Easy and Efficient Bonding of Biomolecules to an Oxide Surface of Silicon

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A new method is described to attach biological molecules to the surface of silicon. Semiconductors such as Si modified with surface-bound capture molecules have enormous potential for use in biosensors for which an ideal detection platform should be inexpensive, recognize targets rapidly with high sensitivity and specificity, and possess superior stability. In this process, a self-assembled film of an organophosphonic acid is bonded to the native or synthesized oxide-coated Si surface as a film of the corresponding phosphonate. The phosphonate film is functionalized to enable covalently coupling biological molecules, ranging in size from small peptides to large multi-subunit proteins, to the Si surface. Surface modification and biomolecule coupling procedures are easily accomplished: all reactions can proceed in air, and most take place under ambient conditions. The biomolecule-modified surfaces are stable under physiological conditions, are selective for adhesion of specific cells types, and are reusable.

Interfacing biomolecules with its surface is a prerequisite for developing sensing devices based on silicon, which can be of significant use in medical, military, agricultural, and environmental monitoring, in bioprocessing industries, and in clinical diagnostics.[1−7] The enormous scope of potential biosensor applications necessitates the interfacing of an equally wide range of biomolecule targets to the Si surface, so it is not surprising that considerable effort is being expended to this end.[8−16] One appealing route to Si surface modification involves covalent attachment of organics, including peptides, to the native or synthesized oxide coating on silicon (SiO_2/Si); this approach has commonly relied on silanization,[7,13,14,17,18] but silanization suffers from the low surface OH group content/unit area of SiO_2/Si[19,20] and siloxane products of silanization can be hydrolytically unstable under physiological conditions.[15,16] Methods for surface modification of Si involving H-terminated,[11,21,22] halogen-terminated,[23] or even diamond-coated Si[12] could address the problem of interface hydrolytic instability; still, a recent review of Si surface processing states, "It remains a challenge to develop strategies for incorporating molecular receptors of a general nature onto the semiconductor surface."[8] We now address this challenge with a simple solution: complete monolayers of functionalized phosphonates can be bonded to SiO_2/Si in a remarkably easy way. These phosphonates can then be used to covalently and stably attach either small or large biomolecules to the Si surface, and the derivatized Si surfaces thus prepared bind their target cells effectively.

Experimental Section

Bonding Biomolecules to the Native Oxide Surface of Silicon. A wafer of native oxide-coated single-crystal Si(100) was cut into ~0.5 cm × 0.5 cm coupons. The coupons were cleaned in a standard way[14] by boiling in 1:3 H_2SO_4−H_2O_2 for 45 min followed by extensive rinsing with water. The coupons were then boiled in a 1:1 solution of HCl−H_2O_2 for 15 min and again extensively rinsed with water. 11-Hydroxyundecylphosphonic acid self-assembles on the cleaned SiO_2/Si by holding the coupon vertically in a 35 μM solution of the acid which was dissolved in dry THF; the solvent is allowed to evaporate slowly so that the meniscus slowly traverses the surface of the coupon, transferring the phosphonic acid to the SiO_2/Si. (The solution reservoir should be large enough so that there is no appreciable change in

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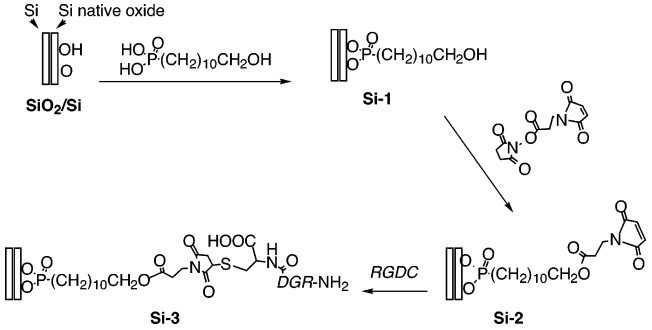

Figure 1. Synthesis of peptide-modified **Si-3**.

concentration of the organophosphonate during this process.) Coated coupons are then gently rinsed, heated in an oven at 130 °C for 48 h, and finally rinsed with sonication in a warm solution (50 °C) of triethylamine (2 mM) in ethanol for 30 min to remove multilayer material to give 11-hydroxyundecylphosphonate/SiO_2/Si (**Si-1**). All QCM measurements were corrected for surface roughness (factor, 1.6). Atomic force microscopy was done using a Nanoscope IIIa (Digital Instruments) in tapping mode.

RGDC was coupled to **Si-1** using the method developed for a Ti surface-bound analogue (Figure 1).[16,24] Coupons of **Si-1** were held in a stirred solution of 1 mM 3-maleimidopropionic acid *N*-hydroxysuccinimide ester in dry acetonitrile for 24 h, rinsed three times with acetonitrile with sonication for 15 min, and analyzed by IR, which showed the maleimido ester derivative **Si-2** (ν_{CO} = 1710 and 1730 cm^{-1}). The coupons were then placed in 0.5 mM RGDC in H_2O, the pH of the solution was adjusted to 6.5 using 0.05 M NaOH, and the reaction mixture was kept at room temperature for 24 h. The coupons were then rinsed with water with sonication and dried in vacuo to give RGDC adduct **Si-3**. For **Si-3**, IR analysis showed a broad peak centered at ca. 1650 cm^{-1}, characteristic of the peptide.

Antibody Coupling to Silicon. ω-Hydroxyl groups of **Si-1** were derivatized using disuccinimidyl glutarate (DSG) (Pierce). Wafers of **Si-1** were stirred in 5 mM DSG in dry acetonitrile under dry, inert atmosphere for 24 h. The wafers were then removed from this mixture and rinsed extensively with dry acetonitrile to give **Si-4**. Rabbit antimouse IgG (Pierce) was then coupled to **Si-4** by incubating for 30 min at a concentration of 100 μg/mL in PBS. The reaction was quenched, and the substrates were washed with 50 mM Tris-HCl pH 7.4. Antibody-coupled surfaces were incubated with 10 μg/mL anti-α4 integrin antibody P1H4 (Chemicon) or anti-α5 integrin antibody SAM-1 (Cymbus Technology Ltd.) for 2 h. Substrates were then washed with PBS and blocked with 1% BSA in PBS for 30 min.

Cell Culture. Human fetal osteoblasts (hFOB 1.19; ATCC) were maintained in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) without phenol red (GIBCO, BRL), 10% fetal bovine serum (Hyclone Laboratories), and 0.3 mg/mL Geneticin (Invitrogen). NIH 3T3 cells were grown in DMEM and 10% calf serum (Hyclone Laboratories). Human melanoma cells (A375), human fibrosarcoma cells (HT1080), and SV40-transformed human fibroblasts (WI38-VA13) were grown in DMEM and 10% fetal bovine serum. Chinese hamster ovary cells expressing α4 (CHOα4), α5 (CHOα5), or α$v\beta$3 integrins were grown in DMEM, 10% fetal clone II serum (Hyclone Laboratories), 1% nonessential amino acids, and 1 mg/mL Geneticin.

Cell Capture Using Modified SiO_2/Si Surfaces. Cells were released from tissue culture dishes using 2.5% trypsin in 0.2 mg/mL EDTA in PBS and resuspended in complete medium. A total of 50 000 cells were added to wells containing Si substrates that had been blocked with 1% BSA in PBS for 30 min. Cells were allowed to attach and spread for the indicated times after which time they were fixed, permeabilized, stained, and visualized as previously described.[25] Where indicated, cells were treated with 10 μM cyclic RGD peptide (The Peptide Institute) for 30 min at 37°C before addition to substrates. To demonstrate sample reusability, fluorescently tagged adherent cells were removed from Si substrates with trypsin as above for 5 min. Substrates were washed with medium and visualized to ensure cell removal, and fresh cells were added. For antibody-modified surfaces, CHOα4 cells were fluorescently tagged with Cell Tracker Orange and CHOα5 cells with Cell Tracker Green (Molecular Probes) for 30 min at 37 °C before release from dishes as described above. Cells were then allowed to interact with antibody/Si substrates and visualized at 2 and 18 h.

Discussion

We recently reported the preparation and structural characterization of a new class of phosphonate monolayers which are formed by self-assembly of phosphonic acids on SiO_2/Si followed by thermal setting.[26] This method circumvents limitations of surface OH content[24] and requires no special pretreatment of the SiO_2/Si. Now, it was important to determine if phosphonate/SiO_2 interfaces were stable under aqueous conditions, as are phosphonate/TiO_2 ones,[16,24] given the imperative for biomolecule functionalization of Si of stability to physiological conditions and the fact of hydrolytic lability of Si−O bonds.

Monolayer films of 11-hydroxyundecyl phosphate/SiO_2/Si (**Si-1**) were prepared, which could provide sites for covalent coupling of biomolecules to the surface through derivatization of the ω-tail groups.[16,24] Hydrogen bonding among phosphonate headgroups and hydroxyl tail groups of the starting 11-hydroxyundecylphosphonic acid can give rise to surface multilayers; happily, sonication in a solution of triethylamine in ethanol removes such multilayered material but leaves the surface-bound phosphonate monolayer intact. A film of **Si-1** was analyzed by AFM (Figure 1) which showed comprehensive coverage of the SiO_2/Si surface by uniform grains ca. 50 nm in diameter with rms roughness ca. 0.4 nm (vs ca. 0.2 nm for cleaned SiO_2) (Figure 2) and with average film thickness of ca. 18 nm. Quartz crystal microbalance (QCM) gravimetric analysis[16,26] using a 10 MHz crystal measured the surface loading of **Si-1** to be 0.82 ± 0.11 nmole/cm^2; no direct, quantitative measurements of film coverage of Si accomplished by other chemical means have been reported. Our measured loading corresponds to a molecular cross sectional area of about 20.5 $Å^2$/molecule, which is about as dense as monolayer octadecylphosphonic acid on mica.[27] Reflectance IR spectroscopic analysis (Figure 2)[28,29] of **Si-1** indicated an alkyl chain-ordered film (ν_{CH_2asymm} = 2914 cm^{-1}) and phosphonate-surface bonding (ν_{P-O} = 936, 1011, 1210 cm^{-1}).

Si-1 is our general interface through which functional biological molecules can be bound to SiO_2/Si by coupling various reactive linkers to the film terminal OH groups. Our first target for such surface attachment was Arg-Gly-Asp (RGD), which is the cell-binding peptide sequence in fibronectin[30] and in other extracellular matrix proteins where it interacts with integrin cell surface receptors to promote cell adhesion.[31,32] First, **Si-1** was reacted with a maleimido group transfer reagent to give **Si-2**. The phosphonate/SiO_2 interface proved stable to water, so it

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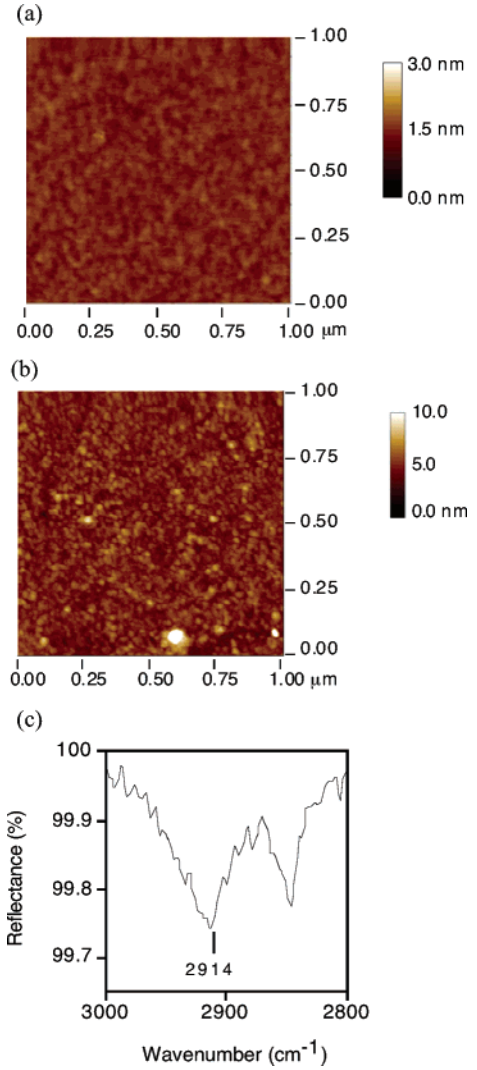

Figure 2. AFM images of (a) cleaned SiO_2/Si (rms roughness 0.2 nm) and (b) 11-hydroxyundecyl phosphate/SiO_2/Si (**Si-1**). The film consists of uniform grains ca. 50 nm in diameter with rms roughness ca. 0.4 nm and with average film thickness of ca. 18 nm. (c) IR spectrum of **Si-1** (ν_{CH_2} region).

was possible to attach the Cys derivative RGDC to **Si-2** under aqueous conditions to give **Si-3** (Figure 1).[16] IR analysis of **Si-3** showed a broad peak centered near 1650 cm^{-1} characteristic of the peptide.

Cell adhesion to RGD modified surface **Si-3** was monitored by counting the number of cells attached to it or to a control at various time points. The extent of cell interactions with these surfaces was determined by examining intracellular organization of the actin cytoskeleton into stress fibers and assembly of focal adhesions, which are protein-rich complexes that connect actin stress fibers to integrin receptors and the extracellular matrix.[33] RGDC-modified **Si-3** was very effective at supporting cell adhesion: 25-fold more cells attached to **Si-3** than to SiO_2/Si after 1.5 h (Figure 3a). Cells on **Si-3** were well-spread and had well-organized actin stress fibers and focal adhesions, as detected by fluorescence microscopy of stained cells (Figure 3b). Cells remained attached to **Si-3** for at least 7 days, and during this time the cells proliferated, as indicated by increased cell numbers (Figure 3a,c). Furthermore, cell binding to **Si-3** was specific for the RGD modification; this binding could be inhibited

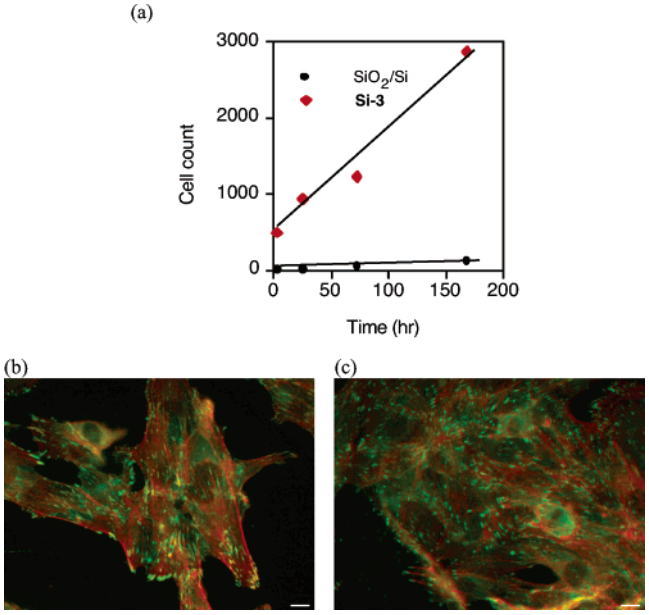

Figure 3. RGD-modified SiO_2/Si surfaces supporting cell attachment, spreading, and growth. (a) Human osteoblast numbers on 0.42 cm^2 modified substrates were counted at the indicated times. Cells spread on peptide-modified **Si-3** were stained with rhodamine−phalloidin to detect actin filaments (red) and anti-vinculin antibodies to detect focal adhesions (green) after (b) 1.5 h and (c) 24 h. Scale bar = 10 μm.

Table 1

substrate	tot. cell no.[a] − cyclic RGD	tot. cell no.[a] + cyclic RGD
SiO_2/Si	13 ± 2	19 ± 2
Si-3	560 ± 23	31 ± 2

[a] On 0.42 cm^2 Si coupons.

by preincubation of cells with a cyclic RGD peptide (Table 1). Thus, **Si-3** is not only stable under physiological conditions, but it lacks cytotoxicity and is a specific substrate for cell adhesion and growth.

A range of cell types attached to **Si-3**, including osteoblasts, fibroblasts, tumor cell lines, and two different Chinese hamster ovary (CHO) cell lines engineered to express distinct integrin cell surface receptors for the RGD sequence, $\alpha5\beta1$ and $\alpha v\beta3$ integrins.[34] This ability of modified SiO_2/Si to support cell adhesion demonstrates that a wide variety of applications, unlimited by cell type or RGD receptor, can be envisaged using our methodology.

The biostability of the interface between the SiO_2/Si surface and a biomolecule can also be key to the lifetime of a Si-based biomonitoring device. It is therefore significant that, in addition to our demonstration of cell growth on **Si-3** (Figure 3a), we find that **Si-3** is stable to proteases and can be reused for cell adhesion. For example, osteoblasts were allowed to attach and spread on **Si-3** for 24 h. Cells were then removed by treatment with the protease trypsin, and the substrate was incubated with fresh cells. Cells adhered and spread equally well on trypsinized substrates as they did on new, unused substrates (Table 2). Substrates were able to undergo several cycles of trypsinization without losing functionality. These data show that phosphonate-modified SiO_2/Si surfaces are stable both hydrolytically and physiologically. The long-term stability of these interfaces and their

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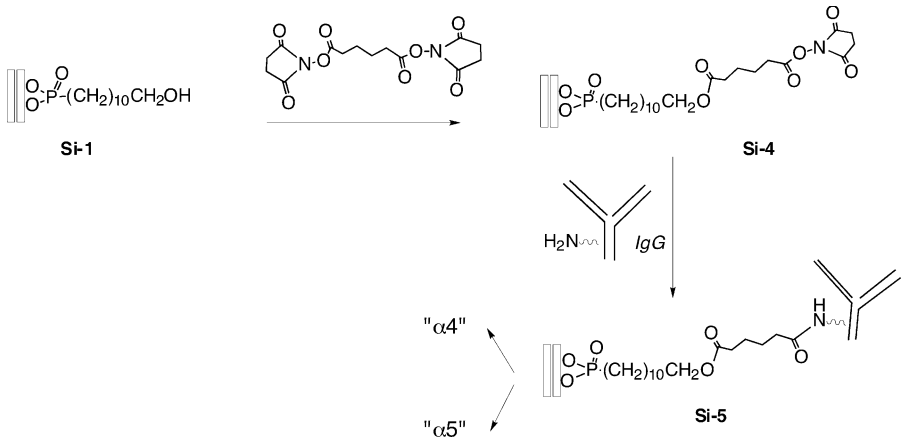

Figure 4. Synthesis of antibody-modified **Si-5**.

Table 2

cell type	tot. cell no. before T[a]	after 1 cycle of T	after 2 cycles of T
human fetal osteoblasts	760 ± 22	840 ±17	790 ± 29
NIH 3T3	580 ± 30	400 ± 26	not done

[a] Adhesion of human fetal osteoblasts to **Si-3** (0.42 cm^2) after 90 min, before and after one or two cycles of trypsinization (T).

recyclable nature is an important economic consideration in the implementation of biosensor devices.

Because of the general nature of reactivity of −OH terminated **Si-1**, surface conjugation of a broad spectrum of biomolecules to SiO_2/Si via this interface can be envisaged. Indeed, we have also found that covalent attachment of large proteins to SiO_2/Si via **Si-1** can also be accomplished in a new and remarkably simple way. Modified surface **Si-1** was treated with a solution of the bifunctional cross-linking reagent disuccinimidyl glutarate (DSG) in anhydrous acetonitrile to give **Si-4**. Incubation of **Si-4** with an aqueous solution of any protein could then enable its linking to the functionalized Si surface through free ω-NH_2 groups of lysine amino acid side chains. Using this approach, we attached purified rabbit antimouse IgG, a 150 kD multi-subunit protein, to **Si-4** (Figure 4). IR analysis of the antibody-derivatized SiO_2/Si (**Si-5**) showed peaks (ν_{CO} = 1620−1650 cm^{-1}) characteristic of peptide linkages.

Rabbit antimouse IgG antibody-modified SiO_2/Si (**Si-5**) is itself a generalized surface derivative that can be made highly specific by incubation with a mouse monoclonal antibody directed against a single protein. To demonstrate specificity, monoclonal antibodies directed against cell surface proteins α4 integrin and α5 integrin were immobilized on two different IgG-modified surfaces. Coupled surfaces were then incubated with a mixture of cells expressing either α4 or α5 integrins and differentially marked with fluorescent dyes (Figure 5a). Cells expressing α4 integrin (red) were specifically recruited onto the **Si-5** surface containing anti-α4 antibody (Figure 5b,d), while cells within the population but not expressing this protein remained in the medium. Similarly, bonding of a mouse monoclonal antibody directed against α5 integrin resulted in significant enrichment of α5-expressing cells on the surface (Figure 5c,d). Thus, attachment of monoclonal antibodies converts rabbit antimouse IgG-derivatized **Si-5** into a surface with exquisite specificity.

We have now demonstrated that the oxide-coated surface of Si (SiO_2/Si) can be modified easily using self-assembled monolayer films of ω-functionalized alkylphosphonates. These reactions can be performed in air, and product films are water-stable. Because the only thermally demanding step involves preparing **Si-1**, and because of the broad range of reproducible coupling possibilities afforded by a well-defined hydroxylated monolayer surface, this new methodology should pave the way for the attachment of biomolecules of a general nature to Si. Indeed, our simple substrate modification techniques may obviate traditional, more complex and lengthy processing. We have shown that these derivatized phosphonate films are stable to physiological conditions and are reusable and that bioactive molecule-derivatized surface phosphonates support the binding and spreading of numerous cell types on that surface. Most significantly, we have also demonstrated the means to bind biomolecules far larger than simple tetrapeptides to derivatized SiO_2/Si. In particular, the linking of antibodies to this surface can enable the selective recognition of a wide range of molecules, including antigens on the surfaces of bacterial

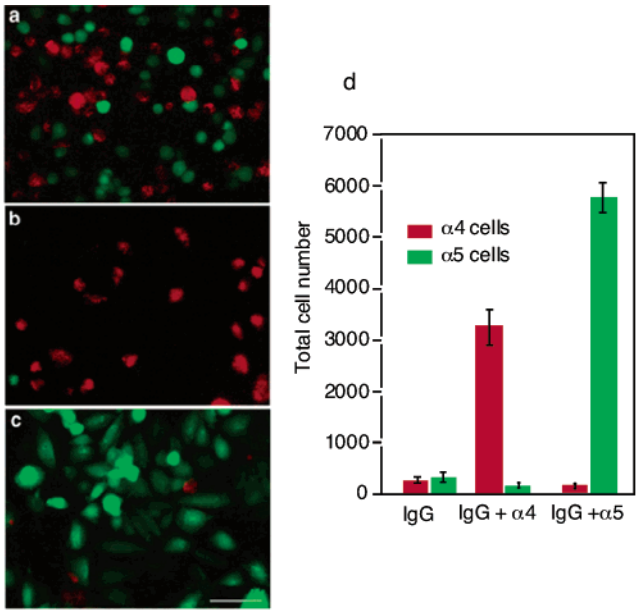

Figure 5. Selective antigen targeting by antibody-derivatized surfaces: (a) a mixed cell population of CHOα4 cells fluorescently tagged red and CHOα5 cells tagged green; (b) CHOα4 adhered to a surface of α4-antibody derivatized Si; (c) likewise, CHOα5 cells adhered specifically to the anti-α5-integrin antibody coupled surfaces. Scale bar = 10 μm. (d) The total number of adherent cells on 0.2 cm^2 substrates was counted from samples after 18 h and expressed as the mean/substrate ± SEM. Similar selectivity was observed at 2 h.

pathogens and parasites. It now remains to demonstrate that our phosphonate-based modification of the native oxide of Si can be an important tool in the rapid, sensitive, and specific detection of protein or pathogen targets by conjugating these interfaces with Si-based transducers.

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