Surface Control of Blastospores Attachment and Ligand-Mediated Hyphae Adhesion of *Candida albicans*

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Supporting Information

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1. Materials and Methods

Chemicals, Media and Strains

1-Pentadecanethiol, (3-*N*-morpholino)propane sulfonic acid (MOPS), sulfuric acid (95-98%), phosphate buffer saline (PBS) and bovine serum albumin (BSA) were purchased from Sigma chemicals (St. Louis, MO). Hydrogen peroxide (30%) used in piranha cleaning and methanol used in drying of piranha cleaned slides were purchased from Fisher chemical (Fair Lawn, NJ). Absolute ethanol (200 proof) used for washing and cleaning of surfaces in the study was purchased from Pharmaco-Aaper (Shelbyville, KY). Glass slide substrates used for deposition of gold were obtained from Fisher's finest premium microscope slides (Pittsburgh, PA). The media that supports budding phase of blastospores was prepared using yeast extract purchased from Fulka (St. Louis, MO), bacto peptone purchased from BD and Co (Sparks, MD), dextrose purchased from Sigma (St. Louis, MO). Hyphae supporting media was prepared from RPMI1640 with glutamine purchased from (Cellgro, Manassas, VA), that was buffered to pH 7.02 using 0.165 M MOPS before being used for assay. 24-well plate used in the study was purchased from Nunc (Thermofisher Scientific, Pittsburgh, PA). Literature reported methods were used in the synthesis of 1-amino undecanethiol,[1] tri(ethylene glycol)-terminated undecanethiol,[2] D-mannitol-terminated undecanethiol[3] and disulfide of 3-(2-(2-(2-(2-(11-mercaptoundecyloxy) ethoxy) ethoxy) ethoxy) ethylamino)-4-phenoxycyclobut-3-3n3-1,2-dione/ phenoxy squarate(PSQ)-terminated[4] SAM molecules, by previous members in the lab. The fungal strain *Candida albicans* SC5314 a clinical isolate was used throughout the experiments to study of adhesion behavior of fungus on chemically designed surfaces.

Piranha Cleaning of Glass Substrates

Fisher's finest premium microscope slides were cleaned with piranha solution prior to gold deposition. Piranha solution (7 parts of concentrated sulfuric acid and 3 parts of hydrogen peroxide (30 % in water)) was used to clean the surface of the glass slides before depositing the gold, the glass slides were soaked in the piranha solution at 70 $^{\circ}$C for 45 min. The piranha solution was poured off after cooling and the slides were repeatedly rinsed 20 times with deionized water, 10 times with ethanol and 10 times with methanol. The slides were later dried in nitrogen and kept in oven overnight at 100 ºC, prior to metal deposition by electron beam evaporator.[5]

Deposition of Gold Films

The deposition of semi-transparent gold films on glass slides cleaned with piranha solution was achieved, by using an electron beam evaporator system (Thermionics, Port Townsend, WA). The glass slides are placed on a stationary holder to ensure uniform deposition of gold at fixed incident angle of 45° to the glass surface. The surface is initially coated with titanium (~70 Å) to enhance the adhesion of the gold, which then was deposited at a rate of 0.1 Å/s to a thickness of (~280 Å). Before being used in the experiment the gold coated glass slides were cleaned in ethanol (200 proof) and dried in a stream of nitrogen.

Preparation of Patterned Molonalyers of Pentadecanethiol Surrounded by phenoxy squarate-, amine-, D-mannitol-, tri(ethylene glycol) terminated alkanethiols

The gold slides were cut into 1 cm x 1 cm squares, washed with ethanol (200 proof) and dried in a stream of nitrogen. The clean dried gold films were later stamped for 20 seconds, with polydimethylsiloxane (PDMS) stamps dabbed with a thin film of 2 mM ethanolic solution of pentadecanethiol. The pentadecanethiol stamped gold slides were cleaned with ethanol and dried

under a stream of nitrogen before immersing them in 2mM ethanolic solution of 1- amino undecanethiol or tri(ethylene glycol) terminated undecanethiol or D-mannitol terminated undecanethiol SAMs for 18h. It should be noted that, for the PSQ-terminated SAMs the solutions consisted of 97% tri(ethylene glycol) undecanethiol and 3% PSQ alkanethiol in ethanol. [6] Following the 18 h immersion the gold slides were rinsed in ethanol and dried under a stream of nitrogen before using them to study fungal adhesion.

Surface Modification by Peptide and Protein BSA Immobilized on Phenoxy Squaramate (PSQ-) and Amine-Terminated SAMs respectively

The surfaces of the PSQ-terminated SAMs were treated with 2mM aqueous solutions of the peptides with cysteine at C-terminus N'–AGRGDSC-C' or N'–AGGRDSC-C' buffered at pH 7.52. The drops of aqueous buffered peptides were placed at desired positions of the PSQ-terminated SAMs surface. The SAM surfaces were placed covered and undisturbed on raised platforms, created within a petridish filled with autoclaved water for 4 h. The SAM surfaces were rinsed with the PBS buffer pH 7.52 before being used for the adherence assay.

Similarly protein bovine serum albumin (BSA) was coated on the amine-terminated SAMs by placing the gold slides with amine-terminated SAMs, for 4 h in vial containing 5% BSA solution prepared in deionized water and rinsed with the PBS buffer pH 7.52 before being used for the adherence assay.

Media Preparation and Growth Conditions

For culturing blastospores, overnight culture of *C. albicans* SC5314 was prepared by inoculating 5 ml of yeast peptone dextrose (YPD) media (1%, w/v yeast extract; 2%, w/v of peptone, 2%, w/v of dextrose in autoclaved water) with *C. albicans* from glycerol stocks maintained at -80°C. The inoculum was placed in orbital shaker (250 rpm) overnight at 37 °C to

produce phase of budding blastospores. The overnight culture of *C. albicans* in YPD media was sub-cultured next day to provide with working culture having optical density of OD_{600} = 0.05 measured by Biotek ELx800 absorbance plate reader which uses Gen 5^{TM} data analysis software.

For inducing growth of hyphae, cells were harvested from 5 ml overnight culture of *C. albicans* in YPD media by centrifugation at 3000*g* for 5min at room temperature. The supernatant was removed and the yeast pellets were re-suspended in 20 ml of sterile PBS pH 7.52. The PBS solution with yeast cells were centrifuged and the process was repeated twice before addition of 20 ml of RPMI 1640 media buffered to pH 7.04 with 0.165M MOPS was pre-warmed to 37°C. The resulting cell suspension was used to obtain a final working cell suspension of 1 x 10^5 cell mL^{-1} of *C. albicans,* by 1:100-fold dilution with RPMI 1640, which was further used in assay to study adhesion of hyphae on SAM surfaces.

Adhesion assay for studying cell adhesion of Candida albicans on SAM modified surfaces in static medium conditions

The slides with SAMs were placed into inner eight wells of the 24-well culture plate for adhesion studies. To study the adhesion of blastospores or hyphae on SAMs 1 ml blastospore culture or hyphal culture were pipetted out into the inner eight wells. The outer 16 wells of the 24-well culture plate were filled with autoclaved water to prevent drying in the inner wells. The culture plate were wrapped in Saranwrap™ and placed on incubator at 37°C. The glass slides placed in the culture were removed from the media at intervals of 2 h and the slides were not washed before taking images using Olympus 4X wide zoom camera; viewed from Olympus

BX51 microscope. It has been observed gentle washing of the hyphae or blastopsore patterned SAMs did not lead to removal of the adhered blastospore or hyphae from the SAM surfaces.

Inhibition of Fungal Cell Adhesion Assay

C. albicans in RPMI1640 were added into eppendorf tubes followed by addition of desired amount of cyclic pentapeptide (RGDFK) containing RGD sequence at 1mM to attain a final volume of 2 mL (were the concentration of cyclic pentapeptide (RGDFK) ranged from 500 to10 μM) and these eppendorf tubes were placed in incubator at 37 ºC for 1h. The SAM patterned surfaces with RGD tripeptide were places in 24 well-plate followed by addition of *C. albicans* cells containing cyclic pentapeptide (RGDFK). SAM surfaces were placed in the presence of static media and observed under optical microscope at intervals of 2h from the time of incubation until 4h.

2. Patterned Chemistry Used for Studying Surface Anchoring of *Candida albicans* Blastospores and Hyphae

The adhesion behavior of *C. albicans* on non-bioinert surfaces of amine-, methyl- and proteins (bovine serum albumin) immobilized on amine-terminated SAMs; were studied using SAMs surfaces as described in (Figure S1).

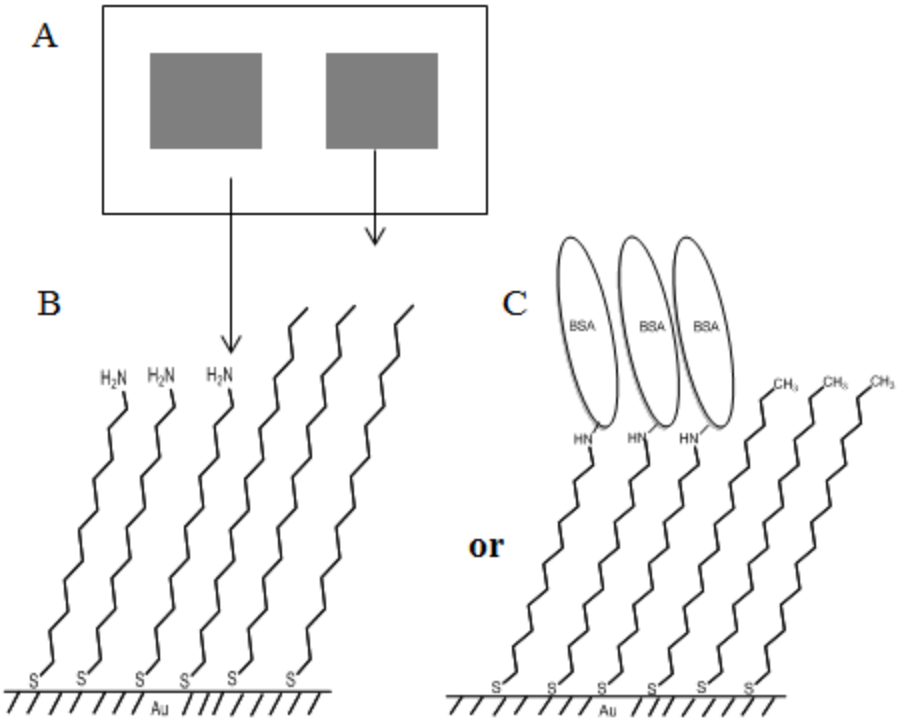


Figure S1. A) Schematic representation of patterned SAMs of pentadecanethiol $HS(CH_2)_{14}CH_3$ (black squares) surrounded by B) undecyl amine-terminated SAMs or C) undecyl amine-terminated SAMs immobilized with bovine serum albumin (BSA).

3. Adhesion of Blastospores and Hyphae of *Candida albicans* on Amine, and Methyl-terminated Monolayers, and on Bovine Serum Albumin Coated Monolayers

The adhesion behavior of *C. albicans* on non-bioinert surfaces of amine-, methyl- and protein (bovine serum albumin (BSA)) immobilized on amine-terminated SAMs reveal that, adhesion of *C. albicans* on non-bioinert surfaces doesn't suffer any resistance in attachment of blastospores and hyphal-forms of *C. albicans* as seen in (Figure S2). The attached biomass of blastospores and hyphal forms remained attached on the SAM surfaces and were not detached when monitored at the end of 24h.

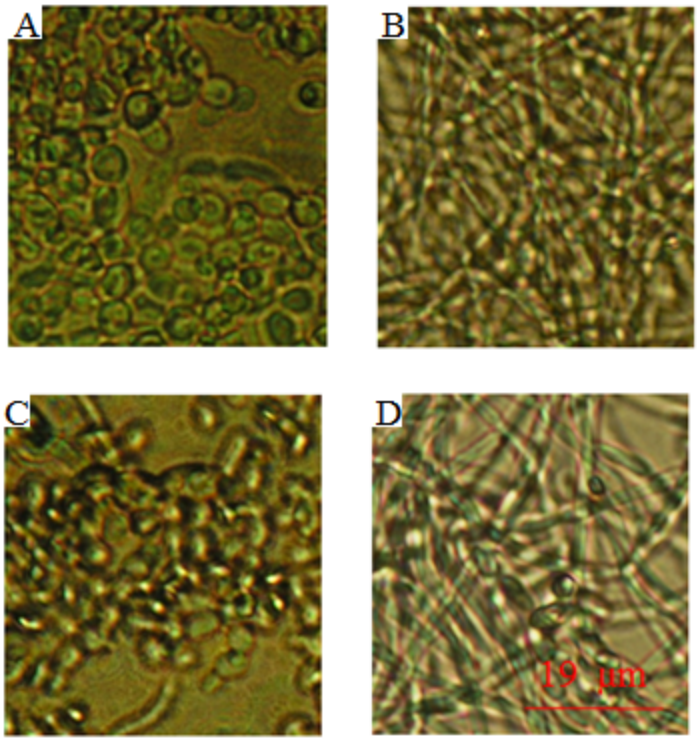


Figure S2. Optical micrograph images for adhesion of *C. albicans* blastospores cultured to OD_{600} = 0.05 in YPD media on surfaces comprising of A) pentadecanethiol square patterns surrounded by undecyl amine-terminated SAMs and C) BSA immobilized on undecyl amine-terminated SAMs that surround pentadecanethiol square patterns; and *C. albicans* hyphae cultured to (1:100) dilution in RPMI 1640 on B) pentadecanethiol square patterns surrounded by undecyl amine-terminated SAMs and D) BSA immobilized on undecyl amine-terminated SAMs that surround pentadecanethiol square patterns.

4. Hyphal Outgrowth Interconnects the Hyphae Patterned by Undecyl tri(ethylene glycol) SAMs on Monolayers of Pentadecanethiol

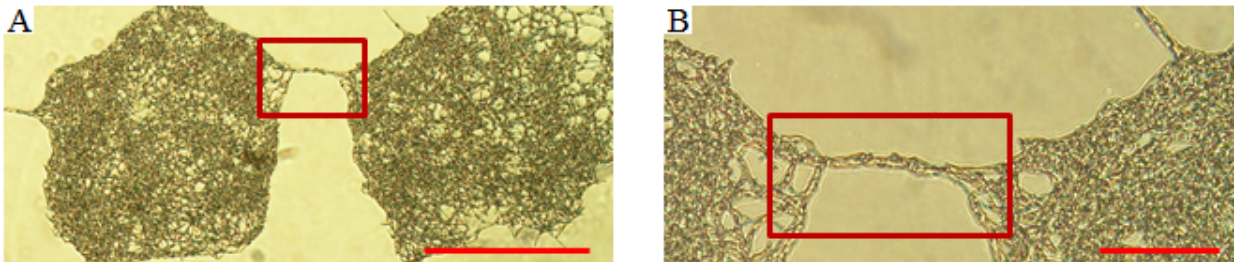
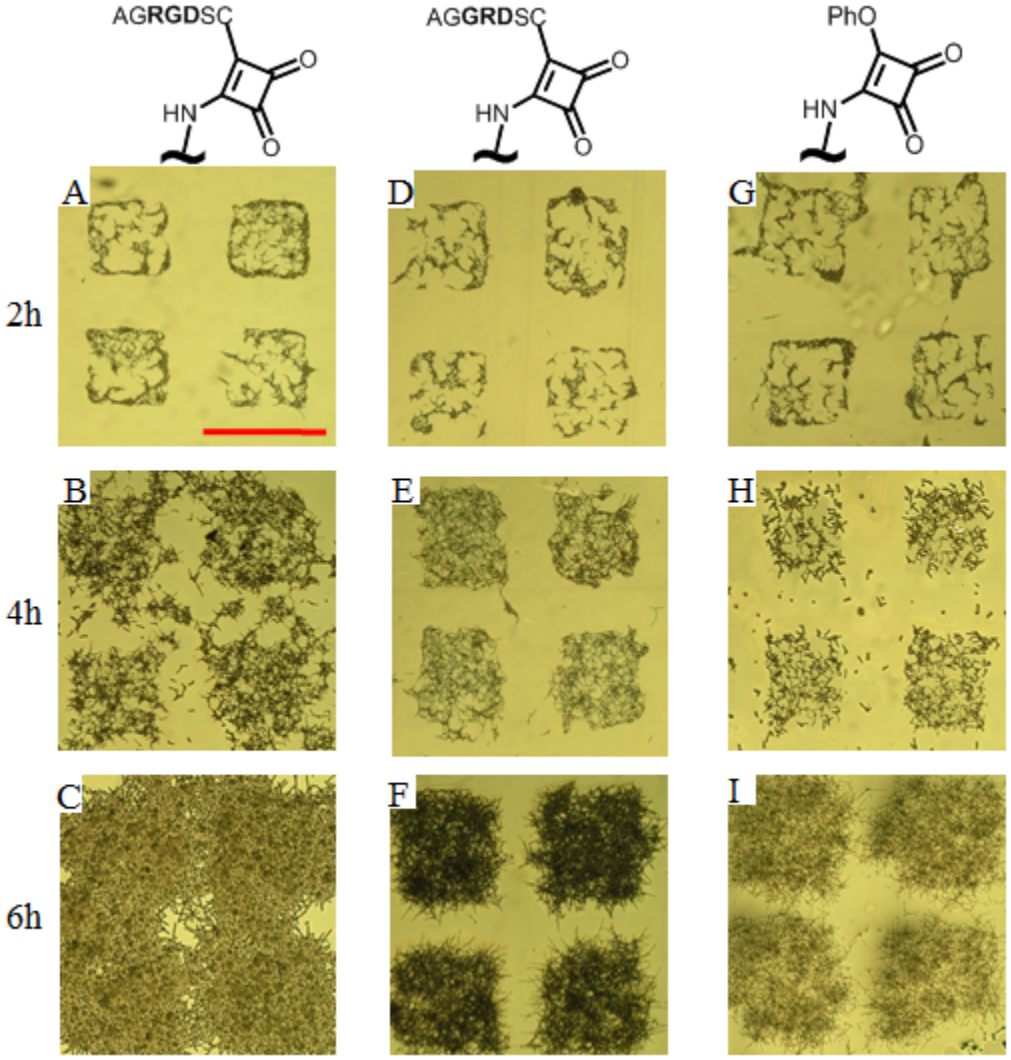


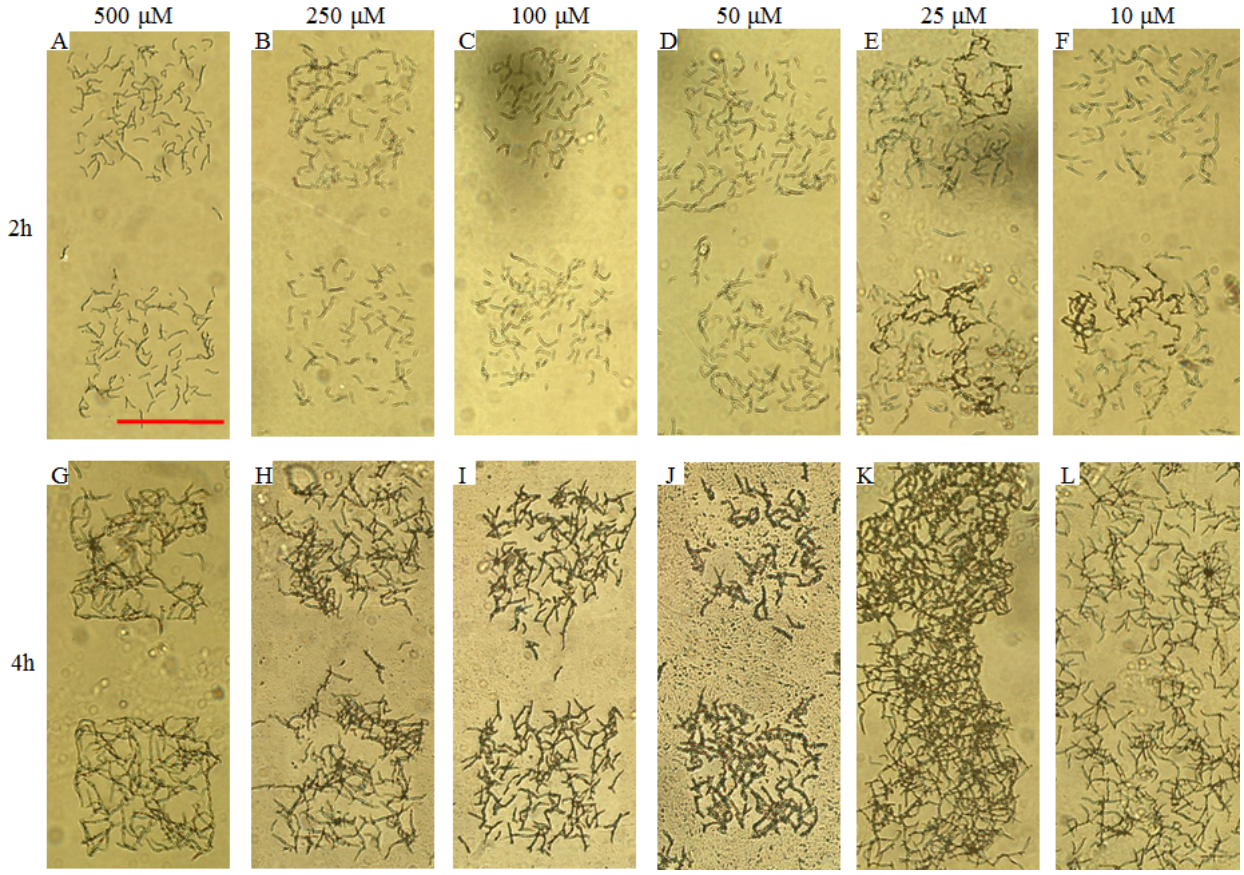
Figure S3. Optical micrograph images for adhesion of *C. albicans* hyphae at end of 6h cultured in RPMI1640 on monolayers of pentadecanethiol square patterns surrounded by undecyl tri(ethylene glycol) A) the patterned hyphal biomass show interconnections between two patterns at scale bar = 380 μm, B) magnified image of the interconnected hyphae scale bar = 76 μm.

5. Surfaces with Immobilized Sequence of Peptide Arg-Gly-Asp (RGD) Fail to Pattern *Candida albicans* Hyphae



S4. Optical micrograph images taken at interval of 2 hours for adhesion of *C. albicans* hyphae (at 1:100 dilution in RPMI1640) on 380 μm-wide square patterns of pentadecane thiol, surrounded by SAMs immobilized with tripeptide sequence of N'-AGRGDSC-C' in (A) to (C), N'-AGGRDSC-C' in (D) to (F) and phenoxy squaramate in (G) to (H). Scale bar = 608 μm.

6. Adhesion of Hyphae resisted by 100 µm of Cyclic Pentapeptide RGD



S5. Optical micrograph images taken at 2h (A to F) and 4h (G to L) for adhesion of *C. albicans* hyphae in RPMI1640 containing cyclic peptide (RGDFK), on 380 µm-wide square patterns of pentadecane thiol, surrounded by bioinert SAMs containing immoboilized tripeptide sequence of N'-AGRGDSC-C'. Scale bar = 380 µm.

7. Hyphae confinement on three pattern geometries

The hyphae of Candida albicans is confined in large, but not small, patterns by bioinert chemistry on self-assembled monolayers. Three pattern geometries were used to study the effect of pattern size and separation for confining and inhibiting hyphae formation on monolayers. These patterns include 600x600 μm^2 of methyl-terminated monolayers separated by 410 μm of either undecyl tri(ethylene glycol) or mixed monolayers presenting the tripeptide (RGD) ligands, 380x380 μm^2 separated by 315 μm, and 84x84 μm^2 separated by 72 μm.

Media RPMI1640 media containing *Candida albicans* were places on these surfaces to study the dependence of hyphae adhesion on size of monolayer patterns. The optical micrographs were recorded following 2h (Figure S6) and 4h (Figure S7) of placing of the patterned slides in the culture media. The patterned monolayers on gold films surrounded by undecyl tri(ethylene glycol) (Figure S6 & S7, column A) or RGD-decorated monolayers (Figure S6 & S7 Columns B & C) were removed from culture media (without washing) and examined under an optical microscope to record adhesion behaviour of hyphae.

To study the effect pattern sizes on hyphae adhesion, methyl-terminated square patterns of 600-μm^2 separated by 410 μm of bioinert regions (Figure S6 & S7, row 1); 380-μm^2 separated by 315 μm of bioinert regions (Figure S6 & S7, row 2), and 84-μm^2 separated by 72 μm of bioinert regions (Figure S6 & S7, row 3) were surrounded by tri(ethylene glycol)- or RGD immobilized SAMs, which were placed in hyphae culture grown in absence or in presence of cyclicpeptide RGDFK.

We observed that hyphae were confined for 4 h in the 600 and 380 μm^2 square patterns tri(ethylene glycol)-terminated SAMs (Fig. S6 & Fig. S7, 1A & 2A). However, no well-defined hyphae patterns were observed for 84 μm^2 square patterns separated by 72 μm. When the RGD tripeptide was decorated on surfaces, hyphae were observed to outgrow into the bioinert regions (Fig. S6 & S7, Column B). When cyclic RGD was introduced into the solution, well-defined hyphae patterns were retained (Fig. S6 & S7, 1C & 2C) for large squares (600 and 380 μm^2).

We note that while no well-defined hyphae pattern was observed for 84-μm^2 squares separated by 72 μm , streaks of hyphae was still vaguely seen at 2 h of culture. Thus, we believe that the primary reason for not maintaining the well confinement of hyphae is due to the narrow separation rather than the small squares.

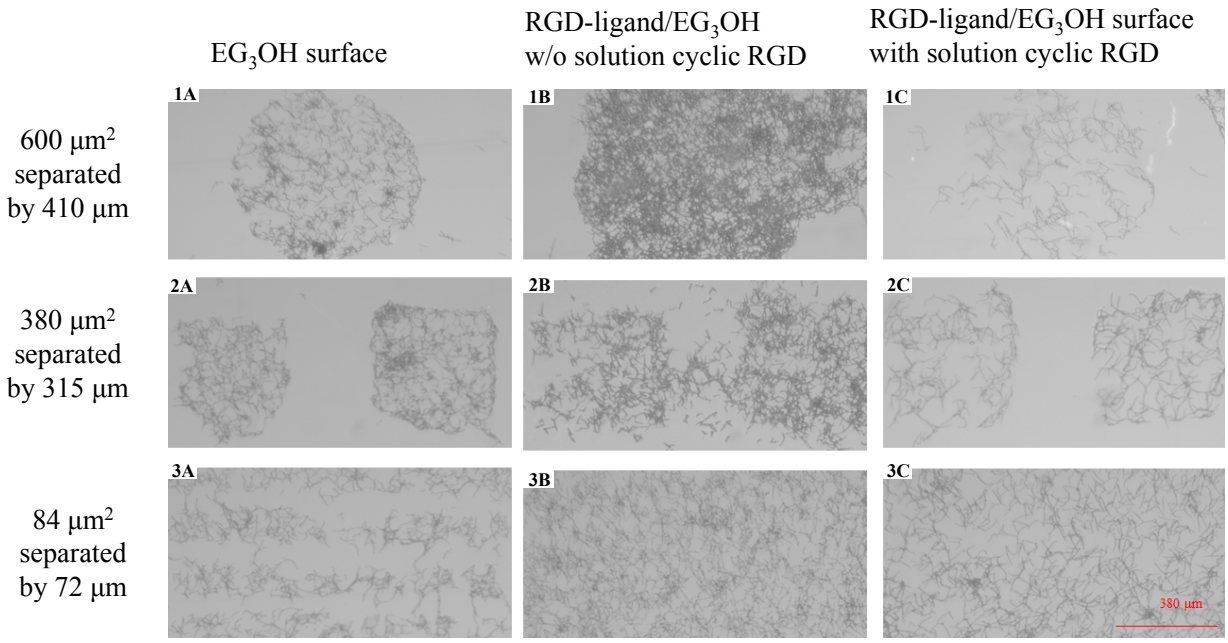


Fig. S6. Hyphae adhesion at 2 h on 600-μm^2 square patterns of separated by 410 μm (row 1), 380-μm^2 squares separated by 315 μm (row 2), and 84-μm^2 squares separated by 72 μm (row 3) on tri(ethylene glycol)-terminated SAMs (column A); RGD-decorated SAMs without (column B) and with (column C) the presence of cyclicpeptide RGDFK in media.

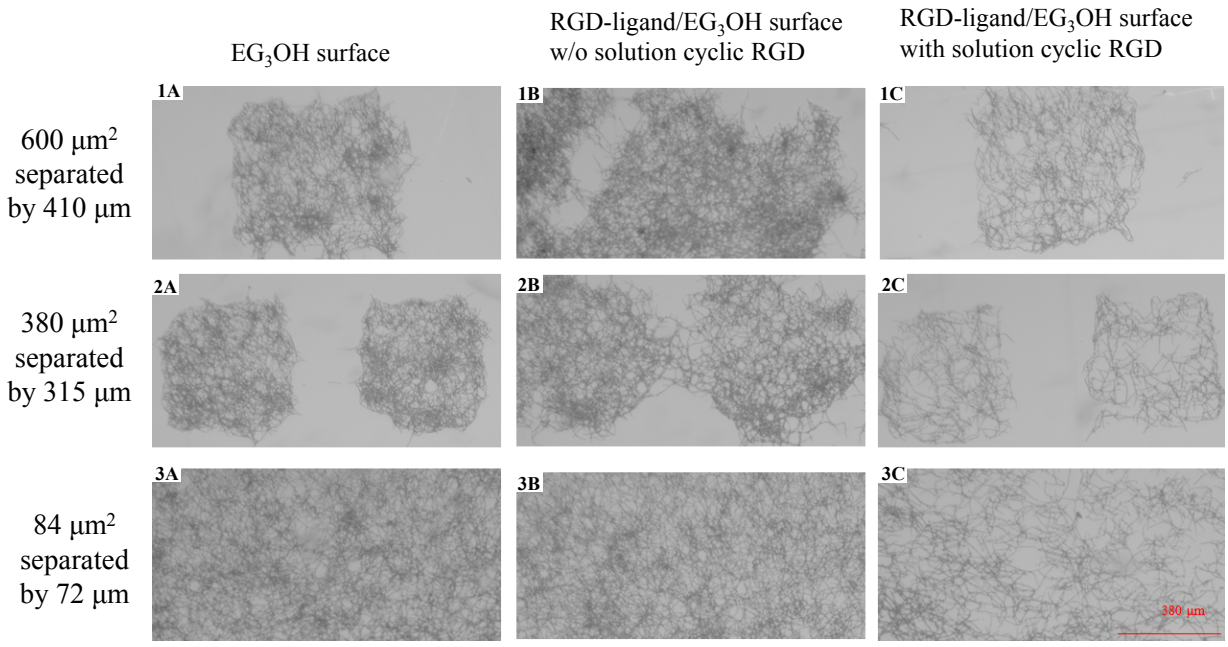


Fig. S7. Hyphae adhesion at 4 h on 600-μm^2 square patterns of separated by 410 μm (row 1), 380-μm^2 squares separated by 315 μm (row 2), and 84-μm^2 squares separated by 72 μm (row 3) on tri(ethylene glycol)-terminated SAMs (column A); RGD-decorated SAMs without (column B) and with (column C) the presence of cyclicpeptide RGDFK in media.

References

(1) Bandyopadhyay, D.; Prashar, D.; Luk, Y.-Y. Stereochemical effects of chiral monolayers on enhancing the resistance to mammalian cell adhesion *Chemical Communications (Cambridge, United Kingdom)* 2011, *47*, 6165-6167.

(2) Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure HS(CH2)11(OCH2CH2)mOH on gold *Journal of the American Chemical Society* 1991, *113*, 12-20.

(3) Luk, Y.-Y.; Kato, M.; Mrksich, M. Self-Assembled Monolayers of Alkanethiolates Presenting Mannitol Groups Are Inert to Protein Adsorption and Cell Attachment *Langmuir* 2000, *16*, 9604-9608.

(4) Sejwal, P.; Narasimhan, S. K.; Prashar, D.; Bandyopadhyay, D.; Luk, Y.-Y. Selective Immobilization of Peptides Exclusively via N-Terminus Cysteines by Water-Driven Reactions on Surfaces *Journal of Organic Chemistry* 2009, *74*, 6843-6846.

(5) Simon, K. A.; Burton, E. A.; Han, Y.; Li, J.; Huang, A.; Luk, Y.-Y. Enhancing Cell Adhesion and Confinement by Gradient Nanotopography *Journal of the American Chemical Society* 2007, *129*, 4892-4893.

(6) Bandyopadhyay, D.; Prashar, D.; Luk, Y.-Y. Anti-Fouling Chemistry of Chiral Monolayers: Enhancing Biofilm Resistance on Racemic Surface *Langmuir* 2011, *27*, 6124-6131.