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Facile Fabrication of “Dual Click” One- and Two-Dimensional Orthogonal Peptide Concentration Gradients

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Abstract

Peptides, proteins, and extracellular matrix act synergistically to influence cellular function at the biotic–synthetic interface. However, identifying the individual and cooperative contributions of the various combinations and concentration regimes is extremely difficult. The confined channel deposition method we describe affords highly tunable orthogonal reactive concentration gradients that greatly expand the dynamic range, spatial control, and chemical versatility of the reactive silanes that can be controllably deposited. Using metal-free “dual click” immobilization chemistries, multiple peptides with a variety of functionality can be immobilized efficiently and reproducibly enabling optimal concentration profiling and the assessment of synergistic interactions.

Graphical abstract

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Notes

The authors declare no competing financial interest.

INTRODUCTION

Optimizing the spatial presentation and concentration of covalently immobilized growth factor peptides on biomaterial surfaces is crucial for tissue function and development.¹ Functional self-assembled monolayers (SAMs) that have easily tunable functions and a wide variety of suitable reaction chemistries provide a suitable platform for testing the bioavailability and bioactivity of the peptides when tethered.^{2–6}

Combined with high-throughput combinatorial strategies, SAMs possessing a gradient in peptide concentration are able to survey a wide range of peptides and peptide combinations in a limited number of substrates while providing rapid, efficient, and precise platforms for quantitative assessments.^{7–10} Methods to generate SAM gradients have been explored widely, for example, microcontact stamping,^{11,12} microfluidic systems,¹³ electric potential stamp,¹⁴ free diffusion,^{15,16} ultraviolet (UV)-induced oxidation,¹⁷ plasma and corona discharge,^{18,19} and so on. However, the ultimate utility is governed by precise control of the slope and functional properties, batch to batch reproducibility, and the versatility in chemical groups on the surface. Gradient methods based on surface soft lithography, for example, microcontact stamping^{11,12} and microfluidic systems,¹³ are limited by the complexity of the stamp device for further development, and the spatial distribution of the functional species are difficult to format into gradient concentration profiles even with stamp depletion methods. Other strategies using plasma, corona discharge, or UV oxidation^{17,20,21} to generate functional gradient surface through controlling the reaction time can only achieve limited surface functionalities, restricting their use for multifunctional systems. Liquid or vapor diffusion, which has no complicated device and reaction involved, is best suited for the fabrication of diverse functions with gradual changes in presentation. Liquid diffusion requires compatibility between the substrate and deposition solvent,¹⁶ so vapor deposition is thought to be more versatile.²² Chaudhury et al.¹⁵ and Epps et al.²² have developed surface concentration gradients based on free gas diffusion or vacuum gas diffusion separately, both of which were adaptable and capable of utilizing diverse chemical functionalities, but the gradient profiles are still poorly tunable.²³ Herein we demonstrate a “vacuum away”

confined channel vapor deposition method, which overcomes many of the aforementioned difficulties. Linear gradient chemical concentration profiles with tunable chemical functionalities were generated on silicon and glass substrates using our simple and easily controllable method. In addition, two-dimensional (2D) orthogonal chemical concentration gradients were achieved by using a sequential deposition protocol that enables the orthogonal engineering of peptide functionalized SAMs.

The method outlined in this paper will be highly useful for the investigation of cooperative interactions of bioactive peptides. One such combination of interest is the synergistic influence of cell adhesion arginine–glycine–aspartic acid (RGD) sequences in combination with osteogenic growth peptide (OGP). RGD is found in numerous extracellular matrix (ECM) proteins, including collagen I, fibronectin, fibrinogen, and vitronectin. RGD peptides are favorable to incorporate into synthetic constructs because they interact with cells through integrin receptors on the cell surface to initiate cell adhesion and cell spreading.^{24,25} OGP is a potent *in vivo* activator of osteogenic activity and has been shown to increase bone density and osteoblast proliferation rates. OGP is a short, naturally occurring 14-mer growth factor peptide (AL-KRQGRTLYGFGG) found in the serum at $\mu\text{mol/L}$ concentrations.²⁶ As a soluble, freely diffusing peptide, OGP regulates proliferation, differentiation, and matrix mineralization in osteoblast lineage cells.^{27,28} We have shown previously that the MAPK stimulating component OGP(10–14) possesses concentration-dependent bioactivity when tethered *in vitro* and *in vivo*.^{21,29} The potential combination of these two highly specific peptides is of interest to enhance cell adhesion while promoting proliferative and osteogenic activity. However, the ability to probe and measure the concentration-dependent effects is critical to maximizing the potential of these molecules. We believe this is the first report to demonstrate orthogonal gradients of two peptide sequences that cover the physiologically relevant ranges for cell–integrin interactions. While the specific biological measurements will follow in a later contribution, the diversity of potential functional species and the control over the concentration profiles will be highly useful to biologists and biomedical engineers who are investigating the cooperative nature of extracellular matrix ligands and growth factor effects of cell behavior.^{30–32}

EXPERIMENTAL SECTION

Materials

4-Chlorobutyldimethylchlorosilane, 5-hexenyldimethylchlorosilane, and (3,3,3-trifluoropropyl)dimethylchlorosilane were purchased from Gelest (Morrisville, PA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless specifically noted. Microscope glass coverslips (75×25 mm for one-dimensional substrates, 25×25 mm for two-dimensional substrates, Fisher Scientific) were used for general gradient fabrication process evaluation; and silicon wafers (Si[100], one side polished, Silicon Quest Int.) were cut to corresponding size (75×25 mm or 25×25 mm) for X-ray photoelectron spectroscopy (XPS) analysis. Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology nor is it intended to

imply that the materials or equipment identified are necessarily the best available for the purpose.

SAM Gradient Fabrication

Silicon and glass substrates were pretreated with ultraviolet light generated ozone (UVO; Jelight Company Inc. Model No. 42A) for 10 min to remove organic contaminants from the surface, washed with methanol and toluene 3 times each, and blown dry with nitrogen prior to use. The Teflon chlorosilane reservoir ($1.5 \times 2.5 \times 1.1$ cm) and substrate support ($7.5 \times 2.5 \times 1.0$ cm) for the glass coverslips were inserted into rectangular glass tubing ($30 \times 2.5 \times 1.3$ cm) and placed in a sealed metal chamber. The dynamic vacuum (4 mPa or 30 μ Torr) was pulled from the side port nearest the reservoir and away from the substrate.³³ Methanol was injected through a syringe port from the opposite side to quench the diffusion process following defined time intervals (Figure 1a). After the vapor deposition, the substrates were removed from the chamber, washed successively with methanol, toluene, methanol, and blown dry under N₂. The samples were stored in vacuum desiccators at room temperature until used.

The functional SAM concentration gradient in our system was achieved using confined gas diffusion while the dynamic vacuum was pulled away (Figure 1) from the deposition substrate. The expected profile can be described using Fick's diffusion laws:³⁴

$$J = -D \partial c(x, t) / \partial x \quad (1)$$

$$\partial c(x, t) / \partial t = -D \partial^2 c(x, t) / \partial^2 x \quad (2)$$

For our specific boundary case, at position $x = 0$, the concentration is constant $c(0) / t = 0$, and for position $x = \pm\infty$, the concentration is almost zero $c(\pm\infty) = 0$. For relatively short distances, $x \ll 2(Dt)^{1/2}$, the solution for eq 2 can be approximately simplified as³⁵

$$c(x, t) = c(0)[1 - x/(Dt\pi)^{1/2}] \quad (3)$$

Orthogonal Two-Dimensional Gradient Formation

2D orthogonal concentration gradients were achieved using a sequential deposition process with a substrate rotation 90° (Figure 1b) following the initial deposition. Chlorine and vinyl functionalized chlorosilanes, 4-chlorobutyldimethylchlorosilane and 5-hexenyldimethylchlorosilane, respectively, were deposited using the following conditions: neat, 30 s and 1 min diffusion time sequentially, and a 2 cm distance away from the chemical reservoir.

Contact Angle Measurements

Advancing contact angles were measured using an Advanced Goniometer (Ramé-Hart Instrument Co., Model 500) at 25 °C using ultrapure water (2 μL ; 18 $\text{M}\Omega\text{ cm}^{-1}$) as the probe fluid and analyzed by a drop shape analysis method (ImageJ, downloaded from the National Institute of Health, Bethesda, MD, U.S.A., <http://rsb.info.nih.gov/ij/>). The surface coverage fractions were calculated based on eq 4,³⁶ which is suitable for heterogeneous monolayers when the size of any patches approach molecular dimensions.

$$(1 + \cos\theta)^2 = f_1(1 + \cos\theta_1)^2 + f_2(1 + \cos\theta_2)^2 \quad (4)$$

$$(f_1 + f_2 = 1)$$

where θ is the contact angle for each point along gradient surface, θ_z is the contact angle for corresponding dense packed surface, and f_z is the component surface coverage fraction.

The standard uncertainty of contact angle measurements at each position was determined by the standard deviation between three independent measurements on three samples prepared under identical conditions.

X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed on a Kratos AXIS Ultra DLD spectrometer using silicon wafers as substrates. The X-ray source was monochromated Al K_{α} , scanning over a binding-energy range of (0 to 1200) eV with a dwell time of 100 ms. The analyzer pass energy was 160 eV for the survey spectra and 20 eV for the high-resolution C_{1s} , N_{1s} , Cl_{2s} , and O_{1s} scans. Each spectrum was collected over a $300 \times 700\text{ }\mu\text{m}$ sample area. Peak area was analyzed and atomistic concentrations were calculated with CasaXPS software using a Levenberg–Marquardt algorithm assuming a linear background. The concentrations for chlorine and fluorine were captured directly from the high-resolution Cl_{2s} and F_{1s} peak fitting, and vinyl concentration was calculated by $(1 - f_{\text{chlorine}} - f_{\text{fluorine}})$ and C_{1s} linear decomposition. For C_{1s} linear decomposition, the final C_{1s} curve was assumed to be the linear combination of three separated components, and the proportions for each component were calculated using Mathematica 6. Control sample was prepared by incubating the substrate in the components mixture (molar ratio 1:1:1). The computation results shown that the final mixture C_{1s} is a linear combination of three components in the ratio 1:1:1, exactly our feed ratio (Figure 4b).

Functional Peptide Synthesis

The peptides RGD (GRGDS) and OGP (YGFGG) were synthesized according to standard solid phase Fmoc chemistry. For thiol-functional peptides, the amino acid cysteine was added to the N-terminus of the peptide sequence. For the OGP-thiol, the peptide sequence was CYGFGG. To synthesize 4-dibenzocyclooctynol (DIBO) functionalized RGD, resin-tethered RGD with a free amine at N-terminus was coupled to DIBO using a method described previously.^{37,38} Alkyne-RGD was synthesized by coupling 5-hexynoic acid to the amine terminus of resin-tethered RGD using standard Fmoc conditions.

Azide-Terminated Concentration Gradient

4-Chlorobutyl-di-methylchlorosilane (150 μ L, neat in reservoir) was deposited on clean glass substrates using a 2 min diffusion time to yield a chlorine concentration gradient surface. Immediately following the gradient profile formation, an SN2 substitution was carried out by incubating Cl gradient substrates in NaN₃ (0.2 g, 61 mM)/DMF (HPLC grade, 50 mL) solution with a small amount of 18-crown-6 as a phase-transfer catalyst. The reaction was carried out at 55 °C for 2 d. The slides were then removed, washed sequentially with methanol, toluene, and methanol, and blown dry under N₂. The near quantitative conversion of -N₃ functionality was verified by the disappearance of Cl_{2p} signaling and the appearance of two N_{1s} peaks that correspond to the azide group in the XPS spectra.³³

Strain-Promoted Alkyne–Azide Cycloaddition (SPAAC) Surface Reaction

Azide-terminated gradient substrates were incubated in RGD-DIBO solution (7.3 mg/mL in PBS buffer, pH = 7.4) at ambient temperature for 48 h. Following the reaction, the substrate was removed, washed with methanol, and blown dry under N₂.

Copper-Catalyzed Alkyne–Azide Cycloaddition

Alkyne-RGD mixture was first prepared (CuSO₄ 1 mg/mL, NaAc 0.5 mg/mL, peptide 7 mg/mL in PBS buffer, pH = 7.4), and azide-terminated gradient substrates were then incubated in the solution at ambient temperature for 48 h. To remove the residual copper catalyst, the substrate was washed in EDTA (10 mmol/L, pH = 7) solution for 2 h, then washed with methanol, and blown dry under N₂.

Surface Thiol–ene Reaction

Vinyl-terminated gradient substrates were generated by using 5-hexenyldimethylchlorosilane as chemical source with a 1 min diffusion time. Peptide OGP-thiol (5 mg/mL) was dissolved in H₂O/DMF (10:1 by volume) solution and Irgacure 2959 was then added as photoinitiator (2.5 mg/mL). The substrate was immersed in the peptide mixture solution and treated with UV (254 nm) for 2 h. Surface-adsorbed residues were removed by a short sonication procedure (2 min) in DMF, washed with methanol, and blown dry under N₂.

RESULTS AND DISCUSSIONS

In our experimental design, the chemical source (organosilane) and substrate (silicon or glass) were placed in a sealed chamber, and a dynamic vacuum was pulled from the opposite direction of gradient formation to create the diffusion concentration gradient (Figure 1a). Previously, to enable gradient formation, reduced pressure was applied in the direction of the gradient. Epps et al. have demonstrated the fabrication of one-dimensional two-component gradient by pulling vacuum from both sides.²² However, we found that slight variations in vacuum when pulling from both sides make reproducibility difficult in our hands and prevent chlorosilane quenching, which is necessary to prevent saturation. The differences in outcome may arise from the extent of vacuum or variation in the diffusion channel parameters, which in our case would change the gas diffusion to turbulent flow instead of viscous flow.³⁹

The physical nature of our confined channel “vacuum away” (VA) diffusion system was relatively straightforward in that the concentration gradient was formed by confined gas diffusion but not free gas diffusion due to the effect of opposing VA. However, Fick’s diffusion laws provide suitable theoretical explanation for our final results, and yield reproducible, well-defined molecular profiles. A series of linear concentration gradients were produced using various diffusion times (Figure 2a) based on the above strategy and measured using water contact angle changes along the substrates. Just as eq 3 predicted, the concentration $c(x,t)$ has a linear relationship as the function of distance x , which can be tuned by the diffusion time t , and the slope of gradient has a linear correlation with $1/\sqrt{t}$ (Figure 2b). This indicates that designed gradient profiles can be calculated, easily achieved, and well controlled by diffusion time in the system.

Initial silane concentration, $c(0)$, is another controllable parameter for tuning the gradient profile according to eq 3. However, experimental alteration to $c(0)$ created an exponentially decreasing curve (Figure 2c), which means the boundary conditions and approximations for eq 3, works when the diffusion distance $x \ll (Dt)^{1/2}$, are not suitable in this case. The solvent fluctuations play an important function in this process and decrease the organosilane effective diffusion constant D .

Our results are different than the theoretical calculations of Douglas et al. on the one side free gas diffusion system.⁴⁰ In their specific case, a sigmoidal gradient was formed by free gas diffusion under air pressure in a one side blocked system, similar to our experimental setup except the employment of one side vacuum. They reported that the gas deposition process was governed by both diffusion and front propagation rate, and the resulting gradient formation was not linear. However, they did mention that there was a linear gradient regime that exists at short diffusion times and in close proximity of the reservoir that was difficult to capture experimentally under free diffusion conditions. Our design expands this linear regime to accessible times and distances through the incorporation of a confined channel evacuated by an active vacuum. The linearity is one of the outstanding features of our gradient, which facilitates straightforward analysis and characterization, especially during biological screening experiments.

Orthogonal 2D concentration gradients were achieved by a sequential deposition process, as shown in Figure 1b. Chlorine and vinyl “dual-click” orthogonal concentration gradients were fabricated, showing the promising potential for two independent immobilization click chemistry methods.^{41,42} Orthogonal reactions conditions (Experimental Section) were carefully chosen for the two sequential reaction steps to eliminate the influence of the first deposited component on the following one, which is common in sequential fabrication methods. To optimize the gradient parameters, precise one-dimensional gradient profiles provided reliable reference data for guiding two-dimensional gradient fabrication.

The final 2D orthogonal gradient formation was identified by the gradually changing contact angle across the 2D surface (Figure 3b), reflecting the components changing in both the X - and Y -axis. The individual component concentrations were measured by XPS. For XPS analysis, the 2D gradient surface was backfilled with (3,3,3-trifluoropropyl)dimethylchlorosilane and high-resolution Cl_{2s} , F_{1s} , and C_{1s} XPS spectra

were measured. The concentrations for chlorine and fluorine can be measured directly from the Cl_{2s} and F_{1s} peak fitting (Figure 3c,e), and vinyl concentration was calculated by $1 - f_{\text{chlorine}} - f_{\text{fluorine}}$ (Figure 3d) and C_{1s} linear decomposition (Figure 4). Vinyl group concentration, which has no distinguishable signals or heteroatoms, could not be measured directly using XPS. An assumption was made that the final C_{1s} curve was the linear combination of three separated components and this assumption was verified by control sample measurements. The results computed by Mathematica 6 shown that the final mixture C_{1s} is the combination of three components in the ratio 1:1:1, exactly our feeding ratio (Figure 4b). The three component fractions at each position within the 2D substrate can be measured by C_{1s} curve linear decomposition (Figure 4c) and are consistent with the results calculated from chlorine and fluorine by $1 - f_{\text{chlorine}} - f_{\text{fluorine}}$.

To assess the utility of the gradient for spatially defined growth factor immobilization, three “click” reactions, thiol–ene, metal free, strain-promoted azide–alkyne cycloaddition, and copper-catalyzed azide–alkyne cycloaddition were employed here for peptide attachment. OGP (YGFGG) and RGD (GRGDS) peptides, two widely studied sequences for cell attachment and osteogenesis, were chosen as examples to generate peptide concentration gradients for bioactive surface studies.

The sequential “click” reactions were carried out under ambient conditions in water and with no residual byproducts. Linear peptide concentration profiles were measured after each of the “click” reactions (Figure 5) and the respective concentrations were calculated using nitrogen–carbon ratio from high resolution XPS elemental analysis. Using our experimental conditions, the thiol–ene click reaction was able to yield 70% peptide surface coverage while using either the copper-free or copper-catalyzed azide–alkyne cycloaddition, maximal peptide coverage was measured to be only ~30%. Considering the significant steric hindrance of the 4-dibenzocyclooctynol (DIBO) and larger volume of space during the cycloaddition reaction of the triazole, the lower reaction conversion efficiency is reasonable and expected.

CONCLUSIONS

In summary, we have demonstrated a versatile and tunable method for generating orthogonal concentration profiles of bioactive peptides. The confined channel deposition method greatly expands the dynamic range and chemical versatility of the reactive silanes that can be controllably deposited. This level of control and versatility is unprecedented and the substrates can be used to identify the concentration dependence and test the bioavailability of surface immobilized bioactive species singly and in combination. When the metal-free “click” chemistry is used, peptides with a variety of functionality can be immobilized efficiently and reproducibly, enabling optimal concentration identification and the assessment of synergistic interactions. Multifactor experiments can be used to elucidate the concentration and synergistic effects of immobilized factors on cell functions, providing basic information for the rational design of future tissue engineering devices and scaffolds.

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Figure 1.

(a) Scheme for fabricating functional self-assembled monolayer concentration gradients using a confined channel diffusion method. (b) A two-step sequential deposition strategy enables a “dual-click” orthogonal concentration gradient. Following the first functional silane deposition, the substrate was rotated 90°, and then second silane was deposited. The vacuum, solute concentration, deposition time, and distance of the substrate from the reservoir define the profile of the resulting gradient.

Figure 2.

Relative concentration of the functional species varies as a function of position (near-linear) on the substrate can be controlled by the deposition time t . Due to the hydrophobicity of 4-chlorobutyldi-methylchlorosilane, we can monitor deposition by measuring the water contact angle (a). The surface coverage fractions were calculated based on eq 4. Well-defined surface coverage and gradient profiles were achieved precisely using various deposition times (a). There is a linear correlation between gradient slope and (b), which correlates with the Fick's diffusion theory. (c) Gradient profiles were varied using solute concentrations from 100 to 10%, as shown. Static water contact angles are measured at each position along the substrates. Error bars reflect standard deviation ($n = 3$) of the mean of three independent measurements on substrates fabricated under identical conditions. 4-Chlorobutyldimethylchlorosilane was used as the solute and toluene as the dilute solvent.

Figure 3.

Orthogonal concentration gradients were generated by sequentially depositing 4-chlorobutyldimethylchlorosilane in the X direction and 5-hexenyldimethylchlorosilane in the Y direction. (a) The final concentration profiles were measured by static water contact angles (b). The static contact angles change gradually across the 2D surface (without backfilling), reflecting the components changing on both the X -axis and Y -axis. For XPS analysis, the sample was backfilled with (3,3,3-trifluoropropyl)dimethylchlorosilane. (c) The concentration of chlorine vs position was analyzed by fitting the Cl_{2s} peak. (e) The substrate was backfilled with (3,3,3-trifluoropropyl)-dimethylchlorosilane to facilitate characterization. (d) Assuming the final total surface coverage of the three components is unique 1, the vinyl silane coverage was calculated by $1 - f_{\text{chlorine}} - f_{\text{fluorine}}$. This result was further verified by high-resolution C_{1s} linear curve fitting of fractional chlorine, vinyl, and fluorine silane C_{1s} curve combinations (Figure 5). (c1), (d1), and (e1) were the perspective images of pictures (c2), (d2), and (e2). The contact angle for each position was the average value based three identical characterization and fabrication process, while the XPS results were just single measurements.

Figure 4.

(a) Scheme of XPS sample data distribution. (b) Control sample C_{1s} curve decomposition as a linear combination of three separated components C_{1s} curves in ratio of 1:1:1, which matches the feed ratio. (c) Curve fitting of C_{1s} for each gradient position as a linear combination of three individual component C_{1s} spectra. The component fractions, as shown in the spectra, were consistent with the results from the Cl_{2s} and F_{1s} elements analysis. The point to point distance is 0.5 cm, and the final 2D gradient substrate dimensions were 2.5 cm.

Figure 5. Orthogonal peptide concentration gradients were achieved using sequential “click” reactions: (a) thiol–ene, (b) strain promoted alkyne–azide cycloaddition (SPAAC), and (c) copper-catalyzed alkyne–azide cycloaddition.