Development and characterization of surface chemistries for microfabricated biosensors

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The high cost and harsh processing conditions associated with microfabricated biosensors demand a new approach to receptor immobilization. We have grafted biotin labeled, 3400 molecular weight poly(ethylene glycol) (PEG) to silicon surfaces to produce a dense PEG monolayer with functionally active biotin. These surfaces have been activated with antibodies through the strong streptavidin-biotin interaction by simply incubating the surfaces with antibody-streptavidin conjugates. The stability of the biotinylated PEG monolayers produces a sensing element that can be regenerated by removal of the streptavidin conjugate and stored in a dry state for extended periods of time. © *1999 American Vacuum Society*. [S0734-2101(99)07605-8]

I. INTRODUCTION

Biosensors are portable diagnostic tools used for the rapid detection of metabolites, drugs, hormones, antibodies and antigens.[1,2] Traditional biosensors are composed of disposable sensor elements containing molecular receptors immobilized by adsorption, covalent crosslinking or entrapment.[3–5] The need for real-time analysis of environments containing minute concentrations of one or more analytes has prompted the development of a new generation of biosensors.[6–9] These biosensors, containing microfabricated silicon sensing elements, promise high sensitivities and the ability to screen large arrays of analytes.

Several new challenges are associated with immobilizing receptors on silicon biosensors. First, the sensing element is fairly expensive to fabricate and assemble, requiring it to be used multiple times. Second, packaging the sensing element in the sensor typically requires drying and heating that degrade many important receptor systems. Third, the sensor element must remain active during long periods of storage.

We have developed a different approach to receptor immobilization in which the receptor is reversibly immobilized at a surface that resists nonspecific adsorption. Previous work has demonstrated the high affinity of the streptavidin-biotin interaction,[10] and the capacity of poly(ethylene glycol) (PEG) to resist nonspecific protein adsorption.[11,12] Based on these results, we have developed an antibody based assay that is performed on a biotinylated PEG surface (Fig. 1). This approach can easily be implemented with other specific molecular receptors and hydrophilic polymer films.

In this article, we describe the chemical and physical properties of biotin (biotin-PEG) and methoxyl terminated PEG (M-PEG) films. These films are formed with a two-step chemistry based on the spontaneous adsorption of poly(ethyleneimine) (PEI) to silicon surfaces.[13,14] We then use immunochemical techniques to characterize the biotin activity of the biotin-PEG films, the activity of antibody streptavidin conjugates on the biotin-PEG films, and the capability of M-PEG films to resist nonspecific adsorption. Finally, we demonstrate that this surface immobilization scheme has two important properties for microfabricated biosensors: (i) chemical treatments can be used to remove the streptavidin complex and regenerate the sensor surface; (ii) the sensor surface can be dried for packaging and storage then spontaneously functionalized with the antibody-streptavidin conjugate.

II. MATERIALS AND METHODS

A. Surface chemistry

PEG immobilization was performed, as shown in Fig. 2, by reacting N-hydroxysuccinimidyl (NHS) ester PEGs with

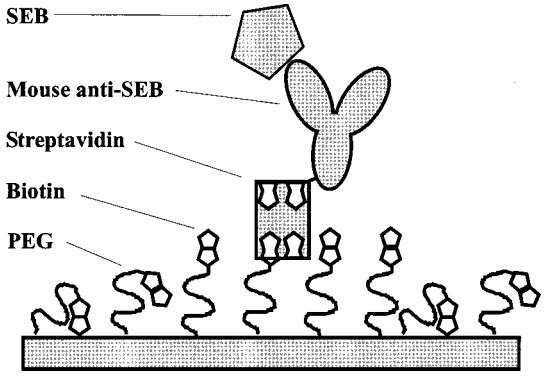

FIG. 1. Assembly of the immunochemical complex on biotin-PEG films. The analyte, staphylococcal enterotoxin B (SEB), is captured by the mouse anti-SEB antibody-streptavidin conjugate, which is immobilized on the biotin-PEG surface.

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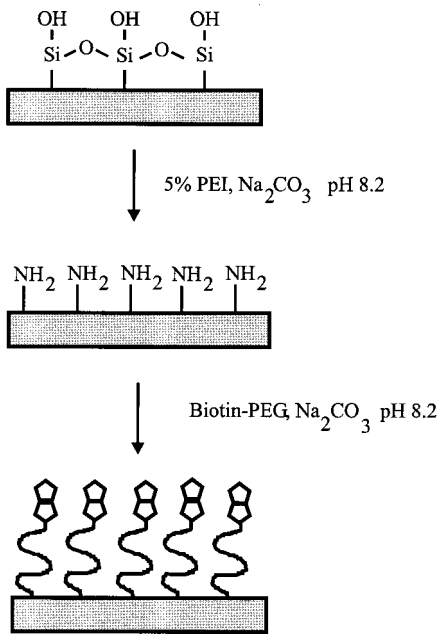

FIG. 2. Chemical scheme used to immobilize PEG derivatives on silicon wafers. In the first step, high molecular weight PEI is spontaneously adsorbed on the silicon wafer. In the second step, a biotin, N-hydroxysuccinimidyl ester of PEG is grafted to the amine functionalized PEI surface.

aminated silicon surfaces that were prepared by a process of adsorption of PEI.[13,14] Silicon (100) wafers with a 30 nm silicon dioxide film (Transition Technology International, Sunnyvale, CA) were used as substrates after ozone cleaning (UVO cleaner, model 42, Jetlight Co., Irvine, CA). All reactions were performed at room temperature unless otherwise stated, and Milli-Q (Millipore, Bedford, MA) water was used. PEI with a mean molecular weight of 500 000 was adsorbed on the substrates by incubation with 5% (w/v) PEI (Polymine SNA, BASF, Rensselaer, NY) in 50 mM Na_2CO_3, pH 8.2, for 2 h. Excess PEI was removed by thorough rinsing in H_2O. PEG derivatives were then reacted with the PEI surfaces in 50 mM Na_2CO_3, pH 8.2, for 2 h at 37 °C. The two PEG derivatives used were α-biotin, ω-NHS poly(ethylene glycol) carbonate, MW 3400 (biotin-PEG), and NHS methoxypoly(ethylene glycol) propionic acid, MW 3000 (M-PEG) (Shearwater Polymers, Huntsville, AL).

B. Streptavidin- antibody conjugates

Streptavidin-antibody conjugates were formed by thiolating primary amines on an antibody and linking then to primary amines on streptavidin with a heterobifunctional crosslinker. Mouse monoclonal antibodies to SEB were thiolated by reaction with N-succinimidyl S-acetylthioacetate (SATA), (Pierce, Rockford, IL) in a 1:10 molar ratio in 50 mM Na_2HPO_4/NaH_2PO_4, 1 mM EDTA (PB), pH 7.5 buffer for 30 min. The sulfhydryl groups on the SATA-antibody were deprotected in 50 mM hydroxylamine-HCl, 2.5 mM EDTA, 62.5 mM Na_2HPO_4/NaH_2PO_4, pH 7.5 buffer for 2 h. The excess SATA and deacylation buffer were removed by ultrafiltration (Microcon 30, Millipore). Streptavidin was reacted with the heterobifunctional crosslinker succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC, Pierce)[15] in a 1:5 molar ratio in 50 mM Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl (PBS), pH 7.2 for 30 min. The excess SMCC was removed from the maleimide functionalized streptavidin by ultrafiltration. The streptavidin-antibody conjugate was formed by reacting the maleimide functionalized streptavidin with the thiolated antibody in a 3:1 ratio in PB, pH 7.5 overnight at 4 °C.

C. Physical characterization

The chemical and physical properties of the PEI, M-PEG, and biotin-PEG films were studied with x-ray photoelectron spectroscopy (XPS), ellipsometry, and atomic force microscopy (AFM). Each PEG film was studied at three (or more) PEG grafting concentrations. The conditions were chosen so as to maintain relative molar concentration, i.e., 0.2, 2, and 20 mg/ml biotin-PEG and 0.12, 1.2, and 12 mg/ml M-PEG. XPS analysis was performed immediately after preparing the surfaces. The ellipsometry and AFM measurements were made on surfaces that were dried and stored for up to one week.

X-ray photoelectron spectra were obtained using a spectrometer equipped with a concentric hemispherical analyzer (SSX301 x-ray photoelectron spectrometer, Surface Science Instruments). The instrument was operated in a fixed analyzer transmission mode using a monochromatic Al $K\alpha$ x-ray source. The pass energy was 50 eV with a 300 μm spot size, the take-off angle was 35°, and the normal operating pressure was 10^{-9} Torr.

Ellipsometric measurements were made in air at 44 wavelengths between 400 and 750 nm (M-44 spectroscopic ellipsometer, J. A. Woollam Co., Inc.). The angle of incidence, phase (Δ), and amplitude (Ψ) were known with an uncertainty of less than 0.005°, 0.02°, and 0.01°, respectively. The complex refractive index ($n=n+ik$) and layer thickness (t) were fitted to the Δ and Ψ profiles using a Newton-Raphson solution of Fresnel's reflectivity equations for multilayer systems. The absorptive contributions embodied in n were very small for all the layers and were not included in the models. Wavelength-dependent values of n for water were obtained by fitting experimentally obtained Δ and Ψ profiles for reflection off a known silicon dioxide substrate in water to the coefficients (A, B, and C) of a three-term Cauchy model:

$$n(\lambda)=A+\frac{B}{\lambda^2}+\frac{C}{\lambda^4}.$$

For the PEG layer, fits of n and t were made using a similar Cauchy model; however, B and C were insignificantly small. The adsorption density (Γ) in air was calculated using the measured t and the simple geometric relationship $\Gamma=\rho t$, where the density (ρ) was set to 1 g/ml.

Atomic force microscope measurements were made using an optical lever AFM equipped with a liquid cell (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA). Tapping mode

TABLE I. Results of XPS study of M-PEG and biotin-PEG films formed on silicon wafers. The peak shifting due to charging was corrected by taking the peak value of the Si $2p_{1/2}$ peak to be 103.3 eV (Ref. 18) and the atomic concentrations were calculated using the standard sensitivity factors (Ref. 22).

Chemistry	Grafting concentration (mg/ml)	% Si $2p_{1/2}$ (103.3 eV)	% C 1*s* (285.6 eV)	% C 1*s* (286.5 eV)	% N 1*s* (399.1 eV)	% O 1*s* (533.0 eV)
PEI	···	24	19	···	7	50
M-PEG	0.12	26	8	11	6	48
	1.2	23	7	19	5	46
	12	17	5	32	4	42
Biotin-PEG	0.2	25	7	14	6	48
	2	19	6	25	6	43
	20	13	5	38	4	38

cantilevers (Nanosensors, Germany) with spring constants near 30 N/m were used for the imaging at approximately 300 kHz in air.

D. Immunochemical characterization

Streptavidin labeled with the radioisotope ^{125}I was used to determine: (i) the activity of biotin in films formed at 0.2, 2, 5, 10, and 20 mg/ml biotin-PEG grafting densities; (ii) the capacity of M-PEG films to resist nonspecific adsorption at 0.2, 5, and 12 mg/ml M-PEG grafting densities. First, the streptavidin concentrations, the labeled-to-unlabeled streptavidin ratio and incubation time, were selected for the optimal signal. The surfaces were then incubated with 50 μg/ml ^{125}I streptavidin (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS for 2 h, which was determined to produce near saturation level binding. The adsorbed and unadsorbed streptavidin fractions were separated by thorough rinsing of the surfaces in 0.05% (w/v) Tween-20 (Sigma, St. Louis, IL) in PBS (PBST). The total amount of ^{125}I in each fraction was measured using liquid scintillation counting (Beckman LS-6500 multipurpose scintillation counter, Fullerton, CA).

A sandwich enzyme linked solid-phase immunoassay (ELISA) for measuring staphylococcal enterotoxin B (SEB) concentrations was used to assess the activity of biotin-PEG surfaces.[16] A mouse monoclonal antibody to the SEB-streptavidin conjugate (1.5 μg/ml) was incubated with dense biotin-PEG films, i.e., films formed at 13 mg/ml biotin-PEG. After incubation with serial dilutions of SEB (Sigma Chemicals), followed by rabbit antibody to SEB (Toxin Technology Inc., Sarasota, FL) and anti-rabbit IgG-horseradish peroxidase (0.07 μg/ml, Caltag Labs, Burlingame, CA), the peroxidase enzyme was identified with the addition of 2,2′azino-di(3-ethyl-benzthiazoline sulfonate) substrate (KPL, Gaithersburg, MD) for 20 min. The wells were incubated for 1 h at each step unless otherwise specified. Between each step, the wells were washed three times with PBST.

III. RESULTS AND DISCUSSION

A. Characterization of the chemical and physical properties of PEG films

The PEG film structure, formed on silicon surfaces, can be derived from XPS characterization of each layer of the film. XPS spectra of PEI films formed on silicon wafers, Table I, contains significant amounts of silicon, oxygen, nitrogen, and carbon. The carbon has a C1s 285.6 eV binding energy associated with PEI.[17] The relative abundance and binding energies of elements associated with both silicon dioxide[18] and PEI are consistent with previous work, which shows that adsorption of the branched high molecular weight PEI gives a thin film of irreversibly bound, amine groups on mica surfaces.[14] Grafting PEGs to PEI reduces the elemental components associated with the PEI-silicon substrate and produces an XPS carbon spectrum that would be expected for a surface containing a large amount of coupled PEGs, i.e., the C 1s region of the spectrum contains a large –C–O– peak at 286.5 eV.[19] The strength of the –C–O– and Si signals allows us to track relative changes in the PEG density through the –C–O– to Si ratio. The density of both the biotin-PEG and the M-PEG films appears to increase as the PEG grafting concentration increases.

Ellipsometry was used to characterize the thickness and refractive index of each layer in the PEG film. Analysis of the silicon wafers following PEI adsorption did not show a significant change in the Δ and Ψ profiles, indicating the film thickness is too small to be measured using ellipsometry. The density and thickness of the PEG films, measured as a function of grafting concentration, are summarized in Table II. A systematic increase in the density of both the M-PEG and the biotin-PEG films is observed as the grafting concentration of PEG increases. At the highest grafting concentrations, the M-PEG and biotin-PEG densities are 2.6 ± 0.2 and 3.1 ± 0.3 mg/m^2, respectively, which is equivalent to the

TABLE II. Results of ellipsometry study of M-PEG and biotin-PEG films formed on silicon wafers.

PEG	Grafting concentration (mg/ml)	Amount adsorbed (mg/ m^2)	Refractive index (*n*)	Layer thickness (nm)
M-PEG	12	2.6 ± 0.2	1.432 ± 0.003	2.6 ± 0.2
	1.2	1.6 ± 0.2	1.42 ± 0.02	1.6 ± 0.2
	0.12	1.2 ± 0.2	1.38 ± 0.01	1.2 ± 0.2
Biotin-PEG	20	3.1 ± 0.3	1.501 ± 0.003	3.1 ± 0.3
	2	1.5 ± 0.5	1.490 ± 0.020	1.5 ± 0.5
	0.2	1.8 ± 0.5	1.490 ± 0.020	1.8 ± 0.5

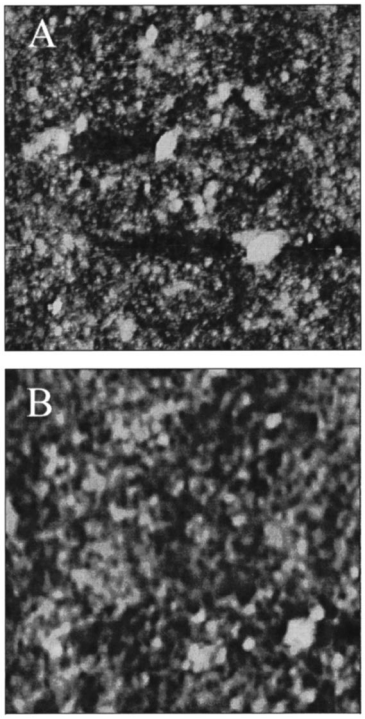

FIG. 3. AFM tapping mode images of M-PEG films. (a) Topographic image of 0.12 mg/ml M-PEG film in air. Image size: 1 μm×1 μm×5 nm. (b) Topographic image of 12 mg/ml M-PEG film in air. Image size: 1 μm×1 μm×5 nm.

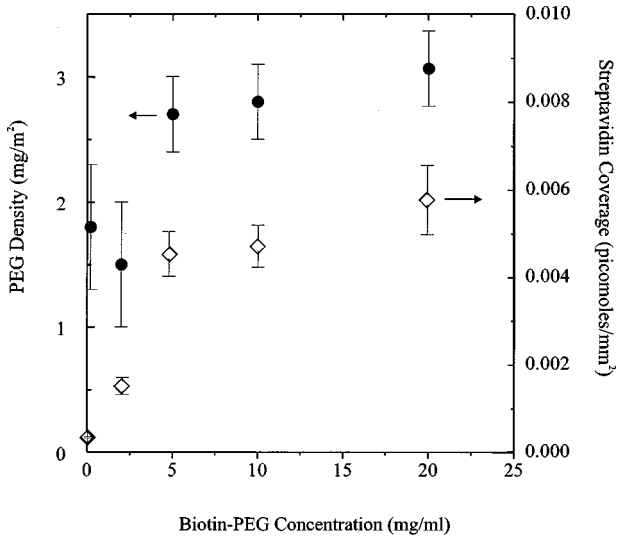

FIG. 4. Biotin-PEG grafting density (closed circles) and streptavidin binding density (open diamonds) on 0.2, 2, 5, 10, and 20 mg/ml biotin-PEG films.

maximum density achieved with similar PEG grafting schemes.[20]

Tapping mode AFM measurements of the PEG films were made in air at the three adsorption concentrations. All films appear to be uniform on the 10–100 μm scales; however, submicron scale topographic images in air revealed they are composed of nanometer scale globular domains. These domains were most distinct in shape in films formed at the lowest grafting concentration [Fig. 3(A)] but become less defined as the PEG grafting concentration was increased [Fig. 3(B)]. The domains appear to be associated with local density fluctuations in the film, and the loss of the domain shape with increased PEG concentration may be associated with denser, more uniform films.

XPS, ellipsometry, and AFM characterization of the PEG-PEI films support the model of the film presented in Fig. 2, in which PEG and PEI form distinct layers on top of the silicon dioxide substrate. The 500 000 MW PEI forms a thin layer that is strongly adsorbed at the interface. The 3000 and 3400 MW M-PEG and biotin-PEG form thicker films that increase in density with increasing PEG concentration. The grafting density and thickness of the PEG films at maximum coverage are consistent with a densely packed, end-grafting monolayer.

B. Characterization of the biotin activity and fouling resistance of PEG films

The capability of free streptavidin to bind to biotin-PEG films was measured with ^{125}I labeled streptavidin at 0.2, 2, 5, 10, and 20 mg/ml biotin-PEG-NHS. Figure 4 shows that both the activity and density of the biotin-PEG film increases with the grafting concentration reaching a maximum coverage of 0.005 picomoles of streptavidin per square millimeter at biotin-PEG concentrations greater than 10 mg/ml. At maximum coverage the area per bound streptavidin molecule is 33 nm^2/molecules, which is approximately one half the geometrically allowed closed packed monolayer density allowed by the 4×4×4.4 nm streptavidin molecule.[21] The area per biotin-PEG-NHS molecule at maximum coverage is approximately 1.9 nm^2/molecule. At the minimum biotin-PEG concentration the streptavidin binding and biotin-PEG density are 249 and 3.2 nm^2/molecule, respectively.

The capability of PEG films to block nonspecific streptavidin adsorption was measured at M-PEG films at 0.2, 5, and 12 mg/ml M-PEG. The amount of streptavidin adsorbed on the surfaces increased from 0.002 ± 0.0006 picomoles/mm^2 at 12 mg/ml M-PEG to 0.004 ± 0.001 picomoles/mm^2 at 0.2 mg/ml M-PEG. These results suggest nonspecific adsorption could account for approximately 30% of the total bound streptavidin on dense biotin-PEG films and all of the streptavidin bound to the biotin-PEG surface at low coverage.

Immunochemical characterization of the biotin-PEG film indicates that a significant fraction of surface immobilized biotin is available for binding with free streptavidin and that this fraction increases with PEG grafting density. The M-PEG film also resists nonspecific protein adsorption at the highest grafting concentrations. Clearly, for biosensor applications PEG grafting concentrations of 10 mg/ml or greater should be used to form a functionally active film.

C. Immunochemical performance of biotin-PEG films

Packaging and marketing considerations require the active element of a biosensor be dried and stored for extended periods of time. The fact that biotin and PEG are relatively

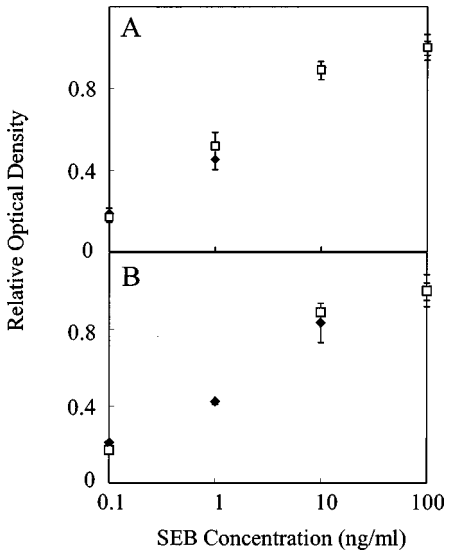

FIG. 5. SEB ELISA demonstrating the behavior of 13 mg/ml biotin-PEG surfaces. (a) The functional activity of freshly prepared and dried surfaces is shown by open squares and closed diamonds, respectively. The background on the freshly prepared and dried surfaces was 0.19 ± 0.08 and 0.15 ± 0.05 optical density, respectively. (b) The functional activity of freshly prepared and regenerated surfaces is shown by open squares and closed diamonds, respectively. The background for the regenerated surfaces exposed and not exposed to the conjugate was 0.02 ± 0.001 and 0.03 ± 0.001 optical density, respectively.

inert suggests the surfaces may be stored dry without losing activity. The physical effects of drying and rehydration were assessed by ellipsometry, and significant changes in Δ and Ψ could not be detected before or after rehydration. The functional activity of the surfaces was tested after drying with a SEB ELISA on 13 mg/ml biotin-PEG functionalized surfaces. The ELISA results from the dried and fresh surfaces, Fig. 5(a), were indistinguishable within the precision of the measurement. These surfaces have now been stored dry for six months without any loss of activity thus demonstrating the potential for long periods of storage.

The stability of biotin-PEG surfaces suggests that it is possible to reuse them, provided the antibody-streptavidin conjugate can be removed without damaging the film. The protocol for protein denaturing and conjugate removal from the biotin-PEG surfaces is as follows: 8 M guanadine-HCl, pH 1.5, 8 h at 65 °C followed by 1 h incubation with 1% sodium dodecyl sulfide (SDS). Biotin-PEG surfaces activated with the streptavidin-antibody conjugate were exposed to this treatment and the state of the surface was probed with anti-mouse IgG peroxidase (Caltag Labs) and biotin-horseradish peroxidase (KPL). Both probes confirm complete removal of the antibody and streptavidin components. The regenerated surfaces were tested with SEB ELISA to determine their activity. These surfaces were actively indistinguishable from fresh surfaces, Fig. 5(b), demonstrating that the biotin-PEG surface can be fully regenerated.

IV. CONCLUSIONS

We have developed a two-step chemistry for functionalizing silicon surfaces with a biotinylated N-hydroxysuccimide 3400 molecular weight PEG monolayer. At biotin-PEG concentrations greater than 10 mg/ml a dense polymer monolayer was formed in which a significant fraction of the biotin groups is accessible for binding with free streptavidin. Antibody-streptavidin conjugates can be used to activate the biotin-PEG surfaces in a simple incubation step. The biotin-PEG films and antibody-streptavidin conjugate produce an antibody immobilization scheme with two important advantages for silicon biosensors; (i) the surface can be stored dry and spontaneously functionalized by specific antibody-streptavidin conjugates; (ii) the films can be treated with denaturing reagents to remove immuonochemical complexes without damaging the biotin or the PEG. This treatment has been used to regenerate the surfaces without a loss in sensitivity. This approach to receptor immobilization may also have several other advantages. First, PEG is known to enhance the chemical stability of proteins and could impart increased stability to immobilized antibodies. Second, the streptavidin-biotin interaction is formed in a single step that could facilitate receptor patterning.

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