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Micropatterned Surfaces with Controlled Ligand Tethering

Timothy A. Petrie¹, Brandon T. Stanley^{1,2}, and Andrés J. García¹

¹Petit Institute for Bioengineering and Bioscience and Woodruff School of Mechanical Engineering

²Undergraduate Research Scholars Program, Georgia Institute of Technology, Atlanta GA 30332

Abstract

Microcontact printing (μ -CP) is a facile, cost-effective, and versatile soft-lithography technique to create 2-dimensional patterns of domains with distinct functionalities that provides a robust platform to generate micropatterned biotechnological arrays and cell culture substrates. Current μ -CP approaches rely on non-specific immobilization of biological ligands, either by direct printing or adsorption from solution, onto micropatterned domains surrounded by a non-fouling background. This technique is limited by insufficient control **over ligand density**. We present a modified μ -CP protocol involving stamping mixed ratios of carboxyl- and tri(ethylene glycol)-terminated alkanethiols that provides for precise covalent tethering of single or multiple ligands to prescribed micropatterns via standard peptide chemistry. Processing parameters were optimized to identify conditions that control relevant endpoint pattern characteristics. This technique provides a facile method to generate micropatterned arrays with tailorable and controlled presentation of biological ligands for biotechnological applications and analyses of cell-material interactions.

Keywords

micropatterning; lithography; microcontact printing; self-assembled monolayers

Introduction

Patterning of ligands with high degrees of density and spatial control has become a powerful approach for applications in tissue engineering 1–3, studying cell-surface interactions 4–6, cell-culture analogues and substrates 7–8, and both cell-based and ligand-based biosensors 9–11. For example, precise spatial control of cell position on 2-D biosensors is vital to the performance of the living circuitry 11. Furthermore, effective manipulation of ligand and cell placement may greatly assist in basic studies of cell function on biomaterials. Recent studies suggest that spatial extracellular matrix cues, including size, geometry, and surface ligand density of adhesive regions, significantly regulate downstream cell responses 12–16. Moreover, creation of controlled gradients of different ligands and cell types may assist in the design of cell culture substrates to direct specific cell function, biomaterials for assembly of multifunctional tissues, or even interfaces to enhance *in vivo* tissue healing 17–18.

In order to pattern ligand islands of prescribed shape, size, and spatial position on substrates on the cellular scale, a variety of direct and soft lithographic techniques have recently been employed – the most widely used being microcontact printing 6–11–19–21. Microcontact

printing (μ -CP) is an easily reproducible, cost effective, simple, and versatile technique to create 2-dimensional patterns of ligand “islands” of specific shape, size, and substrate spatial locale 22-26. Current strategies have utilized μ -CP patterns of “adhesive” hydrophobic self-assembled monolayers (SAMs) of alkanethiolates, typically methyl-terminated, on gold substrates, backfilled with “nonadhesive” SAMs of ethylene-glycol (EG₃)-terminated alkanethiolates 22-27-28. For cell adhesion applications, these islands can adsorb adhesive proteins to promote cell adhesion. Moreover, the size and geometry of these adhesive islands will determine the spatial pattern of adhered cells on the surface. Alternatively, other strategies have focused on using μ -CP to pattern proteins directly onto substrates, relying on protein physisorption to synthetic materials to create localized adhesive regions on a substrate 29-30.

Nonetheless, there are significant limitations associated with using this μ -CP technique with ligand adsorption-dependent alkanethiol SAMs. Since ligands are not covalently immobilized on patterns, but rather passively adsorbed, the ability to implement large ranges of well-controlled ligand density on each pattern is considerably limited. Recently, mixed SAMs of alkanethiolates on gold, consisting of a mixture of reactive carboxylic acid-terminated (EG₆-COOH) and nonfouling tri(ethylene glycol)-terminated (EG₃) alkanethiolates, have exhibited the ability to immobilize controlled surface densities of tethered ligands within a protein-adsorption resistant background 31-28-32. The ligand is immobilized to the surface by peptide bond-forming reactions between the active esters on the alkanethiol and the terminal amino group of the ligand, promoting a stable covalent tethering to the SAM surface.

Here, we have optimized a technique that combines the advantages of mixed alkanethiol SAMs with the patterning utility of μ -CP to afford controlled immobilization of one or more ligands to specific, well-defined patterns of prescribed size, shape, and spatial location. We demonstrate that this easily reproducible μ -CP protocol with mixed SAMs of alkanethiolates generates micropatterns with controlled immobilized ligand densities.

Materials and Methods

Materials

Two alkanethiols were used to produce self-assembled monolayers (SAMs): tri(ethylene glycol)-terminated alkanethiol (HS-(CH₂)₁₁-(OCH₂CH₂)₃-OH; EG₃) and carboxylic acid-terminated alkanethiol (HS-(CH₂)₁₁-(OCH₂CH₂)₆-OCH₂COOH; EG₆-COOH), obtained from Prochimia (Sopot, Poland). AlexaFluor350-conjugated goat anti-mouse (AF350) and AlexaFluor488-conjugated goat anti-mouse (AF488) antibodies were obtained from Invitrogen (Carlsbad, CA). Different size (9mm × 9mm squares, 25 mm diameter circles, No. 1.5) glass slides were used as the underlying substrates for subsequent Ti/Au deposition and SAM assembly. Dulbecco's phosphate buffered saline (DPBS) was purchased from Invitrogen. Peptide tethering reagents, N-hydroysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich (St. Louis, MO). Glycine and 2-mercaptoethanol were also acquired from Sigma-Aldrich. Gel Mount used for fluorescent slide placement was purchased from Biomed (Foster City, CA). PDMS stamps were made from Sylgard 184 and 186 obtained from Dow Corning (Midland, MI).

Stamp and substrate preparation

Master molds of microarrays of different shapes (rectangles, ovals, circles, squares), sizes (including 20 μ m diameter circles, 8 μ m diameter ovals, rectangular patterns [64, 100, 1000 μ m² area], and squares [64, 100, 1000 μ m² area]), and island spacings (75 μ m center-to-

center spacing for circles, 10 – 50 μm for other shapes) were fabricated on Si wafers using standard photolithography methods. Following photoresist spin coating (5 μm thick) and exposure to UV light through an optical mask (consisting of “holes” comprising of the negative stamp pattern desired), exposed patterns were etched away to leave recessed areas of the pre-determined stamp pattern. Poly(dimethylsiloxane) (PDMS) stamps were created using standard methods^{22,19}. Briefly, Si molds were rendered hydrophobic to prevent elastomer-Si adhesion by adding 200-300 μl of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane onto the mold surface. PDMS precursors and curing agents (Sylgard 184 and 186) were mixed (5:1 for precursor elastomers; 10:1 for curing agents), poured onto the microfabricated molds, and placed under vacuum to eliminate bubbles. Following casting on molds, the PDMS was cured overnight at 60°C. After curing, the PDMS stamp was carefully pried off the mold and washed with 70% ethanol. Stamp features were confirmed by microscopy and image analysis. The dimensions of the PDMS stamps used in this study were 30 mm $\times$ 30 mm $\times$ 5 mm.

Gold-coated substrates were created by cleaning glass slides in a custom-made tabletop etcher for 4 min, followed by sequential deposition of titanium (100 Å) and gold (200 Å) films at a deposition rate of 2 Å/sec onto clean glass slides (9 $\times$ 9 mm or 25 mm diameter circles) via an electron beam evaporator (Thermionics Laboratories, Hayward, CA) at a pressure of 2×10^{-6} Torr. The gold-coated glass substrates were stored in a dessicator under vacuum for a maximum of 3 weeks.

μ -CP of mixed alkanethiol SAMs

Before μ -CP, all PDMS stamps and Au-coated substrates were sonicated in 70% ethanol and dried under a stream of nitrogen. Self-assembled monolayers (SAMs) of mixed alkanethiols on Au-coated substrates were used to present well-defined and ordered domains for subsequent ligand tethering. PDMS stamps were “inked” with a specific mixed alkanethiol ratio of EG₆-COOH:EG₃ (1.0 mM total thiol concentration; 0.001 to 0.1 ratios EG₆-COOH:EG₃) and allowed to dry for 10 seconds under a stream of nitrogen to permit excess solution to run off the stamp. Inked stamps were then brought into contact with the Au-coated substrates under various weights (50 – 200 g) and for a range of time periods (15 – 300 s). Only the raised patterns on the stamp contacted the substrates and, therefore, the mixed alkanethiols only transferred to the substrate at these points. After the various stamping times, the PDMS stamp was carefully removed using thin tweezers, and the unstamped, bare gold areas on the substrate were “backfilled” with a 1.0 mM solution of EG₃ alkanethiol for at least 2 h. Model ligands (AF350- or AF488-conjugated antibodies) were tethered onto the mixed alkanethiol SAM islands using standard peptide chemistry³³. Briefly, patterned substrates were washed in 70% ethanol and rinsed multiple times in ultrapure H₂O for at least 15 min. Next, the substrates were incubated in 2.0 mM EDC and 5.0 mM NHS in 0.1 M 2-(N-morpho)-ethanesulfonic acid and 0.5 NaCl (pH 6.0) to “activate” the EG₆-COOH SAMs for subsequent ligand tethering. After 30 min, the activated surfaces were immersed in a 20 mM solution of 2-mercaptoethanol in dH₂O. For single ligand patterns, antibody ligand (either AF488 or AF350) solution at particular concentrations was incubated on the activated supports for various exposure times (15-60 min). For multi-ligand patterns, antibody ligands were sequentially added to activated patterns. This procedure entailed adding the first ligand to activated substrates for a specific length of time, followed by addition of the second ligand to the activated surfaces for another precise time period. Following ligand incubation, unreacted NHS esters were quenched in 20 mM glycine and patterned surfaces were incubated overnight in DPBS to minimize non-specific protein adsorption³¹.

Surface plasmon resonance surface density measurements

Tethered-antibody surface density measurements on μ -CP mixed SAMs were obtained using a Biacore X instrument (Biacore, Piscataway, NJ) 34. μ -CP surfaces were prepared as described as above on in-house glass SPR chips coated with Ti (57 Å) and Au (338 Å). Surfaces were primed in the SPR with sterile DPBS, and the baseline allowed to stabilize for 5-10 min. Surfaces were activated by flowing a 5.0 mM NHS/2.0 mM EDC solution at 4 μ l/min for 10 min. Antibody was subsequently tethered by injection of AF488 at varying solution concentrations at a flow rate of 4 μ l/min for 15 min. Finally, surfaces were washed for 3 min with 0.1% sodium dodecyl sulfate detergent in DPBS to eliminate untethered ligand, and the baseline allowed to stabilize for 2 min thereafter before tethered antibody levels measured. Resonance units (RU) were converted to surface density values (10 RU = 1 ng/cm²). We previously showed that tethered densities determined in situ in the SPR were equivalent to those generated via conventional bench-top incubation methods 34.

Image quantification and analysis

Following overnight incubation in PBS, samples were washed in ultrapure water and mounted on slides with Gel/Mount mounting media. Fluorescence images were taken using a Nikon TE-300 fluorescence microscope and Spot RT digital camera. Different objective lenses (4-100 X) were used. All images for all samples and patterning conditions at each magnification were acquired at the same camera settings to allow direct comparison of fluorescence intensity among all samples. In order to assess relative density of tethered antibody on each sample/condition, the total fluorescence intensity (minus background intensity) was quantified on multiple 10X images from the same sample using ImagePro (Version 6.0, MediaCybernetics). Fluorescence intensity line profiles were also prepared from representative images of various magnifications also using ImagePro. Profiles were normalized to background intensity (reference line).

Statistical analysis

Fluorescence images of patterns presented represent characteristic results from those particular experimental conditions and ligands. Quantified fluorescence intensity and ligand density are reported as mean $\pm$ standard error. All experimental conditions involved at least an N = 5, except mixed antibody tethered patterned surfaces, which involved N = 4 samples/condition, and at least three independent runs were conducted.

Results

Micropatterning with mixed alkanethiol solutions

In order to develop micropatterned substrates with tethered ligand islands of controlled sizes and shapes, we developed a microcontact printing protocol implementing a mixed ratio of specific alkanethiols to covalently immobilize ligands to specific patterned areas. We and others have shown previously that a homogeneous (unpatterned) surface presenting a SAM of a ratio of EG₆-COOH and EG₃- terminated alkanethiols on gold-coated substrates affords controlled tethering of amine-presenting ligands, while preventing significant non-specific adsorption of non-tethered ligands (Fig 1a) 34,35. The carboxyl-terminated alkanethiols can be modified through standard NHS/EDC tethering chemistry to present an NHS ester intermediate, which is readily displaced by freely-presented amine groups, typically on peptides or protein ligands, to form a peptide bond 33. The EG₃ alkanethiols provide for biofouling resistance.

Our protocol for μ -CP mixed ratios of alkanethiols entailed “inking” the cleaned PDMS stamp with a mixed solution of these alkanethiols (Fig 1b-1), bringing it in contact with the substrate (the standard μ -CP technique) (Fig 1b-2), and subsequently backfilling with EG₃ to

preserve non-fouling behavior on the unpatterned areas (Fig 1b-3). Patterns were then “activated” via NHS/EDC chemistry (Fig 1b-4) and ligands were tethered (Fig 1b-5). After quenching unreacted “activated” surface groups, patterned substrates were rinsed overnight in a buffered solution to eliminate non-specific ligand adsorption (Fig 1b-6). For this study, the optimal resulting μ -CP surface resembles circular islands of tethered fluorescent antibody amid a minimally fluorescent background (Fig 1c).

Optimizing the μ -CP protocol for mixed alkanethiols

A central goal of this study was to systematically optimize this μ -CP protocol using an evaluation-based approach. We used two fluorescent antibodies, AF488 (green) and AF350 (blue), as model ligands because of the widespread use of antibodies in biotechnological applications and the ability to easily visualize patterns and eliminate the need for immunostaining of proteins. Early μ -CP patterns were extremely variable, inconsistent, and faint. Therefore, we identified several key experimental design variables/parameters that potentially influence the fidelity of these patterns and qualitatively assessed their impact on pattern quality. These variables included: (1) stamping weight, (2) total stamping time, (3) ratio of alkanethiols used for inking, and (4) ligand (antibody) exposure time post-activation of the stamped substrate, which can have a profound effect for adsorbed or covalently bound ligands. We qualitatively evaluated the relative parameter effect on 4 quality-control outcomes:

1. **surface homogeneity** (corresponding to the overall conformity and consistency of the intensity/shape of all islands over the entire patterned substrate);
2. **pattern homogeneity** (corresponding to the conformity and consistency of the intensity/shape of each individual island from one edge to the other);
3. **pattern integrity** (fidelity of each pattern shape to the original stamp design over the entire substrate);
4. **fluorescence intensity** (ratio of fluorescence intensity, corresponding directly to antibody surface density, of ligand-tethered patterns to background).

The qualitative results for all conditions tested are summarized in Table 1. One specific stamp dimension was used for this assessment (30 mm $\times$ 30 mm $\times$ 5 mm). The best performing condition of each variable was: 90-second stamping time, 5% mixed SAM ratio, and 45-min antibody exposure time. The chosen stamping weight was 150 g, since it exhibited better pattern integrity than the 200 g weight.

Controlling single antibody tethering to μ -CP mixed alkanethiol circular patterns

Relative tethered surface densities were determined by quantifying the fluorescence intensity of patterns from their corresponding fluorescent images (a quantification process we term FIIQ). This FIIQ method entailed quantifying total fluorescence intensity on each image of each sample, using an in-house imaging macros, and normalizing to background fluorescence. To validate this method and compare to absolute measurements of tethered density, we conducted parallel measurements using surface plasmon resonance (SPR). We quantified tethered AF488 antibody densities at two different coating concentrations on both unpatterned (homogeneous 5% COOH SAM surface) and patterned (5% COOH SAM patterns backfilled with EG₃) gold-coated SPR chips (Fig 2a). SPR quantitative antibody densities are in excellent agreement with FIIQ relative density results (Fig 2b,c). For example, the predicted ratio of AF488 (at 100 μ g/ml) surface density on μ -CP compared to unpatterned surfaces (10.3%, based on area) correlates well with the ratio from the SPR runs (11.2%) and the FIIQ analysis (7.8%) (Fig 2c, 1st panel). In addition, AF488 surface density ratios between 100 μ g/ml and 25 μ g/ml coating concentrations for patterned (36.6%) and

unpatterned (30.2%) surfaces were similar to the relative density values obtained by FIIQ (32.2%) (Fig 2c, 2nd panel). Taken together, this data validates the FIIQ method for quantifying relative AF488/AF350 surface density and provides evidence for control of ligand surface density on μ -CP mixed SAM surfaces.

To assess the ability to vary the density of tethered ligand on activated patterns in greater detail, FIIQ was used to quantify AF488 (green) or AF350 (blue) surface density on μ -CP mixed SAM patterns using a range of antibody coating concentrations (Fig 3). For antibody coating concentrations above 50 μ g/ml for AF488 and 75 μ g/ml for AF350, patterns were well-formed and consistent throughout the entire substrate (Fig 3a). However, for lower coating concentrations of 25 and 37 μ g/ml for AF488 and AF350 respectively, pattern fidelity began to degrade both across the substrate and within each pattern (Fig 3). At these low concentrations, tethered antibodies seemed to localize either in clumps or along the periphery of each sample (Fig 4d,h). At high magnification, overall pattern fidelity, as evidenced by line profiles of intensity across representative images, was relatively high using either antibody. At lower magnifications, the intensity line profiles demonstrated patterns of homogeneous intensity across the substrate, even at lower coating concentrations (Fig 4a,b). Moreover, background intensity for all coating concentrations was minimal, indicative of minimal unspecific antibody adsorption to EG₃ backfilled areas. For both AF488 and AF350, tethered antibody surface density increased hyperbolically with antibody coating concentration on activated μ -CP mixed alkanethiol surfaces (Fig 3b,c). This functional dependence of tethered density on coating concentration is in excellent agreement with measurements for tethering onto unpatterned substrates 34. Taken together, this data validates control over tethered single ligand density using this μ -CP mixed alkanethiol protocol.

Controlling multiple antibody tethering to μ -CP mixed alkanethiol circular patterns

We next determined whether multiple ligands (both AF350 and AF488) could be co-tethered to the same μ -CP patterns. We determined through pilot patterns that sequential incubation of activated patterns in each antibody solution would afford greater control over the relative tethered antibody density than one incubation of a single mixed solution of various concentrations of each antibody. Using identical protocol conditions (weight, stamp time, alkanethiol ratio) to the single ligand procedure, different combinations of exposure times of each antibody via sequential incubation were qualitatively evaluated (Table 1). The optimal exposure time ratio was determined to be 40 min for the first ligand, followed by a full 60 min for the second ligand. Next, 25 combinations of different ratios of AF350 and AF488 coating concentrations were incubated on activated patterned substrates, and the total blue and green fluorescence intensity (corresponding to tethered AF350 and AF488 density, respectively) on each sample quantified by FIIQ (Fig 5e). It would be expected that the AF488 green antibody densities would remain constant over all AF350 blue antibody concentrations - as the tethering process for each antibody is independent. However, since the coefficient of variation (standard deviation of the mean divided by the mean) for these measurements is < 10% (data not shown), the observed fluctuations in AF488 tethered densities over the range of blue AF350 coating concentrations are not statistically significant. Representative fluorescent images (Fig 5a-d) at various AF350:AF488 coating concentration ratios reflect FIIQ antibody density data (Fig 5e) and demonstrate our ability to control the density of tethered ligands on multiple ligand-tethered patterns.

μ -CP using mixed alkanethiols is applicable for diverse pattern designs

To further examine the versatility of this system, other PDMS stamp designs were used to create antibody-tethered mixed alkanethiol patterns of different sizes and shapes. Multiple pattern designs were created on a single substrate in localized areas with excellent

consistency and homogeneity (Fig 6a). Rectangular ($16\ \mu\text{m} \times 8\ \mu\text{m}$, Fig 6b) and rod-shaped patterns ($16\ \mu\text{m} \times 2\ \mu\text{m}$, Fig 6c) were stamped, activated, and tethered with AF488, and linear intensity profiles revealed good surface and pattern homogeneity. While slightly fainter and less homogeneous across the surface, rectangular patterns ($20\ \mu\text{m} \times 5\ \mu\text{m}$, Fig 6d) of tethered AF350 were also created using this same optimized μ -CP protocol. Overall, we have demonstrated the utility of this μ -CP mixed alkanethiol system to generate well-defined and customizable patterns (size, shape, spatial locale) with controlled single or multiple ligand densities.

Discussion

Development of effective surface patterning techniques to create micropatterned arrays with adjustable and controlled presentation of biological ligands is critical to the progress of a number of biotechnological applications, including biosensors, drug delivery systems, and *in vitro* analyses of cell-material interactions. Moreover, recent studies have suggested that the surface density and stability of bioadhesive ligands play a central role in cell adhesion, migration, and downstream function, along with adhesive area and geometry.²⁸⁻³⁷⁻⁴¹ Using a microcontact printing technique with mixed alkanethiol SAMs, we developed and optimized a hybrid ligand patterning protocol that affords controlled tethering of ligands in specific geometric patterns. The development of a simple, versatile, and high utility technique to generate micropatterned surfaces presenting tethered ligand is important to the engineering of platforms for rigorous studies of cell-material interactions and biotechnological/biomedical applications. The hybrid mixed alkanethiol μ -CP protocol presented takes advantage of the ligand tethering ability of COOH-terminated alkanethiolates, which can be modified to enable the immobilization of amine-presenting ligands³²⁻³³⁻⁴². Several recently developed 2-D patterning methods, such as the use of polymer films with ink-jet printer technology⁴³ and microfluidics networks⁴⁴, have focused on covalent, rather than adsorbed, ligand attachment on material surface patterns to better control surface density as well as afford control over multiple ligand patterning. However, considerable resources requirements and complex operation limit the everyday and widespread applicability of these methods. In contrast, the simplicity, speed, and minimal resources required of the modified μ -CP protocol presented in this study make it more appealing for many biotechnological and biomedical applications.

Many current ligand and cell patterning methods rely either on selective printing of ligand-adsorbing polymers into geometrically constrained wells/regions or direct printing of ligands onto substrates¹³⁻²⁷⁻³⁰⁻⁴³⁻⁴⁵. These methods generally afford very precise ligand surface placement and are advantageous to screening combinatorial libraries of ligands. However, these techniques also share various key drawbacks, including lack of long-term stable ligand deposition (due to passive ligand adsorption) and precise manipulation of ligand density. These shortcomings hinder their applicability to rigorous studies of ligand-cell interactions, biosensors, and kinetic analyses that require more long-lasting, stable, and ligand-surface parameter controls. The hybrid mixed alkanethiol μ -CP system that is developed and optimized in this study directly solves this lack of control over ligand pattern density while maintaining the aforementioned beneficial features of micro-contact printing. Although not specifically examined in this study, a potential added benefit of this system is more robust control over ligand orientation. Moreover, the covalent tethering of ligands may afford more stable patterned surfaces in physiological media.

Since parameter optimization of this protocol was conducted with one set stamp size (and PDMS composition), it is possible that the optimal stamp time and weight may be slightly altered in systems utilizing alternate stamp size/PDMS volumes. Nonetheless, in our studies, we have typically noticed very little change in pattern integrity and quality with use of stamp

sizes $\pm 50\%$ volume of the stamp used in this study (results not shown). In addition, stamp size did not seem to affect the quality of 5 μm or 20 μm patterns, although a meticulous analysis of these observations was not conducted. Therefore, it may be predicted that the stamping weight and time may need to be slightly increased for use of significantly larger volume stamps; vice versa for smaller volume stamps. Regardless, this study thoroughly delineates the major experimental parameters and general optimal values that should be used with this modified protocol.

This technique is applicable for controlled covalent patterning of a wide variety of ligands, including biomolecules such as proteins and peptides, polymers for drug delivery or sensing applications, antibodies for sensing arrays, and kinetic ligand analyses. This technique also could be easily applied to screening different densities and combinations of ligands on cells and testing potential drug compounds with controlled kinetic analysis. We present optimized parameters to generate spatially defined micropatterns with well defined surface densities of single and dual tethered ligands. While these optimized parameters were developed for two antibody ligands, it is possible that different processing parameters are needed to tether other ligands. A similar optimization strategy as that presented in this report could be easily adopted for other ligands as well as applications requiring more than two ligands tethered on single patterns.

Conclusions

Using microcontact printing techniques with mixed alkanethiol SAMs, we have developed and optimized a hybrid ligand patterning technique that affords controlled tethering of ligands in specific geometric patterns. We present optimized parameters to generate spatially defined micropatterns with well defined ligand densities of single and dual tethered ligands. This technique is applicable for controlled covalent patterning of a wide variety of ligands as well as screening combinatorial libraries of ligands in varying densities for study of cell-material interactions.

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Fig. 1. Microcontact printing (μ -CP) islands of self assembled monolayers (SAMs) of mixed alkanethiols (EG₃ and EG₆-COOH) onto gold-coated glass substrates. **(A)** Schematic of mixed alkanethiol SAM (95:5 EG₃:EG₆-COOH). **(B)** Microcontact printing procedure for mixed SAMs: 1) SAMs "inked" onto PDMS stamp, 2) SAMs stamped and subsequently transferred to substrate, 3) unstamped area backfilled with EG₃ to generate non-fouling background, 4) mixed alkanethiol islands "activated" through conversion of COOH groups into NHS esters via NHS/EDC chemistry, 5) tethering of amine-presenting IgG to ester-converted SAMs, 6) Rinsing of mixed alkanethiol SAM surface overnight to eliminate weakly bound, non-specific ligand (IgG) adsorption to surface. **(C)** Schematic of patterned tethered islands of IgG (white circles) in a non adhesive background (black). Circular islands used in this study were 20 μ m diameter, 75 μ m spacing apart.

Fig. 2. Correlation of AF488 tethered surface density between SPR and fluorescent intensity image quantification (FIIQ) (A) SPR measurement profiles on μ -CP or unpatterned slides using a 5% COOH-EG₆:EG₃ SAM. AF488 coating concentrations of either 100 μ g/ml or 25 μ g/ml were flowed over activated surfaces. SPR steps were: (a) AF488 adsorption on unactivated surfaces, (b) 0.1% SDS wash, (c) NHS/EDC surface activation, (d) AF488 tethering to activated surfaces, (e) 0.1% SDS wash. (B) Table summarizing quantitative AF488 surface density results from SPR experiments, FIIQ, and predicted relative density based on ratio of island:background surface area. (C) Graphs highlighting close correlation between SPR and FIIQ techniques for quantifying tethered IgG ligand surface density.

Fig. 3.

Control over density of tethered ligand on μ -CP islands. **(A)** Fluorescence images of AF488 (green) and AF350 (blue) tethered-antibody on μ -CP circular islands. AF488-tethered island images were at 200, 100, 50, 25 μ g/ml coating concentration, while images of AF350 were at 300, 150, 75, 37 μ g/ml dilutions. Inset graphs are line intensity profiles at the prescribed linear path indicated on each image. Scale bar is 25 μ m. **(B)** Demonstration of control over tethered antibody density via graph of normalized total fluorescence intensity on each sample at varying coating concentrations of AF488. **(C)** Graph of normalized total intensity on each sample at varying coating concentrations of AF350.

Fig. 4.

Pattern homogeneity is maintained over a range of tethered ligand density on μ -CP mixed SAM surfaces. Images are representative of patterns of specific antibody densities, including line intensity profiles at the indicated linear path. Scale bar is 8 μ m. **(A)** AF488, 200 μ g/ml. **(B)** AF488, 100 μ g/ml. **(C)** AF488, 50 μ g/ml. **(D)** AF488, 25 μ g/ml. **(E)** AF350, 300 μ g/ml. **(F)** AF350, 150 μ g/ml. **(G)** AF350, 75 μ g/ml. **(H)** AF350, 37 μ g/ml.

Fig. 5. Multiple ligands can be tethered in a controlled manner to μ -CP mixed alkanethiol SAM islands. Representative fluorescent images of 4 extreme multi-ligand coating concentrations exemplify precise control over each antibody density on the same mixed alkanethiol SAM pattern. Scale bar is 25 μ m. (A) AF350 (300 μ g/ml), followed by AF488 (200 μ g/ml). (B) AF350 (75 μ g/ml), AF488 (200 μ g/ml). (C) AF350 (75 μ g/ml), AF488 (25 μ g/ml). (D) AF350 (75 μ g/ml), AF488 (25 μ g/ml). (E) Controlled ratios of tethered AF350:AF488 on the same islands can be engineered by varying each antibody coating concentration and exposure time on activated mixed alkanethiol SAM patterns. 3-D plot of normalized relative surface density of tethered AF488 and AF350 antibodies on 25 different multi-antibody coating concentration combinations on μ -CP mixed alkanethiol patterns. (mean $\pm$ st. error, N = 4).

Fig. 6. μ -CP mixed alkanethiol SAM patterns of different sizes and shapes can be prepared by modifying the stamp design. Representative images (scale bar is 5 μ m) and corresponding line profiles of: (A) Multi-shaped mixed alkanethiol patterns on one surface (AF488, 200 μ g/ml). (B) AF488-tethered rectangular islands (100 μ g/ml). (C) AF488-tethered rod-shaped islands (200 μ g/ml). (D) AF350-tethered long rectangular islands (150 μ g/ml).

Summary of entire optimization procedure and analysis of each major variable/condition investigated, including stamping time, stamping weight, mixed SAM ratio, and post-surface activation ligand exposure time. Each variable was scored on the relative success of 4 different functional outcomes on a qualitative scale of 1 (poor) to 4 (worked perfectly) “pluses”. The overall best working condition of each variable set is bolded and the corresponding relative scores highlighted in red. (N≥3 was performed for each condition).

Table 1

VARIABLES	OUTCOME	Surface Homogeneity	Pattern Homogeneity	Pattern Integrity	Fluorescence Intensity
<u>Stamping time</u>	15s	+	++	++	+
	30s	++	++	++++	++
	1 m	+++	+++	+++	+++
	1.5 m	++++	+++	+++	+++
	2 m	+++	+++	++	+++
	5 m	+++	+++	+	+++
<u>Stamp weight</u>	50g	+	+++	+++	+
	100g	++	+++	+++	+++
	150g	+++	+++	+++	+++
	200g	+++	+++	+++	+++
<u>Mixed SAM ratio (EG₆-COOH:EG₃)</u>	1%	+++	++	+++	+++
	5%	++++	+++	+++	+++
	10%	+++	+++	++	+++
<u>Ab exposure time</u>	10 m	+	+++	+++	+
	30 m	+++	+++	+++	+++
	45 m	++++	+++	+++	+++
<u>Single-Ab</u>	60 m	+++	+++	+++	+++
	20m-30m	++	+++	+++	++/+++
	40m-30m	+++	+++	+++	+++/+++
	60m-30m	+++	+++	+++	+++/++
	20m-60m	+++	+++	+++	++/+++
	40m-60m	++++	+++	+++	++++/++++
	60m-60m	+++	+++	+++	+++/+++
<u>Mixed-Ab (1STAb-2NDAb)</u>					