

Ultrathin Gradient Films Using Thiol-Ene Polymerizations

VAIBHAV S. KHIRE,¹ DANIELLE S. W. BENOIT,¹ KRISTI S. ANSETH,^{1,2} CHRISTOPHER N. BOWMAN^{1,3}

¹Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, Colorado 80309-0424

²Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309-0424

³Department of Restorative Dentistry, University of Colorado Health Sciences Center, Denver, Colorado 80045-0508

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ABSTRACT: The application of surface-attached, thiol-ene polymer films for controlling material properties in a gradient fashion across a surface was investigated. Thiol-ene films were attached to the surface by first depositing a thiol-terminated self-assembled monolayer and performing a thiol-ene photopolymerization reaction on the surface. Property gradients were created either by creating and modifying a gradient in the surface thiol density in the SAM or by changing the polymerization conditions or both. Film thickness was modified across the substrate by changing either the density of the anchoring thiol functional groups or by changing the reaction conditions such as exposure time. Thicker films (1–11 nm) were obtained by polymerizing acrylate polymer brushes from the surface with varying exposure time (0–60 s). The two factors, that is, the surface thiol density and the exposure time, were combined in orthogonal directions to obtain thiol-ene films with a two-dimensional thickness gradient with the maximum thickness being 4 nm. Finally, a thiol-acrylate Michael type addition reaction was used to modify the surface thiol density gradient with the cell-adhesive ligand, Arg-Gly-Asp-Ser (RGDS), which subsequently yielded a gradient in osteoblast density on the surface. © 2006 Wiley Periodicals, Inc. *J Polym Sci Part A: Polym Chem* 44: 7027–7039, 2006

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INTRODUCTION

Material properties such as modulus, wettability, and surface chemistry are routinely altered and controlled to optimize material design in an efficient manner (e.g., screening cell–material interactions for biomaterial applications). Several novel approaches have been proposed and employed for conducting high throughput research in which a variety of parameters are simultaneously tested.^{1–7} Techniques such as microarrays and microfluidics

have been developed in which small amounts of chemicals are used to perform a wide range of experiments on a single chip and increase the speed of evaluation.^{8–12}

Most of the methods developed for high throughput research change the experimental parameters discretely and hence have limitations in terms of accuracy with which the parameters can be varied. A distinct approach for achieving this goal is to employ methods in which the reaction parameters are changed in a gradual manner. Physical and chemical properties such as temperature, composition, thickness, and reaction conditions are altered gradually across a single sample, making it possible to obtain a vast amount of data

Correspondence to: C. N. Bowman (E-mail: christopher.bowman@colorado.edu)

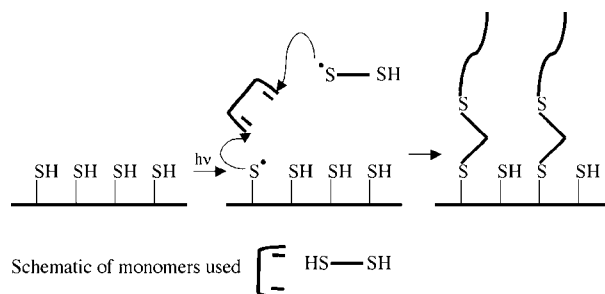
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from a single experiment.^{2,3,13–16} Because of these advantages, various aspects of polymer science, such as polymerization kinetics, polymer microstructure, mechanical properties, and polymer blend properties have been characterized in a high throughput manner by utilizing gradients in monomer composition, initiation conditions, and polymerization conditions.^{1,3,4,6,17,18}

Although these high throughput gradient techniques are more common in experiments investigating bulk physical properties, approaches for studying properties confined to a surface are somewhat limited.^{2,5,14,17,19} The most common technique used to obtain surface chemical gradients involves the creation of a bulk property gradient followed by its replication onto a surface.¹⁹ Other methods involve diffusion through a porous material over a surface, followed by adsorption on the surface.¹⁵ Using these techniques, properties of surface-bound polymers are systematically controlled, and their effect is studied.

Combinatorial methods have also been extended to thin polymer films attached to a surface.^{2,13,18,20,21} Surface-bound polymer films have applications in numerous fields including microelectro mechanical systems (MEMS), biosensing, material science, biotechnology, and nanotechnology.²² Properties such as wettability, corrosion and friction resistance, thickness and surface chemical density are readily varied across the surface to form films and the suitability of the polymer coating is investigated. Various methods of growing polymer films such as atom transfer radical polymerization (ATRP),^{23,24} controlled and living radical polymerization,^{25,26} reversible addition-fragmentation chain transfer (RAFT)²⁷ and photopolymerization²⁸ have been used for surface modification and gradient techniques have been employed to obtain films with variable properties.^{21,29}

Thiol-ene polymerization is a novel scheme used for surface modification where a thiol molecule adds across a carbon-carbon double bond to form a thiol-ene product.^{28,30–35} The thiol-ene reaction is a radical-mediated, step-growth polymerization mechanism, in which the propagation and chain transfer steps occur consecutively. To graft thiol-ene films to a surface, the surface is first functionalized with a thiol-terminated self-assembled monolayer (SAM), and a thiol-ene reaction is carried out on and above the surface. The surface thiols participate in the reaction, and a surface-bound polymer film is formed. This mechanism is shown schematically in Scheme 1. Since polymer chains are formed in the bulk and subse-



Scheme 1. Thiol-ene film attachment on a thiol-terminated SAM surface. When a thiol-ene polymerization is carried out on a thiol-terminated SAM surface, thiyl radicals are formed on both the surface and in the bulk. Surface radicals take part in the bulk polymerization reaction and the thiol-ene film that is formed is attached to the surface.

quently attached to the surface and also formed as a result of direct, surface-initiated polymerization, this grafting scheme is an intermediate between the 'grafting to' and 'grafting from' approaches. This scheme has been employed successfully for controlling film thickness²⁸ and for obtaining spatially patterned films.³⁵ Other characteristics of thiol-ene polymerizations, which are useful for surface modification applications, include initiatorless polymerization, versatile chemistry, and rapid polymerization rates.³⁶

In addition to the attachment of thiol-ene films, acrylate polymerization can also be carried out on the surface, and linear acrylate polymers can be attached to the surface. Chain transfer to surface thiols results in a surface bound radical, which initiates an acrylate polymerization from the surface, forming a covalently attached acrylate film. As is the case with thiol-ene polymerizations, initiation takes place both in the bulk and on the surface in this case as well. Since acrylates react via chain addition mechanism longer chains are formed and a thicker polymer film is attached to the surface.^{32–34,37}

Here, surface modification using thiol-ene polymerizations is extended to controlling surface properties in a gradient fashion. The thickness of the film produced by a linear thiol-ene reaction is controlled by two factors, the density of chains on the surface and the length of each chain. Depending on the potential application, controlling each of these factors becomes important. For example, a change in surface density is desired when a specific functionality like an initiator molecule, a catalyst or a cell-adhesive ligand is presented from the surface. The study of the change in average chain

length is important to test the effectiveness of a coating. For example, the thickness of a coating can determine its effectiveness in applications such as corrosion resistance and biocompatibility.

Here, these two factors are changed independently, and in conjunction with one another, to obtain thickness gradients. A surface thiol gradient was formed by the diffusion of silane across the surface and the surface thiols were subsequently modified with linear thiol-ene polymers to obtain a gradient in the density of thiol-ene chains, leading to a thickness gradient. A gradient in average chain length was obtained by changing the exposure time to ultraviolet light, while maintaining the surface thiol density constant. These two methods were combined in orthogonal directions to obtain a two-dimensional thickness gradient. The formed samples were characterized using ellipsometry and water contact angle measurements.

The surface thiol density gradient can be coupled with the versatile thiol-ene reactions to attach numerous functionalities to the surface for a variety of applications. Specifically, it was used here for investigating cell-material interactions. A cell adhesive ligand, Arg-Gly-Asp-Ser-acrylate (RGDS-acrylate) was attached across the surface in a controlled fashion using thiol-acrylate Michael addition reaction.^{38,39} RGDS, a critical cell adhesive domain of many proteins, has been widely investigated to modulate cell-material interactions.⁴⁰ The RGDS gradient was then used as a rapid test for the effect of RGDS concentration on osteoblast adhesion.

EXPERIMENTAL

Materials

The silanes decyltrichlorosilane (DTS), 3-mercaptopropyltrimethoxysilane (MPTMS), and trichloro (1*H*, 1*H*, 2*H*, 2*H* perfluorooctyl) silane (FSAM) were obtained from Aldrich. Mineral Oil (paraffin oil, heavy) used for gradient SAM formation was obtained from Fisher Chemicals, Fairlawn, NJ. The thiol-ene monomer system used was a mixture of 1,6-hexane dithiol (HDT) (Aldrich) and triethyleneglycol divinyl ether (DVE-3). Acrylic brushes were formed from *tert*-butyl acrylate (tBa) (Aldrich) (Fig. 1). DVE-3 was provided as a sample (Rapi-cure[®] DVE-3) from ISP Technologies (Wayne, NJ). The UV photoinitiator, 2,2-dime-

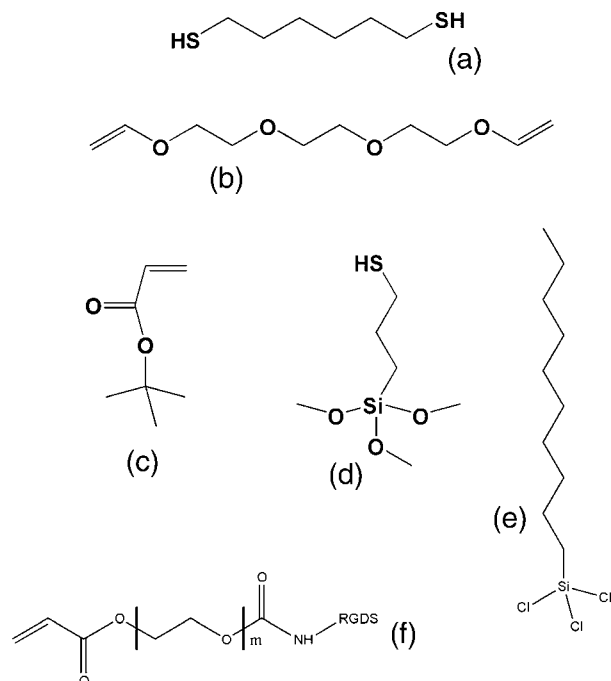


Figure 1. Chemical structure of various monomers used (a) 1,6 hexane dithiol, (b) tri(ethylene glycol) divinyl ether, (c) *tert*-butyl acrylate (d) 3-mercaptopropyltrimethoxysilane (e) decyl trichlorosilane (f) acrylated RGDS ($m = 77$).

thoxy-2-phenylacetophenone (DMPA) was purchased from Ciba-Geigy (Hawthorn, NY).

Substrates

Polished, double-sided silicon wafers (Umicore Semiconductor Processing, Boston, MA) were used for all film growth studies. Wafers were in the (100) orientation and 760 microns thick. Substrates used for cell adhesion studies were precleaned, plain microscope slides obtained from Gold Seal[®] Products, Portsmouth, NH. All substrates were cleaned using “piranha” solution, which is a mixture of hydrogen peroxide and sulfuric acid (1:3 by volume) for 45 min, rinsed with deionized water, and blown dry with a N₂ stream prior to SAM deposition.

Thiol SAM Gradient Formation

A decyltrichlorosilane gradient was formed and backfilled with thiol SAM to obtain a thiol SAM gradient in the opposite direction as detailed below. A 1:10 mixture of DTS and mineral oil by weight was placed in a small container and the cleaned silicon or glass substrate was placed

with the long edge facing away from the container in such a way that the edge of the piece was at the same height as the brim of the silane:oil mixture container. The whole setup was enclosed in a glass Petri dish and placed at room temperature for 30 min. The silane molecules diffused to form a concentration gradient in the vapor phase, which then got replicated on the substrate. The substrate was removed and washed in toluene and acetone to remove any unattached, physisorbed silane from the surface and dried with nitrogen. The substrate was placed in a Teflon container with a vial containing ~ 0.5 mL of MPTMS and placed in an oven maintained at 90°C for 2.5 h. The substrate was washed again with toluene, then acetone, and dried with nitrogen. All substrates were characterized with a water contact angle goniometer and ellipsometry following each of the above steps.

Photopolymer Brush Formation

A stoichiometric mixture of DVE-3 and HDT was initiated with 0.1% photoinitiator, DMPA, to grow thiol-ene brushes from the substrates. The substrates with a gradient of thiol-terminated SAM were treated with a solution of dithiothreitol (DTT), which reduces disulfide bonds to thiols prior to polymer brush formation. Following the method of Jonsson et al., substrates were immersed in a 100 mM DTT solution in 10 mM potassium phosphate buffer for 10 min, then rinsed with deionized water and acetone and blown dry with nitrogen.⁴¹

To grow polymer brushes, a drop of the monomer mixture was sandwiched between the substrate and a glass slide coated with a solution of Teflon Amorphous Polymer (DuPont Fluoroproducts, Wilmington, DE) to minimize adhesion to the cover slide. The sample was exposed to UV light at 365 nm with a light intensity of 60 mW/cm^2 for 10^{-20} s until the polymerization reaction was complete. After the brush formation, a thick coating of the unattached thiol-ene polymer remained on the sample surface. To remove this unattached polymer, the samples were rinsed in a soxhlet extraction apparatus operated using methylene chloride for 48 h.

Gradient Polymer Brush Formation

Polymer brushes with thickness gradients were also obtained by changing the reaction condi-

tions across the surface, namely exposure time. A uniform thiol-terminated SAM was formed by placing a Piranha-cleaned substrate in a Teflon jar with ~ 0.5 mL of MPTMS at 90°C for 2.5 h, followed by washing in toluene and acetone and drying with nitrogen. A stoichiometric mixture of DVE-3 and HDT with 0.05% DMPA was used for polymer brush growth. The same setup mentioned previously was used for growing polymer brushes, with the addition of a syringe pump. The syringe pump was used to move a plate across the surface, thereby changing the exposure time of the sample to UV light. The speed of the moving plate was changed in such a manner that the time taken to travel across the surface was equal to the time for complete conversion to take place, which was ~ 60 s. Gradient acrylate brushes were grown using a similar setup by using a mixture of *tert*-butyl acrylate and 0.1% DMPA. The substrates were washed in methylene chloride soxhlet and characterized using ellipsometry.

Two-Dimensional Gradients

To obtain a two-dimensional thickness gradient, a thiol SAM gradient was obtained as described previously. An exposure time gradient was obtained by moving the plate in the syringe pump in an orthogonal direction during the polymerization. The sample was extracted in a methylene chloride soxhlet and characterized using ellipsometry.

Surface Characterization

Surfaces were characterized for water contact angle and film thickness. Water contact angle measurements were performed on a goniometer using a sessile drop method. An ellipsometer (Multiskop, Optrel GBR, Berlin) was used to make all thickness measurements. All measurements were done at an angle of incidence of 70° . The surface silicon oxide, SAM, and the polymer brush were considered as separate layers; and the variation in SAM thicknesses in gradient SAMs was considered to calculate the brush thickness. Thicknesses in all gradient samples were measured every 0.25–0.5 cm along the direction of change in surface density. Five separate measurements were conducted for every distance as measured from the edge of the sample placed closest to the sample source for gradient SAM samples. Similarly, five separate mea-

surements were conducted for a specific exposure time in the second set of thickness gradient samples (obtained by changing the exposure time across the surface). In all cases, three samples were prepared and the data was averaged.

RGDS Gradient Preparation

Synthesis of Acrylated RGDS

RGDS was synthesized using solid phase methods on an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA) and following procedures for HBTU (2-(1H-benzotriazol-1, 1,3,3-tetramethyluroniumhexafluorophosphate) activation coupling. The peptides, after UV-monitored synthesis, were cleaved from the solid support with a cocktail consisting of 5% phenol, 5% water, and 2.5% triisopropylsilane in trifluoroacetic acid (TFA).⁴² The peptide was then washed with copious amounts of ice-cold diethyl ether, redissolved in distilled water, and dialyzed (Spectrum, 500 MW cutoff) over 24 h with two exchanges of distilled water.

RGDS was coupled to acrylated-PEG following a previously reported method.⁴³ Briefly, RGDS was dissolved in sodium bicarbonate buffer (50 mM, pH 8.4). Acryloyl-PEG-*N*-hydroxysuccinimide (3400 Da, Nektar Therapeutics) was reacted with the peptide while stirring at room temperature for 2 h. The mixture was dialyzed (Spectrum, 1000 MW cutoff) in distilled water over 24 h with two distilled water exchanges. The dialyzed acryloyl-PEG-RGDS was lyophilized and stored at 4 °C until use.

Surface Modification with RGDS

A thiol SAM gradient was obtained using a fluorinated SAM gradient as a template as detailed below. A mixture of trichloro (1H, 1H, 2H, 2H perfluorooctyl) silane (FSAM) and mineral oil in the ratio of 1:10 was placed on one edge of a cleaned glass piece and allowed to diffuse for 1 min at room temperature. MPTMS deposition was carried out as mentioned in the previous sections, thereby depositing a thiol SAM gradient in the opposite direction. An amine-catalyzed Michael type addition reaction was used to react acrylated RGDS to the surface. A 2 mM solution of RGDS-acrylate was prepared by adding 0.0283 g in a 1:1 mixture of diethylamine and 1X phosphate buffered saline (PBS) solution (400 μ L total volume). A drop of the solution

was placed on the thiol SAM gradient, covered with a cover slip and maintained at room temperature overnight. The substrate was washed with deionized water and dried in nitrogen. Substrates were sterilized by treatment with 0.1% sodium azide (Fisher) in PBS.

Adhesion of Osteoblasts on RGDS-modified Surfaces

Osteoblasts were isolated from neonatal (<1-day old) rat calvaria.⁴⁴ The dissected calvaria were stripped of periosteum, minced with a scalpel, and incubated with Type I collagenase and trypsin for three consecutive 10-min digestions. The supernatant from the final digestion was filtered, and the cell pellet was suspended in Dulbecco's modified eagle medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). Osteoblast attachment density was analyzed using a previously described method^{40,45-47} in which they were first trypsinized from culture plates, counted, centrifuged, resuspended, and seeded onto sterile surfaces at a density of 20,000 cells/cm². After 2 h, the cells were rinsed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Phase contrast images (Nikon Eclipse TE300) were taken of the fixed osteoblasts. Attached cells were counted on a minimum of three random fields (0.01 cm²) every 1.5 mm along the gradient.

RESULTS AND DISCUSSION

Thiol SAM gradients were obtained by modifying the method first proposed by Chaudhury and Whitesides.¹⁹ Conceptually, the method involves diffusion of silane molecules above a surface creating a concentration gradient in the vapor phase, which is replicated on the surface. The steepness of the concentration gradient depends on the diffusivity of the silane in the vapor and its reactivity with the surface. Chlorosilanes have a very high reactivity with a hydroxy-terminated surface as compared to methoxy and ethoxy silanes⁴⁸ and, hence, have been used to obtain controlled gradients. The thiol SAM gradients were obtained as detailed in the Experimental section.

The water contact angle (WCA) after each of these steps used in obtaining the thiol gradient

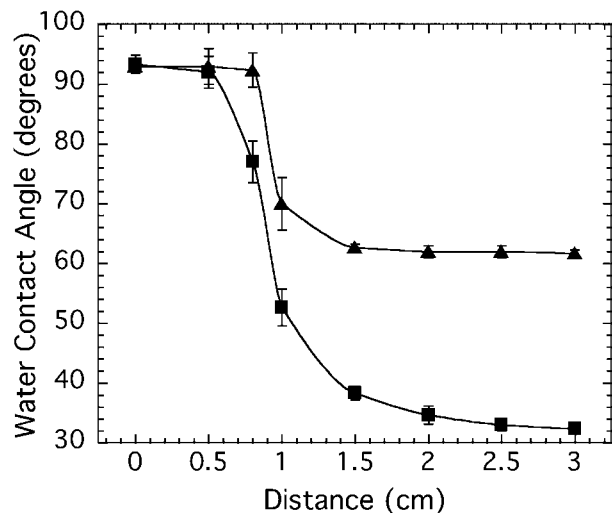


Figure 2. Water contact angle of the gradient SAMs. DTS SAM gradient obtained by diffusing 1:10 mixture of DTS silane and mineral oil for 30 min at room temperature (■). DTS SAM gradient backfilled with thiol SAM for 2.5 h at 90 °C (▲). Data points are the average of three samples, with three measurements per sample. Error bars are the standard deviation of all measurements. Lines are a guide to the eye.

is shown in Figure 2. The DTS deposition produced a gradient at a distance of 0.5 and 1.5 cm from the surface. Upon deposition of the thiol SAM, the water contact angle increased because of the partial deposition of MPTMS molecules between the DTS SAMs. The region of the substrate beyond 2 cm showed an almost constant value of 63° for WCA after backfilling, which compares well with the value of 61° found for a surface completely covered with thiols. Figure 3 shows the fractional coverage of the end groups after both the steps. The fractional coverage of the DTS SAM was calculated using Cassie's equation, assuming a wca of 111° for the DTS SAM and 0° for the hydroxyl groups on the surface.^{49,50} The WCA of the thiol-terminated SAM was assumed to be 61°, and the surface after backfilling was assumed to be covered only with DTS and MPTMS (thiol) molecules, with all the surface hydroxy groups covered with one of the two SAM molecules.

Figure 4 shows the ellipsometry data for the samples covered with the thiol gradient. The thickness of the samples increases after the deposition of thiol SAMs throughout the sample. Thus, there was incomplete DTS SAM formation after the first step, which is also supported by

the WCA data in Figure 2. The difference in the thicknesses of the samples in the first two steps was calculated and plotted as thiol SAM thickness in the graphs. The thiol SAM thickness increased from 2 Å and plateaued at around 6.5 Å, which is close to the value of around 7 Å for a complete MPTMS SAM, indicating a nearly complete monolayer. Figures 2–4 show conclusively that a surface thiol gradient has been deposited on the surface using the DTS gradient as a template.

The surface thiol gradient was utilized for growing thiol-ene films from the surface. As shown in Scheme 1, when a thiol-ene polymerization is carried out on a surface presenting thiol groups, a thiol-ene polymer chain is anchored to the surface. Photoinitiated polymerization of a stoichiometric mixture of DVE-3 and hexane dithiol was carried out on the substrates having the thiol SAM gradient. Figure 5 shows the ellipsometry data for the substrates with formed thiol-ene brushes. As expected, there was a thickness gradient with the thickness changing from 4.5 to 6.5 nm across the surface.

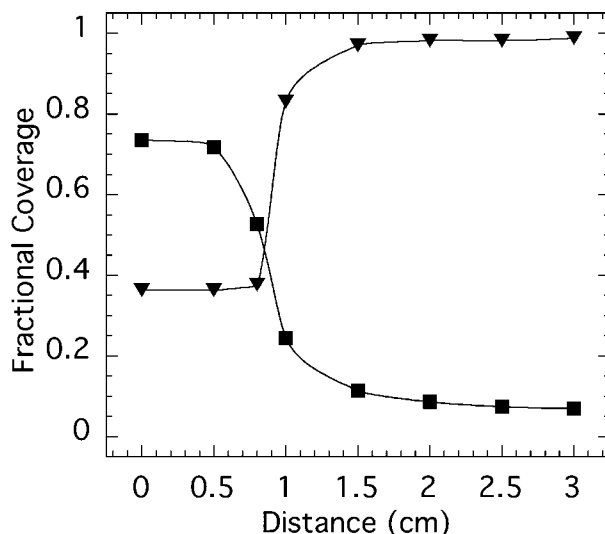


Figure 3. Fractional coverage of the DTS SAM (■) and the thiol SAM after backfilling (▼) as calculated using Cassie's equation. The surface with the DTS gradient is assumed to be covered with a mixture of DTS molecules and hydroxy groups, whereas the surface after backfilling is assumed to be covered with a mixture of DTS and thiol (MPTMS) molecules. The WCA of DTS is assumed to be 111° and that of hydroxy groups on surface is assumed to be zero. The WCA of the thiol SAM is assumed to be 61°. Data points are the average of three samples, with three measurements per sample. Lines are a guide to the eye.

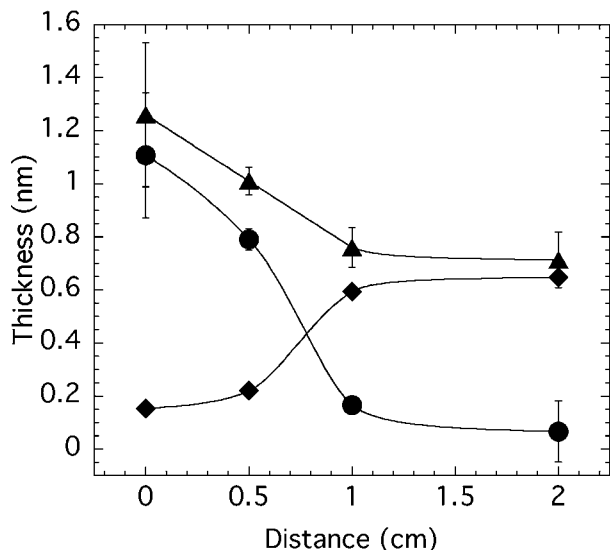


Figure 4. Ellipsometric thickness of the gradient SAMs. DTS SAM gradient thickness (●) and DTS SAM backfilled with thiol SAM (▲). Difference between the thickness before and after thiol SAM deposition (thickness of the thiol SAM, ◆). Data points are the average of three samples, with five measurements per sample. Error bars are the standard deviation of all measurements. Lines are guide to the eye.

Since the entire sample was exposed to identical polymerization conditions (i.e. light intensity, exposure time, monomer concentrations), the average chain length of the attached chains is the same, and the difference in film thickness across the surface is due to the difference in surface thiol concentration across the surface. The SAM thickness before the polymerization varied across the surface and its variation with position were taken into consideration when calculating the thickness of the thiol-ene layer presented in Figure 5.

As mentioned earlier, polymerization initiation takes place both in the bulk and on the surface. As a result, the obtained surface chain densities are intermediate between the ‘grafting to’ and ‘grafting from’ processes. Higher surface chain densities are obtained in the case of ‘grafting from’ process where all initiation takes place on the surface and the processes are typically slower due to the low concentration of initiation sites. On the other hand, if the process follows a purely ‘grafting to’ mechanism, the obtained densities are lower since long chains in the bulk compete for anchoring sites on surface. It has been shown earlier by Harant et al. that

film formation in thiol-ene polymerizations takes place as an intermediate between these two schemes.²⁸

Thickness control is a desired characteristic of a surface modification technique and was obtained by varying the surface chain density as shown in Figure 5. A thickness gradient was also obtained by changing the average chain length, while keeping the density constant. This outcome is achieved by changing the polymerization conditions across the surface, while keeping the density of anchoring groups on the surface constant. A thickness gradient was obtained by changing the UV exposure time for the thiol-ene polymerization. The advantage of this method, as opposed to the controlled surface density, is that the slope of the gradient is controlled relatively easily. Control of the slope in the SAM gradient technique is obtained by changing either the silane concentration or temperature of SAM deposition. On the other hand, the exposure time gradient is easily controlled by monitoring the reaction conditions.

A stoichiometric mixture of thiol and ene monomers was sandwiched between a silicon substrate covered with a uniform thiol-termi-

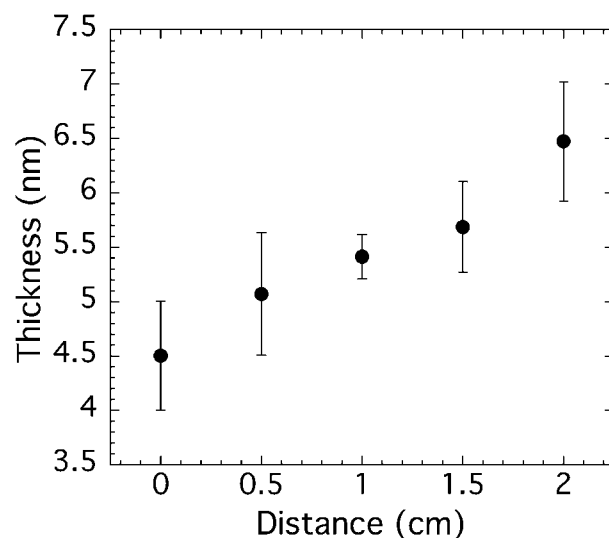


Figure 5. Thickness of the gradient thiol-ene brushes prepared by polymerizing a stoichiometric mixture of hexane dithiol and divinyl ether with 0.1% DMPA on the gradient thiol-terminated SAMs under 365 nm UV light with an intensity of 60 mW/cm² measured using ellipsometry. The samples were polymerized to complete conversion. Data points are the average over three samples, with five measurements per sample. Error bars denote standard deviation.

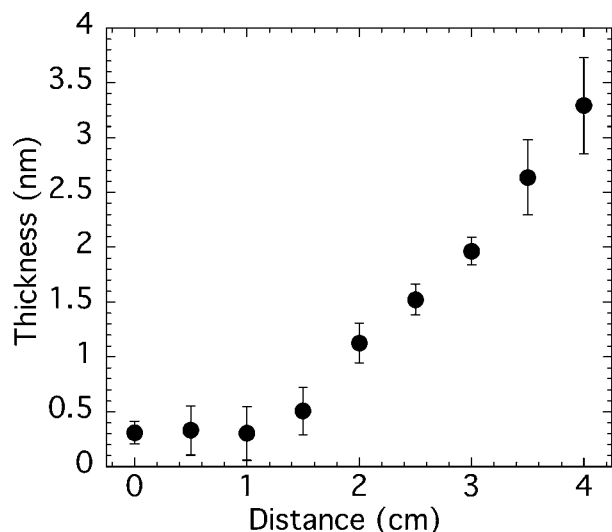


Figure 6. The thickness of the thiol-ene film obtained using a gradient in exposure time measured using ellipsometry. A stoichiometric mixture of hexane dithiol and divinyl ether with 0.05% DMPA was polymerized under 365 nm UV light with an intensity of 60 mW/cm². The exposure time was varied from 0 to 60 s across the sample using a syringe pump, with the smallest exposure time being for distance 0 cm. Data points are the average over three samples, with five measurements per sample. Error bars are the standard deviation of all measurements.

nated SAM and a cover slide. A syringe pump was used to move a plate across the surface, thereby changing the exposure time from 0 to 60 s, and hence the functional group conversion. The ellipsometry results for this sample are shown in Figure 6. From 0 to 4 cm along the surface, film thickness increased from 0.4 to 4 nm. The thickness was constant for the first 1 cm across the surface as the conversion in the first 1 cm is very small and results in the formation of a minimal amount of thiol-ene polymer. The thickness increased nearly linearly to 4 nm for a maximum exposure of 60 s.

The thickness scale in Figure 5 is higher than that in Figure 6, which can be due to various reasons. When the ratio of the two reacting species in a step growth polymerization is very close to unity, even small changes in the stoichiometry affect the average chain length to a significant extent. Another possible explanation could be the different extents of loop formation in the two systems. Harant et al. proposed loop formation as a possible reason for lower thicknesses in surface-attached thiol-ene linear systems.²⁸ Loop formation is a result of either

bimolecular radical termination of two growing polymer chains or the addition of a molecule across two chains and hence decreases with decreasing surface density. As a result, in Figure 5, where the density of surface grafts is less than that for the samples in Figure 6 (except for the regions near to the end where the thiol SAM fractional coverage is close to unity), the loop formation could be reduced, resulting in thicker films.

A thickness gradient was also obtained for acrylate brushes by changing the exposure time. *Tert*-butyl acrylate with 0.1% DMPA was polymerized on the surface under changing exposure time and reacted to complete conversion at the maximum exposure times. Acrylate brushes react through a chain addition polymerization mechanism and form thicker films as compared to step-growth thiol-ene films. Acrylate films are particularly attractive since several biologically important groups, such as antibodies, are readily incorporated in acrylate brushes.⁵¹ The thickness of the gradient acrylate brushes is seen in Figure 7. The film thickness increases from 1 to 12 nm over the surface.

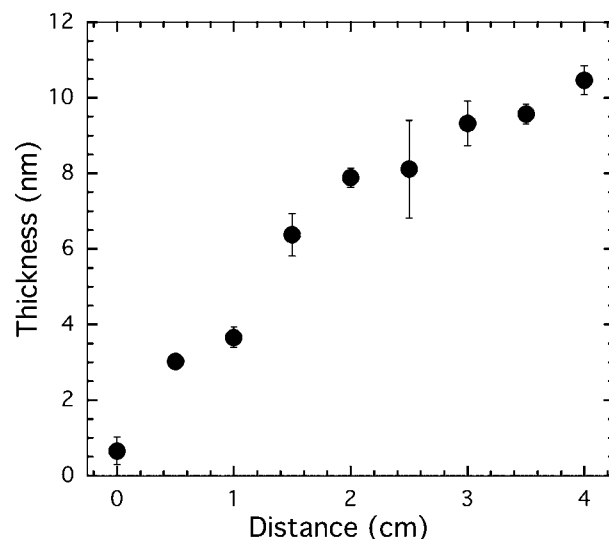


Figure 7. Thickness of gradient acrylate brushes obtained by varying the exposure time measured using ellipsometry. *Tert*-butyl acrylate with 0.1% DMPA was polymerized under 365 nm UV light with an intensity of 60 mW/cm² with the exposure time changing from 0 to 60 s across the surface. The lowest exposure time is for the point distance 0 cm. Data points are the average of three samples, with five measurements per sample. Error bars are the standard deviation of all measurements.

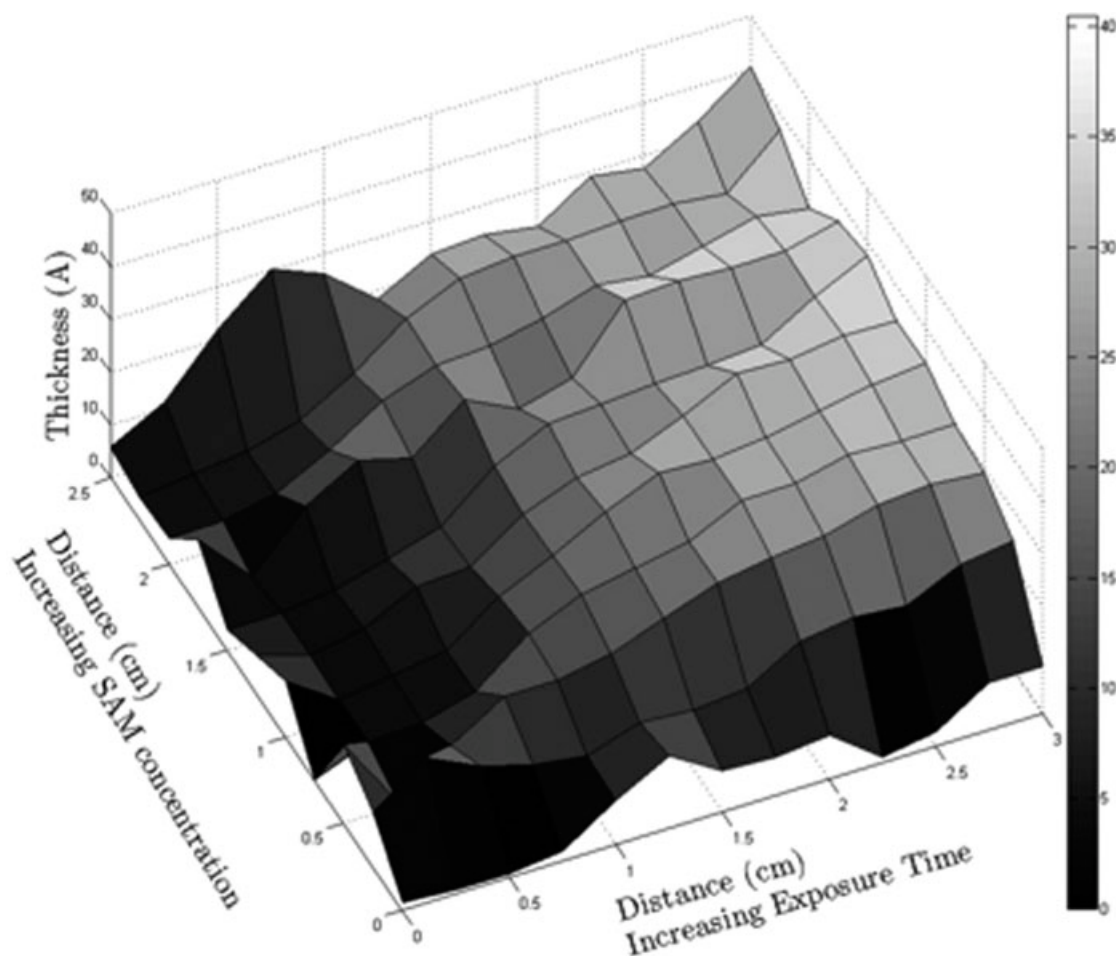


Figure 8. Two-dimensional gradient of thiol-ene brushes. The thiol SAM gradient is used to obtain a thickness gradient in one direction as in Figure 5, and the exposure time for the thiol-ene polymerization is varied in the orthogonal direction as in Figure 6. A stoichiometric mixture of hexane dithiol and divinyl ether with 0.1% DMPA is polymerized with 365 nm UV light with at an intensity of 60 mW/cm². The maximum thickness is ~40 Å.

The two techniques for obtaining thickness gradients (i.e., changing the density of chains attached to the surface and changing the average chain length) were varied across the surface in orthogonal directions to obtain a two-dimensional thickness gradient with the results shown in Figure 8. A thiol SAM gradient was obtained in one direction, and a thiol-ene reaction was carried out on the surface while the exposure time was changed in the perpendicular direction. The resultant two-dimensional surface gradient had a thickness varying from 0.1 to 4.0 nm.

An important advantage of this method mentioned above is the ability to design surfaces with variable thiol densities. This surface thiol gradient can be coupled with the versatile thiol-

ene chemistry to attach several functionalities on the surface in a controlled manner to obtain systematically varying properties. One such application is the attachment of biologically important functionalities such as peptides, which play an important role in cell-material interactions. Such a system is investigated here.

The manner in which a cell interacts with a material depends on a range of factors including surface chemistry, cell type, surface geometry, and material properties of the substrate.^{5,16,40} Often, an interplay of one or more of these factors determines cell adhesion characteristics and the ultimate cell function. Studying each of these factors in decoupled settings is often a prohibitively time consuming task. Accordingly,

gradients as proposed here are useful to perform a large number of experiments on a single sample. The surface thiol gradient obtained in the previous sections was used to change the cell adhesive nature of the surface in a spatially controlled manner.

A gradient of a fluorinated SAM was prepared on the surface by diffusing a fluorinated silane on the surface to obtain a noncell adhesive surface on which to study specifically the interaction of cells as a function of RGDS attachment concentration. The surface was then backfilled with thiol SAM, and a Michael type addition reaction was carried out on the surface and used to present RGDS to rat calvarial osteoblasts. The amine acts as a catalyst in the addition of thiol across the acrylate double bond.³⁸ The acrylated RGDS reacts with the surface thiols attaching a single acrylated RGDS molecule to the surface. The most commonly researched adhesive peptide, RGDS, is found ubiquitously in cell-binding domains of extracellular matrix proteins such as fibronectin, collagen, and osteopontin, among others. Integrins on the surface of cells bind to the RGDS and allow cells to adhere very specifically to otherwise nonadhesive surfaces. To test the gradient in RGDS concentration, osteoblasts were seeded on the substrates and allowed to adhere for 2 h.

Figure 9(a–f) shows light micrographs of osteoblast attachment along an increasing gradient of RGDS after 2 h of interaction at the surface. In general, cell density increased in the direction of increasing RGDS concentration, from ~ 2000 cells/cm², where the RGDS concentration was lowest, to $\sim 20,000$ cells/cm², where the RGDS concentration was highest. The extent of cell attachment on RGDS-modified materials has been determined to be highly correlated with RGDS concentration, where concentration of 10 fmol/cm² was sufficient to support fibroblast adhesion but at 1 fmol/cm², cells were spread but did not form focal contacts and exhibited abnormal actin cytoskeletal organization.^{46,52,53} The cell density as a function of distance is plotted in the adjoining graph [Fig. 9(g)]. The increase in the cell number is nearly linear within experimental error. Since the profiles of the gradients in SAMs are not linear, this indicates that within the RGDS surface density range presented, the resulting adhesion does not respond linearly to the ligand. It is possible that the adhesive strength (e.g. focal contact formation) is altered through the fluori-

nated surface chemistry, which could impact cell–material interactions. Also, during the short time for cell adhesion (2 h), the cells were allowed to adhere, and not proliferate; therefore, complete coverage did not result, even at the highest RGDS ligand availability. By allowing for proliferation, a nonlinear cell density gradient might result. It should also be noted that the initial seeding density (defined as the number of cells seeded initially per unit area) of cells was 20,000 cells/cm², which is close to the highest value of cell density as observed in Figure 9(g).

Gradient SAMs, therefore, are useful for studying the relationship between adhesive ligand concentration and cell attachment. Further, this methodology could be applied to study synergistic effects of two or more adhesive peptide sequences, as the ligand gradient is readily varied in two dimensions simultaneously. For instance, in fibronectin, PHSRN (Pro-His-Ser-Arg-Asn) is known to be a synergistic adhesive peptide in combination with RGDS. In addition, investigation of cell migration on gradient surfaces could be performed, as insoluble, haptotactic agents (proteins, peptides, hormones, etc.) could be covalently coupled to the free thiols. Subsequent monitoring would enable the rapid evaluation of optimal ligand concentration, type, and spacing of ligands from the surface to achieve optimal adhesion or migration or any other cell function. The main advantage of this particular method is that a wide range of conditions are simultaneously evaluated with a single sample, enabling one to collect rapidly a diverse array of information rapidly from a single experiment.

CONCLUSIONS

Ultrathin thiol-ene polymer films with a thickness gradient were chemically attached to a surface. A thiol SAM gradient was obtained by using a chlorosilane gradient as a template, which was further modified with thiol-ene brushes. Thiol-ene brushes and acrylate brushes with a thickness gradient were also obtained by changing the exposure time for thiol-ene polymerizations carried out on a surface. A two-dimensional thickness gradient was obtained by changing the chain density and the average chain length in orthogonal directions to obtain a sub-5 nm thickness gradient. The thiol SAM gradient was used to obtain an RGDS gradient to change the cell adhesiveness of the surface

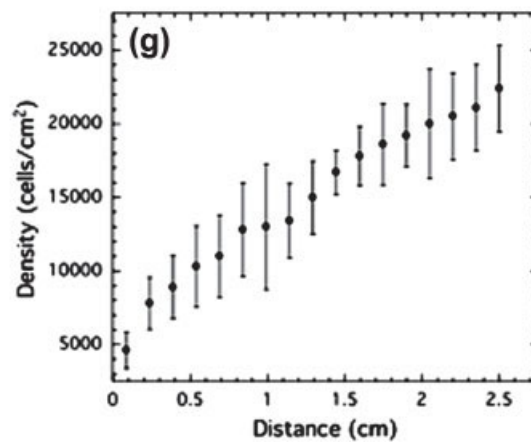
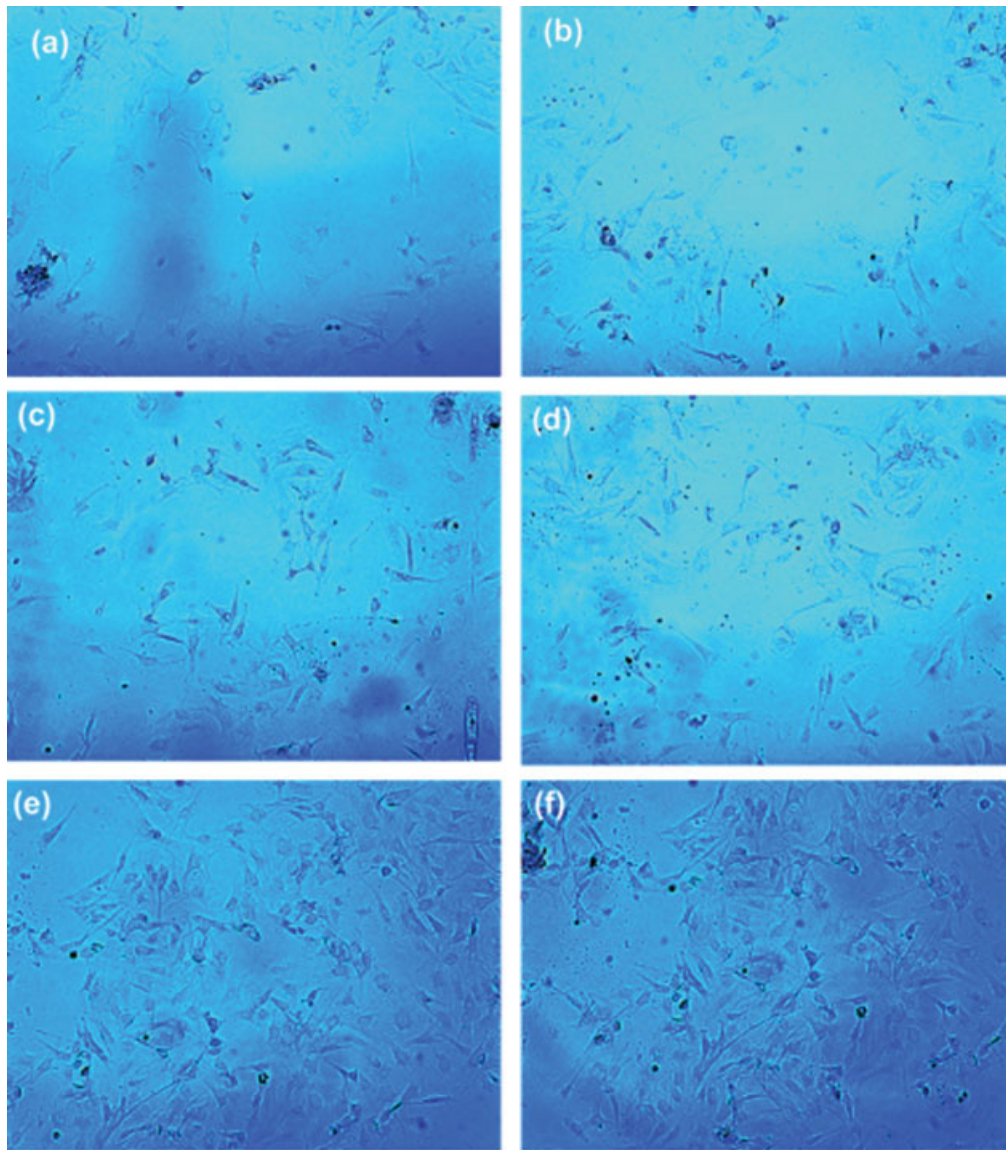


Figure 9. Cell adhesion studies on samples containing an RGDS gradient (details of the preparation given in text). A few representative light micrographs of osteoblasts attached to gradient surfaces at approximately equal distances (at ~0 cm, ~0.5 cm, ~1 cm, ~1.5 cm, ~2 cm, and ~2.5 cm across the sample) are shown (a–f) in the direction of increasing amount of RGDS with Figure 9(a) having the least amount of RGDS and Figure 9(f) having the most. (g) shows the quantification of all the images from 0 to 2.5 cm, spanning the entire RGDS gradient.

and was used to obtain an osteoblast density gradient across the surface.

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