Bonding Organics to Ti Alloys: Facilitating Human Osteoblast Attachment and Spreading on Surgical Implant Materials

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Introduction

Titanium and its alloys (notably Ti−6Al−4V ELI grade) have high mechanical strength and are resistant to chemical attack; thus they enjoy widespread use as surgical implants which are often in contact with bone.[1−5] Paradoxically, this resistance to chemical attack can be problematic: Osteointegration[6] can be weakened by the absence of strong chemical bonding at the bone−implant surface interface. Consequently, implants can fail under shear stress, requiring revision surgery.[1,2] Clearly, enhancing osteoconductivity of implant material surfaces[7] will have a high quality of life impact for millions.[1]

Bonding the fibronectin cell attachment peptide, arginine−glycine−aspartic acid (RGD), to a surface through an organic tether[8−12] might enhance its osteoconductivity by providing sites for cell attachment and spreading,[13] but conventional methods for such surface peptide attachment to Ti alloys are often problematic. In particular, silanization, long considered the benchmark method for attaching organics to Ti or Ti−6Al−4V via their native oxide coatings, is limited by surface hydroxyl group content, and the OH group content of the Ti native oxide surface accounts for only about 15% of total surface oxygen.[14] Low yields of direct surface silanization result,[15] and silane reagent cross-linking can predominate over surface attachment under silanization conditions.[9,10,12] Also, since siloxane products of silanization can be hydrolytically unstable, surface coverage by key organics can be further reduced when silanized surfaces are exposed to aqueous conditions.[9]

We have described two methods for surface modification of Ti or Ti−6Al−4V with organics. The first involves silanization of a heavily hydroxylated Ti(III) phosphate layer ("TiP") which is strongly bound to the Ti or Ti−6Al−4V surfaces.[16] The second entails bonding alkylphosphonate films which are formed on these surfaces by self-assembly/heating.[17] Neither method is limited by the low native oxide OH group content of the Ti or Ti−6Al−4V surfaces.[14,15] We now report that both interfaces can be used to accomplish covalent bonding with high surface coverage of RGD on Ti−6Al−4V; surface coverage by derivatized phosphonate films can be measured directly, using a novel quartz crystal microbalance (QCM) technique. Neither type of interface is cytotoxic; indeed their RGD derivatives promote considerable attachment, spreading, and proliferation of human fetal osteoblasts on Ti−6Al−4V. However, even though initially high surface coverage by silanization and subsequent RGD derivatization of TiP are readily effected, the inherent hydrolytic instability of surface siloxanes under physiological conditions[9] ultimately limits the useful lifetime of this class of interfaces. In sharp contrast, RGD-modified surface-bound phosphonate films remain robust under physiological conditions, and cell proliferation on them is extensive.

Experimental Section

General. Aminopropyl(triethoxy)silane (**1**) (Gelest), 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (**3**) (Aldrich, 99%), and RGDC (American Peptide) were used as received. Acetonitrile was distilled from CaH_2.

Titanium(III) Dihydrogen Orthophosphate on Ti−6Al−4V (TiP). Disks of Ti−6Al−4V ELI ($^3/_8$ in. diameter) were sanded first with 240 grit and then 600 grit silicon carbide paper, rinsed, and sonicated successively with methylene chloride, methyl ethyl ketone, and methanol, dried, and stored in an oven at 200 °C. The disks were placed in a solution of aqueous phosphoric acid (1.4 M) as previously described for pure Ti.[16] TiP was identified as the sole surface species present, by X-ray diffraction analysis.

Attachment of RGD to Ti−6Al−4V ELI via the TiP Interface. Disks of Ti−6Al−4V ELI coated with TiP were placed in a Teflon well, treated with a solution of **1** (10 mM in THF), and then solvent rinsed with sonication to give surface-bound **2** (IR: ν_{CH_2} = 2925, 2860 cm^{-1}; residual ν_{OH} = 3200 cm^{-1}; ν_{NH_2} group = 1575 cm^{-1} [9]). The disks were then placed in solutions of **3** (5 mM in acetonitrile) for 18 h at room temperature. The disks were removed from solution, solvent evaporated, and analyzed by IR. They were then rinsed in acetonitrile with sonication and dried in vacuo (0.1 Torr) to give surface-bound **4** (IR: $\nu_{CO(asymm)}$ = 1709 cm^{-1}; absorbance intensity ≈0.1% vs ≈0.003% for the adduct prepared by direct silanization of the native oxide[9]). A solution of RGDC (5 mM) was prepared in 5 mL of purified water (Millipore), with the pH adjusted to 6.5 using 0.1 M NaOH. Disks of **4** were left in the stirred solution of the peptide at room temperature for 24 h to give surface bound RGDC (**5**). They were then removed, dried, and analyzed by IR. The disks were then rinsed with water, dried, subjected to tape peel testing, and reanalyzed by IR (for **5**, ν_{CO} = 1700, 1650, 1560 (shoulder) cm^{-1} [9]).

Attachment of RGD to Ti−6Al−4V ELI via an 11-Hydroxyundecylphosphonate Interface. Disks of Ti−6Al−4V were placed in a Teflon well and covered with a solution of

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Table 1. Surface Coverage of Ti by Alkylphosphonates and Derivatives As Measured by QCM: (a) Preparation of Phosphonate Films; (b) Derivatization of 11-Hydroxyundecylphosphonate (6)

(a) Preparation of Phosphonate Films

preparation of phosphonate film	Δf (Hz)	coverage, nmol/cm^2 [a]	preparation of phosphonate film	Δf (Hz)	coverage, nmol/cm^2 [a]
octylphosphonate (**11′**)	109	2.98 (2.29)	11-hydroxyundecyl-	141	2.84 (2.18)
	115	3.15 (2.42)	phosphonate (**6**)	115	2.31 (1.78)
average		(2.36)		140	2.82 (2.16)
				135	2.71 (2.09)
				106	2.13 (1.64)
				135	2.71 (2.09)
			average		(1.99)

(b) Derivatization of 11-Hydroxyundecylphosphonate (**6**)

coverage by **6**, nmol/cm^2 [b]	conversion to **7**, Δf (Hz)	coverage, nmol/cm^2	yield (%)	conversion to **8**, Δf (Hz)	coverage, nmol/cm^2	yield (%)
2.72 (2.09)	37	1.08 (0.83)	40			
2.13 (1.64)	31	0.90 (0.69)	42	69	0.67 (0.52)	74
2.72 (2.09)	27	0.79 (0.61)	29	47	0.46 (0.35)	58
average			37			66

[a] Corrected value for surface roughness factor measured to be 1.3 by AFM analysis of the sputtered Ti electrode given in parentheses. [b] Corrected value for surface roughness given in parentheses.

11-hydroxyundecylphosphonic acid[18] dissolved in dry tetrahydrofuran (10.0 mM). The solvent was allowed to evaporate, the disks were warmed in an oven at 120 °C for 48 h and were rinsed, with sonication, in THF. Analysis by IR showed an alkyl-chain-ordered[19] monolayer film of **6** ($\nu_{CH_2(asymm)} = 2917$ cm^{-1} and $\nu_{CH_2(symm)} = 2948$ cm^{-1}). Disks of **6** were placed in a solution of **3** in acetonitrile (5 mM) and were kept at room temperature under N_2 for 24 h. The disks are removed from the solution, dried, and analyzed by IR, which shows peaks corresponding to both the maleimide adduct **7** ($\nu_{CO} = 1707$; 1734 cm^{-1}) and excess **3**; the latter could be removed by rinsing with acetonitrile. The disks coated with **7** were then immersed in an aqueous solution of RGDC (2 mM in water, Millipore) at room temperature for 24 h, removed from solution, dried, and analyzed by IR (1650 cm^{-1}). The persistence of this peak following several solvent rinses indicates irreversible attachment of the coupled tetrapeptide to the surface as **8**.

Attachment of 11-Carboxyundecylphosphonic Acid to Ti−6Al−4V. Disks of Ti−6Al−4V were placed in a Teflon well and were treated with a solution of 11-carboxyundecylphosphonic acid[20] in THF, as described for 11-hydroxyundecylphosphonic acid ($\nu_{CH_2(asymm)} = 2915$ cm^{-1}, $\nu_{CH_2(symm)} = 2948$ cm^{-1}, and $\nu_{CO} = 1694$ cm^{-1}).

QCM Determination of Alkylphosphonate Surface Loadings on Ti. Piezoelectric quartz crystals (International Crystal Manufacturers (ICM); AT-cut, 1000 Å Ti electrodes, 10 MHz, overtone polished, 0.201 in. electrode diameter) were used for film deposition and as references. The QCM was allowed to stabilize for 30 min after start-up, before experimental measurements were made. In each experimental run, the fundamental frequency (f_o) of an untreated QCM was measured. The QCM was then removed from its holder, and both electrodes were treated with a solution of the phosphonic acid, dried, and then heated at 120 °C for 3 days. The new frequency (f_c) was then measured. The QCM was then subjected to rinsing followed by evacuation ($\leq 10^{-2}$ Torr), until a constant frequency was measured (±2 Hz), which was assumed to represent monolayer coverage of the Ti electrodes. The difference between frequencies for the treated and the untreated QCMs was then related[21,22] to the amount of material chemisorbed on the Ti electrode active area. Loadings measured by QCM for octylphosphonic and 11-hydroxyundecylphosphonic acid on Ti are given in Table 1. Similar procedures were used to measure chemical yields for conversion of **6** to **7** and of **7** to **8**.

Modified Surface Interactions with Human Osteoblasts. 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 G418 (GIBCO, BRL). Cells were labeled with 10 μM Cell Tracker Orange (Molecular Probes, OR) for 30 min at 34 °C. After this time, the medium was removed and replaced with fresh medium and serum for an additional 30 min at 34 °C. Cells were released from tissue culture dishes using 0.2 mg/mL EDTA in PBS, washed with PBS, and resuspended in serum-free medium at 1×10^5/mL, and 500 μL of the cell suspension was added to wells containing the alloy substrate disks which had been blocked with 1% BSA in PBS for 30 min before cell addition. Cells were allowed to spread on the substrates for 90 min, 24 h, and 3 days. Samples taken at each time period were washed with PBS and visualized using a Nikon Optiphot-2 microscope. Images were captured using a Photometrics Coolsnap camera and analyzed using Coolsnap and IP labs software. A quantitative assessment of cell adhesion was carried out by counting the number of cells from three random fields per substrate (0.52 mm^2). Values (see Figure 2) are expressed as the mean number of adherent cells. To visualize the actin cytoskeleton, cells were allowed to adhere to substrates for 90 min before staining with rhodamine conjugated phalloidin (Molecular Probes Inc.) at 1:1000 in 2% ovalbumin (Sigma Chemical Co.) in PBS at 34 °C for 30 min.

Results and Discussion

Silanization of a surface is limited by its hydroxyl group content, which is low for the Ti native oxide.[14] We have reported[16] the preparation of surface-bound $Ti[H_2PO_4]_3$[23] (TiP) on Ti foils by heating thin films of surface-adsorbed phosphoric acid. We note here that TiP is prepared on Ti−6Al−4V ELI grade with equal ease. TiP has a layered structure; phosphate OH groups lie above and below the layers in a titanium phosphate polymer. Thus forming TiP on Ti−6Al−4V effectively converts a low OH surface into a high OH content one. Accordingly, we had found that simple protolytically labile organometallics react more extensively on TiP than on the Ti native oxide.[16] TiP also reacts with functionalized silane aminopropyl(triethoxy)-silane ("APTES", **1**) to give the corresponding surface-bound aminosiloxane, **2**. Apparently, reaction of the phosphate groups at Si is somewhat favored versus simple proton transfer from a surface OH to the amino group of

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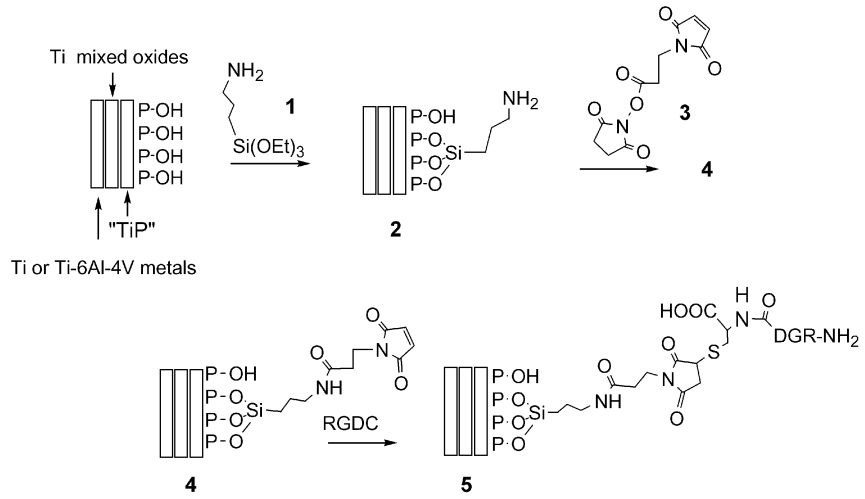
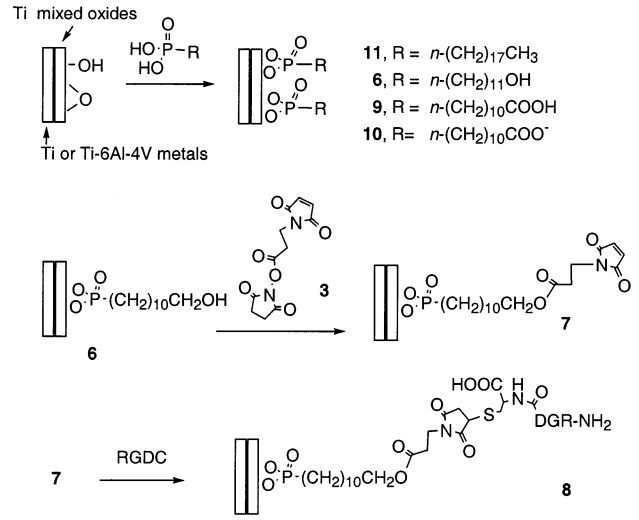
Scheme 1. Silanization of the TiP-Modified Surface[a]



[a] Silanization of the TiP-modified surface is enhanced by its high OH content. Elaboration to a surface-bound RGD product occurs with high surface coverage.

Scheme 2. Phosphonic Acid Films Formed by Self-assembly and Baking on Ti or Ti−6Al−4V



1. Surface coverage of TiP by **2** is far higher than that which can be accomplished on the native oxide of Ti as determined by direct comparison of IR spectra; in the latter case, **2** could not be detected by IR.[9] In a key observation, we note that **2** and 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (**3**) react to give an adduct (**4**), for which comparative IR analysis shows at least a 30-fold increase in surface coverage on TiP compared to that which is obtained on the native oxide of Ti.[9] When surface-bound **4** was treated with an aqueous solution of arginine−glycine−aspartic acid−cysteine (RGDC), rinsing and evaporation of the water gives the surface-bound peptide, **5** (Scheme 1).

We have also reported that alkylphosphonic acids self-assemble on the native oxide surfaces of Ti and Ti−6Al−4V. Heating bonds the acids strongly to these surfaces as ordered phosphonate films (Scheme 2).[17] Atomic force microscopy (AFM) imaging of a film formed from octadecanephosphonic acid shows comprehensive surface coverage by aggregated islands, with elevations of each island consistent with monolayer formation (film thickness 2.9 nm, "tilt angle" of 33° [17,24]) and with total coverage at least as good as has that observed for alkanethiols/gold.[25] X-ray photoelectron spectroscopy analysis[26] of a film of butanephosphonic acid/Ti suggests that two of the three oxygens of the phosphonate headgroup are surface bound, consistent with film thickness measurements.[17,24]

Alkanephosphonic acids substituted at the ω-position with hydroxyl (**6**) or carboxylic acid (**9**) "tail" groups can also be bound to the native oxide surfaces of Ti and Ti−6Al−4V by self-assembly/heating. These tail groups however can be problematic: Surface multilayer deposition can occur, likely through H-bonding networks. The surface−phosphonate bond is apparently stable to both aqueous and nonaqueous media; thus adlayers can be removed by copious washing and sonication with organic solvents or aqueous base to give surface-bound monolayers. IR analysis for these monolayers is consistent with surface attachment through the phosphonate headgroups;[27] hydrogen bonding of the tail group (by IR) apparently helps to make strong alkyl-chain-ordered, robust films (for **9**, ν_{CO} = 1694 cm^{-1}). Washing **9** with aqueous buffer (pH 10) causes ν_{CO} to shift to 1575 cm^{-1}, indicating deprotonation to the ω−carboxylate-terminated film (**10**).

H-bonding interactions among tail groups in films such as **6** or **9** can also compromise reactivity of these groups; for **9**, this factor appears to be more acute than for analogous thiol/Au films. For example, carboxyundecanethiol/Au reacts readily with $SOCl_2$ to give the corresponding acid chloride,[28] but **9** does not react even with neat $SOCl_2$. Conditions could be developed, though, for conversion of OH group-terminated film **6**, via maleimido derivative **7**, to the RGD adduct, **8**.

That phosphonic acid coverage on the native oxide surface of Ti is not limited by surface OH content[14] was demonstrated by QCM measurements. The QCM technique allows accurate, gravimetric determination of mass changes on an electrode which is deposited on a piezoelectric quartz crystal. It is thus ideal to monitor surface reactions of target metals when they are used as such electrodes:[21,22] The QCM oscillates with a resonance

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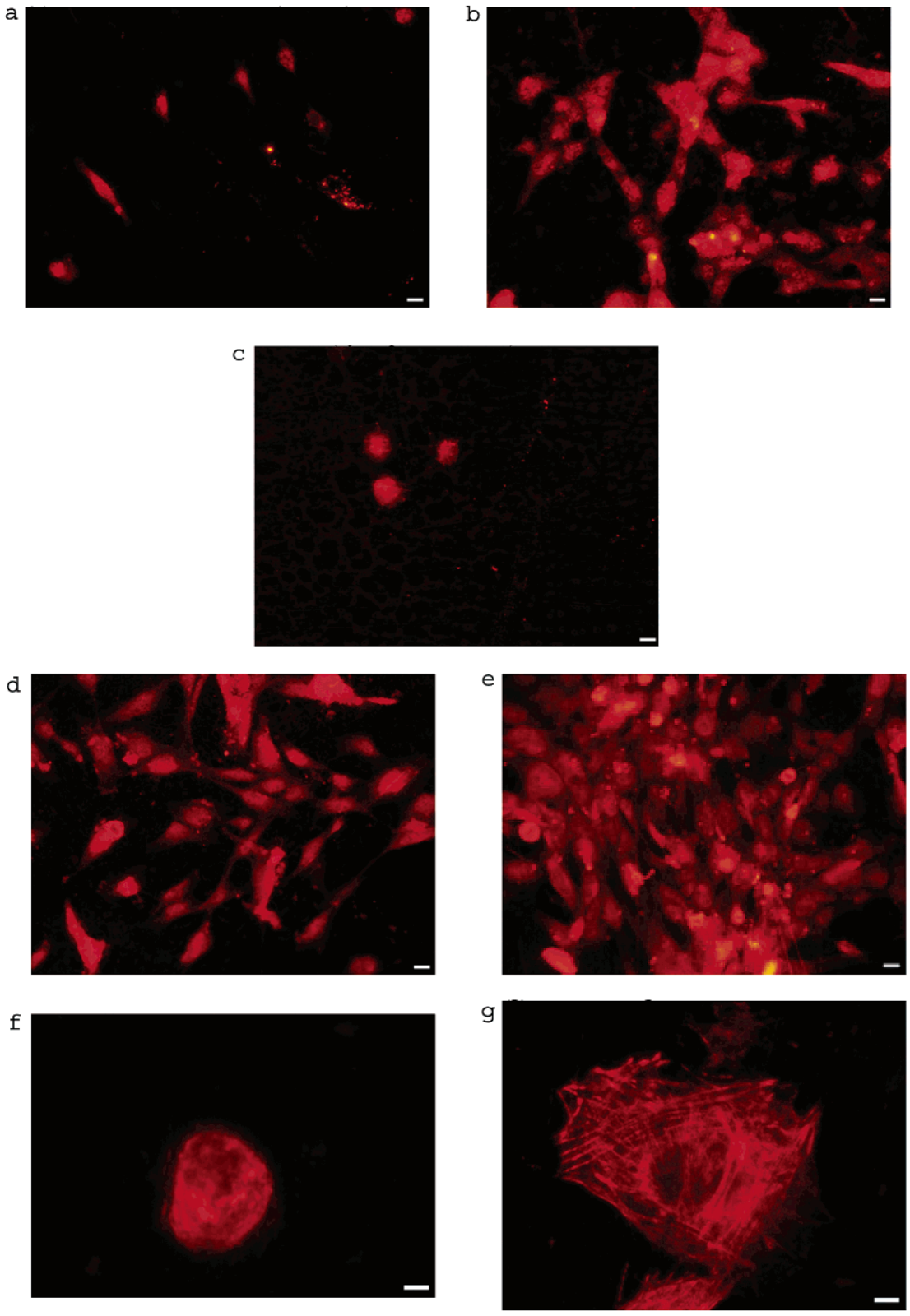

Figure 1. Human fetal osteoblasts on: (a) clean Ti (control), 24 h; (b) RGDC-derivatized alkylsilane on TiP, **5**; (c) ω-hydroxyalkylphosphonate, **6**, 24 h; (d) RGDC-derivatized phosphonate, **8**, 24 h; (e) **8**, 3 days; (f) actin staining of osteoblasts on **6**, 90 min; (g) actin staining of osteoblasts on **8**, 90 min. Size marker bars 12 μm for (a−e) and 4 μm for (f−g).

frequency which is determined by the cut and mass of the crystal, and just as for a classical oscillator, changes in electrode mass result in changes in crystal resonance frequency. Since our experiments necessitate detaching active crystals from the QCM oscillator for extended periods of time followed by reattachment, control measurements had to be made of reference crystals which were subjected to similar handling, but without surface treatment. Several reference crystals were used as received to calibrate the QCM. Careful handling of the active and reference crystals was imperative: Any change in stress on a crystal in its holder would result in an unacceptably large (>10 Hz) frequency change from its initial value, invalidating an experimental run. To ensure

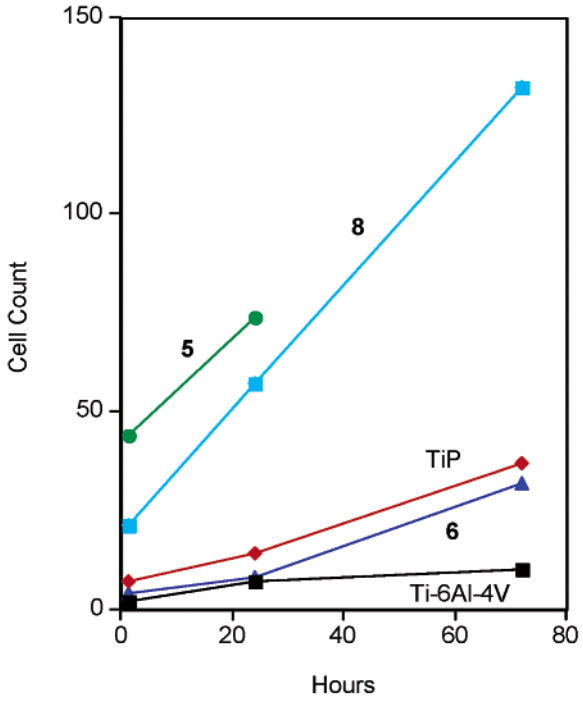

Figure 2. Cell growth on surfaces: Ti−6Al−4V native oxide (control); TiP (control); RGDC-derivatized alkylsilane on TiP, **5**; *ω*-hydroxyalkylphosphonate, **6** (hydrophilic control); RGDC-derivatized phosphonate, **8**.

that monolayer coverage (at most) occurred on Ti, films of **6** were subjected to copious rinsing (without sonication) followed by evacuation ($\leq 10^{-2}$ Torr), until a constant crystal frequency was established (±2 Hz). QCM measurements were also made for simple substrate **11′**, in which multilayer formation through head and tail group interactions is obviated. Direct gravimetric analysis measured surface loadings of both **6** and **11′** on Ti to be on the order of 2 nmol/cm^2 (see Table 1). This loading is corrected for surface roughness of the sputtered Ti electrode surfaces (roughness factor 1.3, as measured by AFM) and is higher than that measured for alkanethiols on gold by similar methods.[29] Significantly, phosphonate film coverage of Ti is nearly 10 times greater than that reported for siloxane **2** (of comparable headgroup size) on the native oxide of Ti.[9] QCM gravimetric measurement of the yield for conversion of **6** to **7** was close to 40%, and of **7** to **8** was on the order of 60% (Table 1). A simple calculation (MM2, Chem3D) shows the cross-sectional area of **7** as projected on the *ω*-OH terminated organic surface of **6** to be about 42 Å^2. The cross-sectional area for a close-packed alkyl chain structure with a 33° tilt angle is calculated to be ca. 22 Å^2.[30] These relative size considerations suggest that the maximum possible yield for converting **6** to **7** would be on the order of only 50−60%. Since **8** is sterically larger than **7**, a chemical yield less than 100% would also be expected for this step.

To test the stability of our interfaces under physiological conditions and to determine their efficacy for cell adhesion and spreading, human fetal osteoblasts (HFOB 1.19; ATCC) were incubated with unmodified or variously surface-modified Ti−6Al−4V ELI for 90 min, 24 h, and 3 days. Cells were previously tagged with a fluorescent marker so that cell adhesion, spreading, and counting could be monitored by fluorescence microscopy. Osteoblast images taken at various times are shown in Figure 1. Very little osteoblast adhesion occurred on unmodified Ti−6Al−4V ELI (Figure 1a) or on alkyl (**11**, not shown), *ω*-hydroxy (**6**, Figure 1c), *ω*-carboxylic acid (**9**, not shown), or *ω*-carboxylate (**10**, not shown) group-terminated alkylphosphonate-modified surfaces, similar to observations made for modified alkanethiols/Au.[31] A striking observation was made for TiP-modified surface **5** (Figure 1b), where cell adhesion was initially quite efficient (Figure 2): It may be that the presence of exposed phosphate groups of TiP in conjunction with chemically bonded RGD creates an especially attractive mixed-function environment for the osteoblasts. Unfortunately, the inherent hydrolytic instability of surface siloxanes limits the long-term viability of this interface; after 3 days, loss of surface material was visually apparent. In contrast, adhesion and spreading of the osteoblasts on RGD-modified surface **8** were quite substantial after 24 h (Figure 1d) and even more so after 3 days (Figure 1e). Indeed cell proliferation continued unabated throughout the test period on this surface (Figure 2). The morphology and actin cytoskeleton of cells were observed by staining with rhodamine phalloidin. Cells remained small and rounded with no organized actin cytoskeleton on control substrates (Figure 1f). However, more than 90% of cells adherent to RGD-modified substrates became well spread and organized their actin filaments into robust stress fibers (Figure 1g).

Conclusions

We have shown that surface coverage and subsequent RGD derivatization of the Ti−6Al−4V surface can be readily effected by silanization in amounts far higher on a surface-bound Ti phosphate interface than can be accomplished on the native oxide by standard methods. Nonetheless, the inherent hydrolytic instability under physiological conditions of surface siloxanes ultimately limits their utility. In sharp contrast, RGD-modified surface-bound phosphonate films, which are also easy to prepare with high surface coverage, remain robust under physiological conditions, and cell proliferation on them is extensive. Thus, a successful surface chemistry approach for creating stable osteoconductive surfaces on Ti and Ti−6Al−4V is now at hand.

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