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Rapid Prototyping of Heterotypic Cell-Cell Contacts

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Abstract

Disparities in cellular behaviour between cultures of a single cell type and heterogeneous co-cultures require constructing spatially-defined arrays of multiple cell types. Such arrays are critical for investigating cellular properties as they exist *in vivo*. Current methods rely upon covalent surface modification or external physical micromanipulation to control cellular organization on a limited range of substrates. Here, we report a direct approach for creating co-cultures of different cell types by microcontact printing a photosensitive cell resist. The cell-resistant polymer converts to cell adhesive 0 with light exposure, thus the initial copolymer pattern dictates the position of both cell types. This strategy enables straightforward preparation of tailored heterotypic cell-cell contacts on materials ranging from polymers to metallic substrates.

Introduction

Synergistic interactions between different cell types in co-culture reveal markedly different cellular behavior than that observed in homogeneous cultures. This phenomenon extends beyond neural stimulation of electroactive cells, such as ventricular myocytes¹ or muscle cells.² Mesenchymal cells promote liver-specific functions in hepatocytes,³ glial cells facilitate neuron regeneration,⁴ and fibroblasts mediate attachment of cancer stem cells.⁵ To mimic these *in vivo* behaviors *in vitro* requires control over heterotypic cell-cell contacts.

Elegant approaches to forming co-culture assemblies^{6, 7} have been developed using microfluidics,⁸ electron beam lithography,⁹ or properties unique to a specific cell type, such as migration¹⁰ or differential adhesion on proteins.¹¹ However, these methods are limited to cell types with sufficiently different behaviors or require specialized equipment. Recent and more accessible techniques include sequential removal of Parafilm sections from surfaces,¹² construction of microscale barriers for cell confinement,¹³ and culturing in aqueous two-phase systems.¹⁴ Scope and throughput are restricted in these examples by manual micromanipulation of the surface or the second cell type. Here, we develop a noninvasive approach to generate co-cultures by synthesizing a light-switchable, cell-resistant ink that can be contact printed directly on a wide range of substrates and switched on to become cell adhesive. The geometry of a silicone stamp and unmasked flood illumination creates spatially-defined arrays of two cell types. This strategy facilitates rapid assembly of two different cell types over arbitrarily large areas on chemically-diverse culture surfaces, from polymers to metals.

Results and Discussion

The microcontact-printable copolymer comprises poly(ethylene glycol) and poly(lactic acid) blocks separated by a light-sensitive nitrobenzyl linker (PEG-L-PLA). The PEG block resists nonspecific protein adsorption, while PLA is cell adhesive.¹⁵ By virtue of its insolubility in aqueous solutions, PEG-L-PLA and PLA adhere to a variety of surfaces in the presence of culture media. Upon illumination of a solution of PEG-L-PLA in dichloromethane (Figure 1a), gel permeation chromatography (GPC, Figure 1b) demonstrates pseudo first-order (Figure 1c) cleavage of the copolymer into cell-resistant PEG-L (5 kDa) and cell-adhesive PLA with rate constant $k_{\text{obs}} = 0.027 \text{ min}^{-1}$.

The amphiphilicity of the light-cleavable copolymer PEG-L-PLA suggests that detachment of the copolymer from the surface by aqueous self-assembly could occur. Protein adsorption precedes cellular attachment, thus the relative fluorescence intensity between printed and unprinted areas (Figure 2a, b) after incubation with fluorescent protein can be used as a metric to predict differential cellular adhesion. After standing in saline for two weeks, printed PEG^{5k}-L-PLA^{25k} yielded significant protein contrast, whereas PEG^{5k}-L with PLA blocks of 5 and 10 kDa did not display any protein resistance. This is consistent with observations that PEG^{5k}-PLA copolymers form stable micelles in water when the PLA block is less than 15 kDa.¹⁶ Though the PEG-L-PLA ink does not create binary protein patterns (some protein also deposits on the printed pattern), 70 % or less fluorescent bovine serum albumin contrast successfully localizes cell adsorption exclusively to the unprinted areas (Figure 2c).¹⁷ The protein fluorescence contrast between printed and unprinted regions for varying PLA-block molecular weight, pattern thickness and substrate are summarized in Figure 2d.

Figure 3 schematically depicts the strategy for assembling multicellular ensembles and its application to a polystyrene substrate. A patterned poly(dimethylsiloxane) stamp coated with PEG-L-PLA transfers a pattern of convertible cell resist to the culture surface (Figure 3a). These micropatterns limit attachment of the first cell type to the unprinted areas (Figure 3b). Illumination triggers cleavage of the copolymer, converting the printed regions to cell adhesive PLA by loss of PEG-L into solution, resulting in the second cell type occupying the printed area (Figure 3c).

Circumventing covalent linking of the photosensitive cell resist to the culture surface allows micropatterned assemblies of two cell types to be generated independent of pre-existing surface chemistry. The photosensitive ink can thus be applied to any substrate that is bioadhesive in its native state. In addition to polystyrene culture dishes, PEG-L-PLA organizes two cell types on commonly-used polylysine and gelatin substrates, as well as indium tin oxide for use with electroactive cells (Figure 4).

Cell-substrate interactions¹⁸ have been regulated using external cues such as light,¹⁹ enzymes,²⁰ temperature,²¹ electrochemical potential,²² polyelectrolyte assembly²³ or micromechanical forces.²⁴ Important advances have employed light to micropattern a single cell type with a photomask, though extension to patterning two cell types requires multiple photolithographic steps, often in conjunction with specific chemical functionality residing on the substrate. For example, siloxane-bound,²⁵⁻²⁷ triazole-linked,²⁸ spin-coated²⁹ or self-assembled monolayers³⁰ of photosensitive cell resist afford patterns of a single cell type after masked irradiation. Although two cell types can be spatially organized by a spin-coated photoresist using sequential microscope projection lithography,³¹ the patterned area is throughput-limited to the field of view.

The various methods for appending caged arginylglycylaspartic acid (RGD) peptides to extend spatial control over cell arrangement from one culture surface to another illustrates

the need for synthetic modification of photocleavable cell resists to accommodate differences in substrate functionality. Nitrobenzyl-appended RGD is cell resistant; release of the photosensitive caging group triggers adhesion. Nitrobenzyl-RGD siloxanes, thiols, maleimides, and carbodiimide coupling enables attachment to glass,³² gold,³³ polylysine,³⁴ and hyaluronic acid,³⁵ respectively. In these reports however, masked irradiation led to patterns of only one cell type.

Instead of sequential photolithographic steps and/or substrates with specific chemical functionality, printing a photosensitive cell resist followed by unmasked (flood) illumination enables straightforward preparation of two different cell types arrayed according to the reliefs of the stamp. This relates conceptually to conversion of printed cell-resist patterns by adsorption,³⁶ but avoids *in situ* use of cytotoxic³⁷ polycations. Diblock copolymer PEG-PLA is amenable to microcontact printing³⁸ for use as a cell-resistant ink, affording spatially-defined arrangements of a single cell type.³⁹ The PEG block confers bioresistance, while hydrophobic PLA is cell adhesive.¹⁵ Incorporation of a photocleavable linker between blocks of PEG and PLA creates a photoswitchable ink that is initially cell resistant. Among photosensitive linkers, the methoxy-substituted nitrobenzyl functionality has high photolytic efficiency,⁴⁰ reducing the UV-A dose required to release PEG-L (365 nm for 20 min @ 0.9 mW cm⁻², 1.1 J cm⁻²) considerably below the apoptosis threshold for a variety of cell types.⁴¹

Experimental

Materials

Reagents were purchased from Aldrich or Acros. Methoxy PEG (mPEG) was dried at 130 °C under vacuum for four hours. Dichloromethane (DCM) for the lactide polymerization was distilled from phosphorus pentoxide under argon. The lactide polymerization and acylation were carried out in oven-dried glassware under an atmosphere of argon. Proton NMR spectra was obtained on a Bruker AMX400 spectrometer operating at 400 MHz; chemical shifts were referenced to the residual solvent peak. Gel permeation chromatography (GPC) was conducted using an Agilent 1100 HPLC system equipped with a refractive index detector. For analysis, a solution (5 wt % in DCM, 0.5 μ L) was injected onto a Jordi Gel 500 Å poly(divinylbenzene) column (#15309) at 25 °C having dimensions 4.6 mm $\times$ 150 mm using 10 vol % acetic acid in acetone as the mobile phase at 1.3 mL min⁻¹. Green and red CellTracker were purchased from Invitrogen. NIH 3T3 cells and human epidermal keratinocytes (HEK) cells were obtained from Lonza. Cell adsorption was visualized with a Nikon TE2000 fluorescent microscope. Images were collected using Metamorph software (Universal Imaging) and processed using Mathematica (Wolfram). Fluorescence images were acquired in black and white. White in each image was replaced with false color (green for observed green emission and red for observed red fluorescence). To enhance cell contrast with respect to background noise, a piecewise linear function instead of gamma encoding was applied to transform the original image brightness.

Synthesis of the block copolymer (Scheme 1)

PEG Nitroacetophenone 1—To a suspension of 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoic acid⁴² (4.00 g, 13.4 mmol, 1.2 eq.) in DCM (50 mL) and dimethylformamide (3 drops from a 22 gauge needle) was added oxalyl chloride (1.10 mL, 13.4 mmol, 1.2 eq). The reaction became homogeneous upon completion. After one hour, the solvent was removed, leaving a yellow solid. This crude nitroacid chloride was redissolved in DCM (50 mL) and cooled to 0 °C. A solution of monomethyl PEG (Mn = 5000, 56 g, 11 mmol, 1 eq) and triethylamine (1.81 mL, 13.4 mmol, 1.2 eq) in DCM (200 mL) was added *via* cannula over 30 minutes. The solution was warmed to room temperature

overnight and transferred to a separatory funnel with additional dichloromethane (150 mL). The organic layer was washed with saturated sodium bicarbonate (2×200 mL) and brine (1×400 mL), dried on magnesium sulfate and stripped of solvent, yielding **1** as a yellow solid (55.18 g, 93 %). ^1H NMR (400 MHz, CDCl_3) δ 2.06 (2H, pentet, $J = 7.2$ Hz), 2.50 (3H, s), 2.59 (2H, t, $J = 6.7$ Hz), 3.38-3.97 (427H, m), 4.17 (2H, t, $J = 6.2$ Hz), 4.26 (2H, t, $J = 5.0$ Hz), 6.77 (1H, s), 7.61 (1H, s).

PEG nitrobenzyl alcohol 2—Sodium borohydride (240 mg, 6 mmol) was added to a solution of **1** (2.26 g, 0.45 mmol) in isopropanol/DCM (40 mL, 1:1) and stirred for twelve hours. Excess sodium borohydride was quenched by dropwise addition of aqueous ammonium acetate (ca. 1 M). The solution was transferred to a separatory funnel with DCM (100 mL) and washed with water (1×100 mL) and brine (1×100 mL). The organic layer was dried (MgSO_4) and stripped of solvent, furnishing **2** as a light yellow solid (1.60 g, 71 %). ^1H NMR (400 MHz, CDCl_3) δ 1.51 (3H, d, $J = 5.9$ Hz), 2.18 (2H, pentet, $J = 6.4$ Hz), 2.45 (3H, s), 2.59 (2H, t, $J = 6.6$ Hz), 3.38-3.97 (445H, m), 4.11 (2H, t, $J = 5.8$ Hz), 4.25 (2H, t, $J = 4.8$ Hz), 5.54 (1H, q, $J = 5.9$ Hz), 7.34 (1H, s), 7.56 (1H, s).

PEG-L-PLA—Alcohol **2** (760 mg, 0.152 mmol) and lactide (ca. 3.7 g, 25 mmol) were dissolved in DCM (50 mL), followed by addition of DBU (50 μL , 0.33 mmol, 1.3 mol % with respect to lactide).⁴³ The reaction was stirred under argon for three hours, after which a solution of benzoic acid in DCM (1.2 mL of a 0.6 M solution, 0.72 mmol) was added. The reaction volume was reduced to approximately 20 mL and precipitated dropwise into stirring isopropanol (300 mL). The supernatant was decanted and the residue triturated with isopropanol (2×50 mL) to reveal copolymer PEG-L-PLA as a white solid (3.65 g, 82 %). ^1H NMR (400 MHz, CDCl_3) δ 1.37-1.62 (1418H, m), 3.54-3.66 (445H, m), 5.08-5.21 (474H, m).

Cell Patterning

Pattern generation—Micropatterns consisting of parallel grooves 30 μm wide with ridges of width 60 μm were fabricated on silicon wafers using standard photolithographic techniques. From this silicon master, complementary poly(dimethylsiloxane) replicas⁴⁴ were generated and used as stamps in subsequent microcontact printing steps to form patterns of copolymer PEG-L-PLA directly on culture dishes. A solution of the polymer (10 μL of a 0.8 wt % solution in dichloroethane for a stamp approximately 1 cm^2) was spread evenly over the stamp. After the solvent had dried, the stamp was pressed gently against the surface of the culture dish for a few seconds and peeled away. The printed patterns were aged for one day at 60 $^\circ\text{C}$ before use.

Screening Copolymers by Protein Adsorption—Fluorescein-conjugated bovine serum albumin (10 $\mu\text{g mL}^{-1}$) was incubated with the patterned dish for 30 minutes prior to washing with phosphate-buffered saline (PBS). Protein intensity ratios were calculated by selecting one hundred random points away from the pattern edges in the printed and unprinted areas of three positions on each of three separate patterned culture dishes using a custom script written in Mathematica.

Cell Culture—Human epidermal keratinocytes were cultured in keratinocyte cell basal medium (KBM, Lonza # 192151) with growth medium kit (KGM, Lonza # 192152) including epinephrine (0.05 %), hydrocortisone (0.1 %), epidermal growth factor (0.1 %), insulin (0.1 %), transferrin (0.1 %), gentamicin sulfate amphotericin (0.1 %), and bovine pituitary extract (0.4 %). NIH 3T3 fibroblasts were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO # 12440053) with serum (10 %). Cultures were maintained at 37 $^\circ\text{C}$ in a humidified atmosphere containing CO_2 (5 %).

Micropatterning Cells—In order to distinguish sequential cell seedings, a solution of green or red CellTracker ($3 \mu\text{g mL}^{-1}$) in medium not containing serum was used to incubate cells at 37°C for 45 minutes, after which the dye solution was replaced with growth media. These cells were seeded on the printed pattern at a density of $10,000 \text{ cells cm}^{-2}$ (for the first cell deposited) or $200,000 \text{ cells cm}^{-2}$ (for the second seeding). The culture dish was exposed to UV light for 20 minutes to convert the cell-resistant ink to cell-adhesive. After two hours, the next cell type was seeded on the pattern. After four hours, the pattern was washed with PBS to remove non-adhering cells and replaced with fresh media.

Conclusions

Cellular arrays incorporating heterotypic cell-cell contacts have vast applications in studies of cell function, disease progression and drug screening. Organization of two cell types in co-culture heretofore relied on chemical functionalization in conjunction with specialized equipment or techniques, which impedes universal application to larger areas on a wider range of substrates. The microcontact-printed PEG-L-PLA ink obviates the need for any apparatus or expertise beyond that required to generate and use silicone stamps. A more versatile printing approach extends the gamut of light-tunable biomaterials for cell patterning by avoiding covalent surface modification. This methodology can be used to create complex cell ensembles with micrometer resolution over sizeable areas, allowing construction of arrays for *in vitro* study of cell-cell interactions.

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Fig. 1.

(a) Light-induced fragmentation of PEG-L-PLA into PEG-L (5 kDa) and PLA. (b) GPC monitoring of photolysis (0.9 W cm^{-2} , 365 nm) reveals photodegradation of PEG-L-PLA by growth of the 5 kDa peak as a function of time (c, fit shown is to first-order kinetics; error bars are the intensity multiplied by the baseline correction).

Fig. 2.

Protein contrast used as a prognosticator of cell adhesion. (a) Fluorescent protein patterns from incubation of fluorescent albumin with microcontact-printed PEG-L-PLA^{25k} ink, shown above the corresponding vertically-averaged protein intensity (b). Printed fluorescence intensity less than 70 % of the unprinted intensity portends successful cell patterning (c). Protein patterning to optimize ink molecular weight and pattern thickness (d; gelatin contrast not shown due to autofluorescence). Scale bars 200 μm; a piecewise linear function was used to transform the brightness of fluorescence image (a) after addition of false color.

Fig. 3.

Directed arrangement of two cell populations with light-switchable ink PEG-L-PLA. (a) Phase contrast and AFM images of a culture surface printed with PEG-L-PLA to create a patterned surface. (b) Fluorescence image showing the cell-resistant ink guiding adhesion of the first cell type (human epidermal keratinocytes labeled with CellTracker green) to the unprinted area. (c) Global illumination converts the ink to cell adhesive, creating a patterned co-culture of the second cell type (fibroblasts labeled with CellTracker red) with the first. Scale bars 200 μm ; fluorescence images in (b, c) processed in the same way as Figure 2a.

Fig. 4.

Assembly of human epidermal keratinocytes (green) and fibroblasts (red) on polystyrene culture dishes, gelatin, polylysine, and indium-tin-oxide substrates following the methodology employed in Figure 2. Scale bars 200 μm ; images processed in the same way as Figure 2a.

Scheme 1.

Synthesis of the light-cleavable block copolymer. a) $(\text{COCl})_2$, DMF, DCM the mPEG^{5kDa}, Et_3N ; b) NaBH_4 , DCM, iPrOH; c) racemic lactide, DBU, DCM.