hot plate at 95 °C for 10 min to further condense the silane layer. The array was stored in a vacuum desiccator until use.

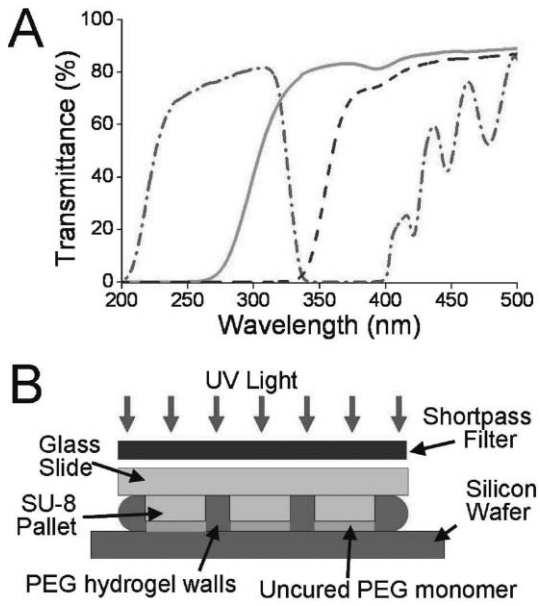

Fig. 1 Fabrication of a pallet array with PEG walls. (A) UV-visible spectra of a 1 mm thick glass slide (solid line), a 30 μm thick SU-8 film on a glass slide (dashed line), and the 325 nm shortpass filter used for PEG wall fabrication (dash-dot, line). (B) Schematic of the assembly used to fabricate of PEG walls in the inter-pallet spaces.

Photopolymerization of PEG walls in the region between pallets

Fig. 1B shows the arrangement for the fabrication of PEG walls on the inter-pallet space. The micropallet array was fabricated and silanized as described above. The array was then placed on a silicon wafer (3 inch diameter) which had been treated with (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane, with the pallet side facing the silicon wafer. This coated silicon wafer was used as a non-stick surface so that the PEG gel could be easily detached from the wafer. PEG precursor composed of 2-hydroxy-2-methylpropiophenone : PEG-DA : water (0.5 : 50 : 50 wt : wt : wt, 100 μL) was added by pipet to the edge of pallet array. The liquid was wicked by capillary action into the gap between the glass slide and silicon wafer, forming a thin and uniform liquid layer. The excess liquid around the edge was removed with a cotton swab. A 325 nm shortpass optical filter (Asahi XUS0325, Asahi Spectra USA Inc., Torrance, CA, USA) was placed on top of the glass slide. This optical filter/pallet array/PEG precursor/silicon assembly was placed inside an Oriel collimated UV source (6.8 mW cm^{-2}) and exposed to UV radiation for 43–47 s (290–320 mJ cm^{-2}). After irradiation, the optical filter was removed and the pallet array was carefully detached from the silicon wafer by inserting a razor at the corners. The pallet array was rinsed with deionized water and incubated in water for 5 min to dissolve non-polymerized PEG precursor. The array was purge dried with a nitrogen stream, and a chamber was constructed by using poly(dimethylsiloxane) (PDMS) to attach a silicon O-ring (24 mm outer diameter) to the pallet array which was then cured in a 80 °C oven for 20 min.

UV-visible spectra

The UV-visible spectra of a 1 mm thick glass slide, a 30 μm thick SU-8 film on a glass slide, and a 325 nm shortpass optical filter were measured using a spectrophotometer (JASCO model V-530).

Laser-based pallet release

Laser-based pallet release was similar to that described in prior reports.[13,15,16,29] Briefly, a frequency-doubled Q-switched Nd:YAG laser (MiniLase, New Wave Research, Fremont, CA, USA) was steered into the back port of an inverted fluorescence microscope (TE300, Nikon). The laser pulse was focused at the pallet–glass interface by a microscope objective (20× 0.5 NA). Pallets were released with single pulses of 2–20 μJ.

Cell culture

A pallet array was immersed in 75% ethanol for sterilization, and then rinsed with PBS buffer five times to remove the ethanol. To coat the pallet surface with collagen, 1 mL of 100 μg mL^{-1} collagen in water was added to the pallet array chamber for 2 h at room temperature. The pallet chamber was then rinsed five times with PBS buffer. A suspension of HeLa cells (20 000 cells) was added to the chamber, and the cells allowed to settle. The cells were cultured on the array in DMEM supplemented with FBS (10%), and L-glutamine (584 mg L^{-1}) at 37 °C in a humidified, 5% CO_2 atmosphere. Penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}) were added to the media to inhibit bacterial growth. Immediately prior to use, the growth medium was removed from the cell chamber and replaced with PBS.

Cell viability testing

Cells cultured on the array were washed twice with PBS. The cells were then incubated in PBS with glucose (10 mM) plus Oregon Green diacetate (10 μM) for 10 min. The cells were washed twice with PBS and examined on an inverted fluorescence microscope (ex/em 488 nm/514 nm).

Scanning electron microscopy (SEM) of cells

Cells plated on pallet arrays were rinsed gently with PBS and then fixed with 2.5 wt% glutaraldehyde in PBS for 30 min. This sample was washed with PBS, and dehydrated with a series of ethanol/water mixtures of increasing ethanol concentration (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol, 10 min in each mixture). The fixed cells were observed by SEM (S-4700-2 FESEM, Hitachi, Japan).

Cell collection after pallet release

Pallets with single cells attached were released and collected in a manner similar to that described previously.[13] Prior to laser release, the pallet array was rinsed with fresh culture medium three times to remove non-adherent and dead cells. HeLa-conditioned medium (1 mL) was added to the pallet array.[13] Prior to use, the collection plate was rinsed with fresh culture medium and placed directly above the pallet chamber in a sterile environment with the two O-rings of the multiwell plate and the pallet array opposed. The assembly was placed on the microscope stage and selected cells/pallets were released with the pulsed laser. The assembly was then inverted to transfer the media and released pallets into the collection plate. For these

experiments, pallets were selected so that an average of no more than one pallet was present in a well of the multiwell plate. The collection plate and pallet array components were separated in a sterile environment. The multiwell plate containing the released cells/pallets and conditioned media was placed into a polystyrene Petri dish and transferred to a standard tissue culture incubator. The growth of the collected cells was observed over time by transmitted light microscopy.

Results and discussion

Photopolymerization of PEG within the inter-pallet space

PEG hydrogels can be formed from the free radical polymerization of the monomer, poly(ethylene glycol) diacrylate. Illumination of a photoinitiator such as 2-hydroxy-2-methylpropiophenone with UV light (220–360 nm) is typically used to generate the free radicals needed to initiate the polymerization reaction. When a pallet array was overlaid with poly(ethylene glycol) diacrylate and a photoinitiator and then illuminated from above with UV light, a PEG hydrogel formed in the inter-pallet spaces and across the surfaces of the pallets. The PEG hydrogel blocked pallet release. Two strategies were possible to restrict the PEG hydrogel formation to the regions between the pallets. The first is the use of a photomask to block the access of UV light to the solution above the pallet but not between the pallets. This strategy, however, requires an additional alignment step and mask. A second potentially simpler method is to exploit the absorption of SU-8 in the ultraviolet wavelengths using the pallets themselves to block the access of UV light to the solution above the pallet. If successful, this strategy might yield an easy, maskless process to form a PEG hydrogel restricted to the inter-pallet regions.

SU-8 has an excellent optical transparence at a wavelength above 400 nm, but becomes opaque at wavelengths below 350 nm (Fig 1A).[28] In contrast, the glass substrate on which the SU-8 pallets are fabricated transmits UV light above 270 nm (Fig. 1A). Therefore, glass but not SU-8 will be transparent to light at wavelengths of 270 to 350 nm. When an array is illuminated from below with UV light, the SU-8 pallets should serve to block the light, while the light should be transmitted through the inter-pallet area. As a result, the pallet array itself might act as a photomask to permit the UV-initiated polymerization of PEG precursor only in the inter-pallet space.

To determine whether the pallets could act as their own mask, the pallet array was placed on a silicon wafer with the pallet side facing the silicon wafer (Fig. 1B). The pallet array was previously coated with 3-(*N*-allylamino)propyltrimethoxysilane which provided C=C moeties to which the PEG diacrylate could form a covalent bond during polymerization. The silicon wafer was previously coated with a perfluoroalkylsilane ((heptadecafluoro-1,1,2,2-tetrahydrodecyl)trichlorosilane) to create a "non-sticky" surface. Poly(ethylene glycol) diacrylate (PEG-DA; MW 575) mixed with a photoinitiator in water was added at the edge of the array and capillary action distributed the liquid between the array and silicon wafer. To block the high-intensity, long-wavelength UV light from the lamp, a short-pass optical filter was placed between the glass and the UV light (Fig. 1A & B). The assembly was then exposed to collimated UV light, disassembled, and washed. In the regions where UV-transmission occurred, *i.e.* between the pallets, the UV-initiated polymerization of PEG occurred. Since each PEG monomer has two functional C=C groups, a highly cross-linked three-dimensional hydrogel was formed. The PEG hydrogel was easily visualized between the pallets due to its refractile appearance compared to arrays without PEG hydrogels. The pallets were similar in appearance to pallets on arrays without PEG hydrogels suggesting that the PEG monomer did not polymerize above the pallets.

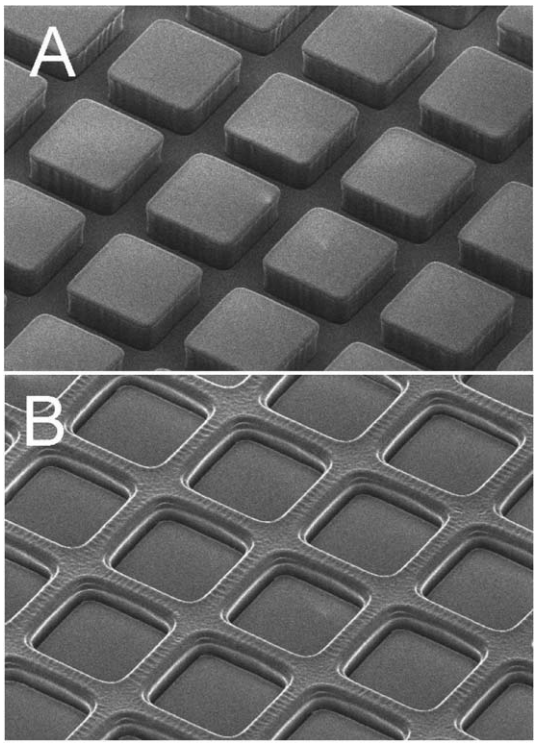

Fig. 2 SEM images of a micropallet array without (A) and with (B) PEG hydrogel walls. The square pallets possess a side of 70 μm and a height of 30 μm. The spacing between the pallets is 30 μm.

To further assess the location of the PEG hydrogel, the pallet arrays were imaged by SEM. The PEG hydrogel was clearly visualized between the pallets and was taller in height than the adjacent pallet (Fig. 2A & B). The PEG hydrogel was not present over the pallets. Instead the PEG wall appeared to be retracted from the edge of the pallet with a small gap between the pallet and the hydrogel (see also Fig. 4D). Since the PEG diacrylate solution was dissolved in 50% water, the cross-linked PEG gel also contained a substantial amount of water. In preparation for SEM, the pallet arrays were dried and the shrinkage of the PEG from the pallet was most likely due to removal of the water from the PEG hydrogel. When these dried pallet arrays were re-submerged in an aqueous solution, the small gap between the PEG walls and pallet was no longer visible suggesting that the PEG gel was easily rehydrated.

The photopolymerization of PEG was extremely sensitive to the exposure time to the UV light. High quality PEG hydrogels formed in the inter-pallet regions but not above the pallets when the UV illumination time was 43–47 s (6.8 mW cm^{-2}).

The PEG monomer did not cross-link when the exposure time was too short (0–35 s). At long UV exposure times (>55 s), PEG diacrylate polymerized in the regions above the pallets. The formation of a hydrogel above the pallets was likely due to the diffusion of free radicals from the UV-exposed regions into the SU-8-masked regions and to partial transmission of UV light through the thin SU-8 pallet. Further optimization of the viscosity of the monomer solution, the pallet height, and the UV illumination intensity and wavelength would likely result in improved spatial control over the polymerization reaction.

A variety of PEG monomers, monomer concentrations, and photoinitiators were tested for the ability to form a PEG hydrogel on the pallet arrays. PEG diacrylate with average molecular weights of 258 and 700, and PEG dimethacrylate of molecular weight 330, 550, and 750 were studied. The water content of the PEG monomer solution was also varied (0–80 wt%). Other photoinitiators including 2,2′-dimethoxy-2-phenylacetophenone were tested as well. In each case, PEG hydrogel walls were obtained. For each PEG precursor composition and condition, only a small time window for UV exposure yielded high quality PEG hydrogel walls between the pallets but not above the pallets.

Laser-based release of individual pallets surrounded by PEG walls

Previous work has shown that the adherence of SU-8 pallets to their glass substrate is weak; therefore, individual pallets can be detached with a mechanical force generated by a pulsed laser.[15] Cells attached to the released pallet have a high rate of viability due in part to the minimal energies required to release the pallet.[13] These prior studies were performed with a "virtual air wall" surrounding the pallets, which could reasonably be expected to have little to no influence on the energy required to dislodge the pallet from the array.[12,15] However, the hydrated PEG walls surrounding each pallet might provide a frictional force opposing the release of the pallet. This force could be of sufficient magnitude to prevent pallet release or require high laser energies that are detrimental to cell viability. To determine whether individual pallets could be released from the surrounding PEG hydrogel, a single pulse (5 ns duration) of a Nd:YAG laser (532 nm) was focused at the interface between the glass and individual SU-8 pallets. This laser pulse generated a plasma-induced cavitation bubble between the pallet and glass to create a mechanical force that dislodged the overlying pallet.[13,15,16] Under transmitted light, single pulses (8–10 μJ per pulse) appeared to release pallets surrounded by PEG walls, but the transparency of the pallets made the assessment of pallet removal a challenge. In some cases the released pallet settled in the vicinity of the empty site or a bubble was formed in the empty site confirming pallet release. Since SU-8 is fluorescent, the release of a pallet can also be assessed using fluorescence microscopy.[29] Under epifluorescence imaging, pallets that had been targeted by the laser were confirmed to be absent. For all pallet releases with PEG hydrogel walls ($n = 20$), no pallets bordering the released pallet were detached. Multiple pallets in the array could be released by moving the microscope stage to sequentially place individual pallets at the point of the focused beam. SEM images of the array in the area of pallet release showed that while targeted pallets were missing from the array, their surrounding PEG walls remained undamaged (Fig. 3A).

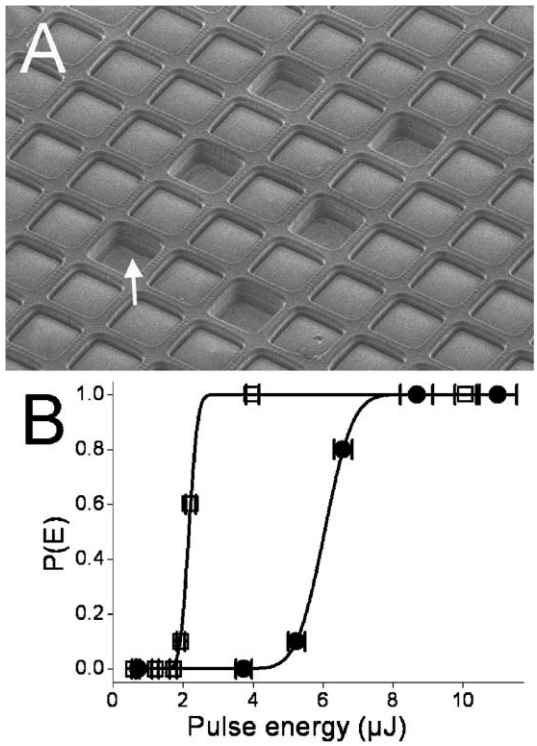

Fig. 3 Laser-based release of SU-8 pallets. (A) SEM image of an array with square pallets (70 μm side, 30 μm height, 30 μm spacing) surrounded by PEG walls. Six pallets were each released with a single laser pulse (8 μJ). The white arrow marks a single release site. (B) The probability of pallet release (P(E)) is plotted against the laser pulse energy for arrays with (solid circles) and without (open squares) PEG walls. P(E) is defined as the probability of the pallet becoming dislodged by a single focused pulse of energy E. Ten pallets were released at each pulse energy. Since the pulse to pulse energy was slightly variable, the energy of each pulse was measured and the average pulse energy with the standard deviation (error bar) was plotted. The lines are the best fits of the data to a Gaussian error function. The threshold energy was defined as the pulse energy required to release 50% of the targeted pallets. The array geometry was the same as that in panel A.

To compare the energy required to release pallets in the presence or absence of PEG walls, the probability of pallet release with respect to the laser pulse energy was measured for arrays with and without PEG walls (Fig. 3B). The data were fitted to a Gaussian error function to determine the threshold energy for pallet release or energy at which 50% of the pallets release.[15] The threshold release energy for pallets surrounded by water or PEG hydrogel was 2 and 6 μJ, respectively. Thus, the energy needed to release pallets surrounded by PEG was increased by 3-fold, likely as a result of frictional forces imparted by the PEG wall.

The release threshold energy is dependent on the type of PEG monomer utilized to form the walls. Pallets on arrays with walls formed from PEG dimethacrylate (MW 750) could not be released with energies as high as 20 μJ, the highest energy tested. Since SU-8 is hydrophobic, the additional methyl groups on the monomer may have enhanced the hydrophobic interactions between the SU-8 and gel. In addition, the release

threshold energy depended on the water content of the initial monomer solution. When the monomer solution possessed no added water *i.e.* only PEG monomer and photoinitiator, pallets on the arrays could not be released after immersion of the array in water. This was most likely due to the swelling of the PEG wall after immersion in water which held the pallets rigidly in place. It is possible that optimization of the chemical properties of the monomer as well as the water content of the PEG hydrogel could further lower the threshold energy for pallet release.

Culture of cells on a pallet array with PEG walls

To determine if pallets surrounded by a PEG wall could be used to create a cell-based array, arrays were coated with collagen to provide a surface for cell attachment.[12,27] To determine the location of the collagen on the array, arrays were initially coated with collagen conjugated to Oregon Green 488. When viewed by fluorescence microscopy, pallets on the array coated with collagen–Oregon Green 488 exhibited substantially more green fluorescence as compared to uncoated arrays (Fig. S1).‡ The PEG walls were nonfluorescent. These data suggest that the collagen adsorbed to the pallet surfaces but not the PEG walls.

In prior work we have shown that when cells cultured in the absence of air (or PEG), walls grow in random locations on the glass surface or the side walls of the pallets.[12] HeLa cells were cultured on arrays ($n = 3$ arrays) of pallets (70 μm) coated with collagen and incubated in a standard tissue culture medium. The array was examined by microscopy after 6 h, then daily for 3 days. The cells remained localized exclusively to the top surfaces of the pallets (Fig. 4A). This finding persisted even after 72 h in culture. No cells were identified on the PEG walls ($n = 500$ cells). SEM images (Fig. 4C & D) corroborated these findings. Cells were also cultured on arrays of collagen-coated pallets of 30 μm size with PEG walls. On these arrays the vast majority of pallets possessed either 1 or 0 HeLa cells (Fig. S2).‡ These results also suggested that the collagen adhered only to the pallet tops and not to the PEG gel. The PEG is known to be a poor surface for cell attachment so that the walls served as barriers to cell adhesion.[27] Notably, as seen by SEM, the level of the PEG walls were higher than that of the top of the pallet. Thus, a microwell was formed around each pallet so that gravitational forces also assisted cell localization to the pallet surface.

Cells grown on the pallet arrays appeared healthy on the basis of their morphology. To confirm that the cells were viable, a live-cell assay was carried out using the viability dye Oregon Green. In this assay, viable cells take up the diacetate form of the dye and metabolize it to the membrane impermeant free acid which is fluorescent.[30] Nonviable cells are unable to metabolize the dye and remain non-fluorescent. HeLa cells grown on the array for 16 h took up and retained the fluorescent dye, indicating their viability (Fig. 4B).

Single-cell sorting from pallet arrays with PEG walls

To determine whether living cells could be selected and released from the pallet arrays with PEG walls, HeLa cells were cultured on the arrays. Pallets with single cells were identified by microscopy and released using a single laser pulse as described (8 μJ) (Fig. 5A,B). Following laser-based release, detached pallets were collected in a multiwell plate.[13] The cells were imaged by microscopy within 1 h of collection and then at varying times thereafter. Within 1 h after collection, the HeLa cells were seen to remain attached to the pallet tops (Fig. 5C, $n = 5$). After 20 h in culture, the released cells had undergone cell division (Fig. 5D, $n = 5$). Within 2–6 days following culture, small colonies were present with cells attached to the pallet and surrounding area (Fig. 5E). These data clearly demonstrate the feasibility of viable cell sorting using the pallet array with PEG wall structures.

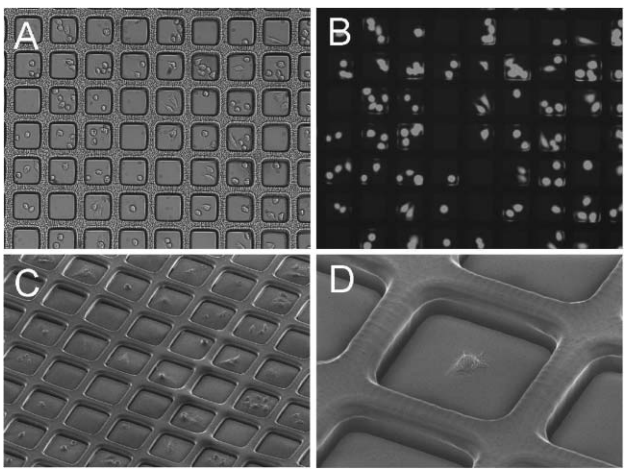

Fig. 4 Patterning of cells on pallet arrays with PEG walls. HeLa cells in suspension were placed on the arrays (70 μm side, 30 μm height, 30 μm spacing) and allowed to grow for 16 h. (A) Transmitted light image of HeLa cells on the array. (B) Fluorescence image of cells loaded with the vital dye Oregon Green. The image corresponds to the transmitted light image in panel A. (C) SEM image of cells grown on an array. (D) A close-up of an SEM image of a HeLa cell on a pallet surrounded by PEG walls.

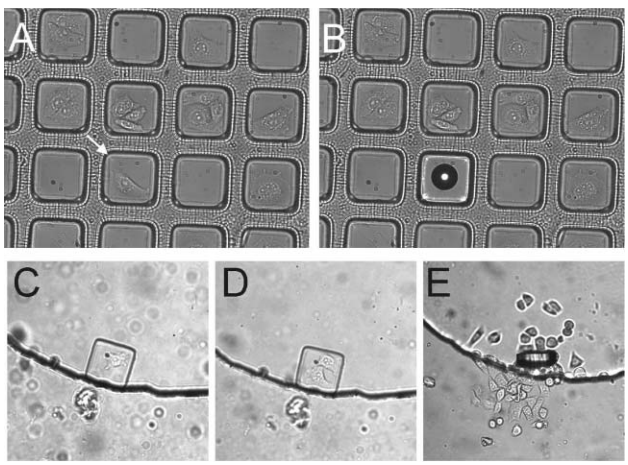

Fig. 5 Selection and cloning of single cells on pallet arrays with PEG walls. (A) HeLa cells were grown on an array for 24 h. The pallet with a single cell (white arrow) was selected for release. (B) Image of the array in panel A with the pallet/cell released from the array. (C)–(E) The released pallet from panel B was collected, and imaged at 0 h (C), 20 h (D), or 6 d (E) after the initiation of culture. At 6 d, some cells are growing out of the collection well and the pallet has rotated onto its side.

Conclusion

A PEG hydrogel was fabricated in the inter-pallet regions of an array of pallets. A simple one-step and mask-free process capitalized on the differential absorption of UV light by glass and SU-8 photoresist. Pallets on this array were releasable by a single laser pulse without release of nearby pallets. In addition, the PEG walls formed microwells which localized cells exclusively to the surfaces of the pallets. The base of the microwell or pallet could be released to collect selected cells from the pallet array. Importantly, these cells survived release and could be cultured to yield a clonal colony. The PEG walls offer a number of advantages compared to the virtual air walls. The stability of the PEG wall is independent of the geometry of the pallet array. PEG walls but not virtual air walls are stable on arrays with widely spaced (>50 μm) pallets. The height of a pallet does influence the fabrication of the PEG walls by this mask-less method. Very short pallets (<10 μm) may not have sufficient absorption of the UV light. However, PEG walls in the presence of very short pallets could also be fabricated using a mask. In contrast, virtual air walls are generally not stable on arrays with very short pallets (<50 μm). The stability of the PEG walls will also be largely independent of the properties of the overlying solution; whereas, the stability of the virtual air walls is critically dependent on the solution properties. The microwells formed by the PEG walls around the pallets also act to initially localize the cells to the pallets as well as contain the cells during their growth. The pallets with PEG walls do require higher laser energies for pallet release relative to that with air walls. These higher release energies may negatively impact fragile cells, for example, primary cells cultured on the pallet surfaces. It is likely that further optimization of the monomer properties, the gel water content, and the cross-linking density of the gel might further reduce the threshold energy for laser-based pallet release.

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