Self-assembled Monolayers of α,ω-Diphosphonic Acids on Ti Enable Complete or Spatially Controlled Surface Derivatization

Michael P. Danahy,† Michael J. Avaltroni,† Kim S. Midwood,‡ Jean E. Schwarzbauer,‡ and Jeffrey Schwartz*,†

Department of Chemistry, Princeton University, Princeton, New Jersey 08544-1009, and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

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α,ω-Diphosphonic acids self-assemble on the native oxide surfaces of Ti or Ti-6Al-4V. Heating gives strongly bonded phosphonate monolayers. Infrared and X-ray spectroscopic and water contact angle data show that the films are bonded to the surface by one phosphonate unit; the other remains a phosphonic acid. Surface loadings were measured by quartz crystal microbalance procedures. Mechanical shear strengths for the films were also measured; these do not correlate simply with surface loadings. Films formed from 1,12-diphosphonododecane were treated with zirconium tetra(*tert*-butoxide) to give surface Zr complex species; derivatives of these surface complexes are stable to hydrolysis under physiological conditions and are mechanically strong. The complexation reaction can be accomplished over the entire surface; alternatively, dropwise application of the alkoxide to the surface enables spatial control of deposition. The cell attractive peptide derivative RGDC can be bound to these surface Zr alkoxide complexes through (maleimido)-alkylcarboxylate intermediates. Surfaces modified with RGDC were shown to be effective for osteoblast binding and proliferation.

Introduction

Spatially controlling surface modification of a substrate can in principle define zones for simultaneous bonding of different types of reagents. For example, such controlled modification of an implant with both cell adhesion peptides[1,2] and other bioactive groups[3] might enhance device biointegration.[4] For such spatially controlled modification to succeed, the surface attachment of each reagent must be irreversible and must occur rapidly with regard to their diffusion across the surface. Elegant methods have been developed to pattern substrates[5,6] using variously functionalized alkanethiols on Au[7,8] or siloxanes on SiO_2[9] by stamping onto the surface. Simple synthetic methods can then be employed to immobilize, for example, bioactive species on these surfaces.[6,8,9] However clever these technologies, they cannot satisfy the first criterion for modification of devices based on commonly used metal or metal alloy materials: Alkanethiols do not form strongly bound monolayers on metal native oxide surfaces,[10,11] and siloxanes (which can be bound to these surfaces) can be unstable to hydrolysis under physiological conditions.[12,13] We have reported that self-assembled monolayers of phosphonates can be strongly bonded to Ti or Ti alloy surfaces via reaction of phosphonic acids with their native oxides,[13−15] but our attempts to make spatially controlled films of two different phosphonates on the same substrate have not been successful:[16] Thermal setting of phosphonate films on the alloy surface is required, and mixing of the precursor phosphonic acids on the surface is apparently faster than is their bonding to that surface.

We now report that α,ω-diphosphonic acids (**1**) can form hydrolytically stable, strongly adhered films of the corresponding phosphonates (**3**) directly on the native oxide surfaces of Ti and Ti-6Al-4V (Scheme 1). We further report that these films can be activated in a way that is, in principle, amenable to patterning. This method gives a new type of compound interface between a surface and a biomolecule. In particular, we find that zirconium tetra(*tert*-butoxide) (**4**) can react rapidly with these films, either across the entire surface or at determined places by controlled deposition. Finally, we show that these surface-

* To whom correspondence should be addressed.
† Department of Chemistry.
‡ Department of Molecular Biology.

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(16) A coupon of Ti-6Al-4V was treated[17] with a solution of 11-carboxyundecylphosphonic acid and octylphosphonic acid (1:1). IR analysis of the subsequently prepared monolayer film indicated that the 11-carboxyundecylphosphonate was apparently in a non-hydrogen-bonded environment (ν_{CO} = 1708 cm^{-1} c.f. ν_{CO} = 1690 cm^{-1} for a homogeneous film of 11-carboxyundecylphosphonate), suggesting that islanding of the components does not exist to an appreciable extent. These results are comparable to that which has been noted for mixed alkanethiol films on gold.[18,19]
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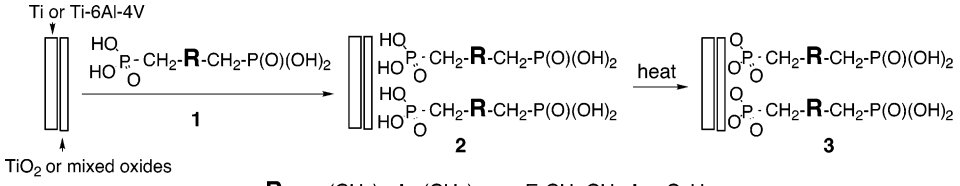
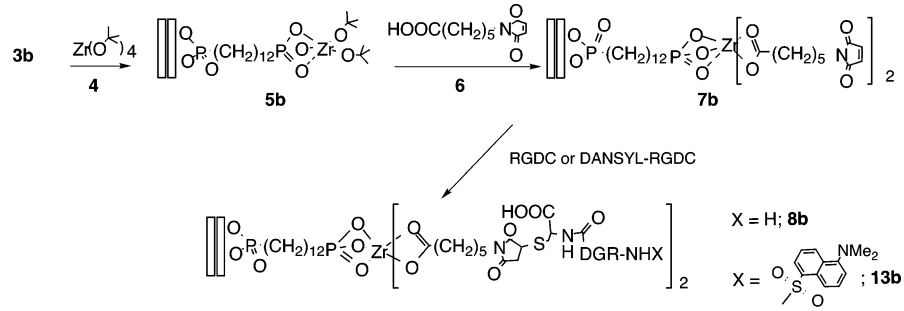
Scheme 1



Scheme 2



bound alkoxides can be further derivatized to bond the biomolecule to the surface (Scheme 2). Derivatized Zr complex-modified surfaces are hydrolytically stable, and they can retain the high mechanical shear strength of the parent diphosphonate film−metal oxide surface interface.

Experimental Section

General. All reagents were used as received unless otherwise noted. Titanium grade 5 alloy (Ti-6Al-4V) (Titanium Industries) rod (10-mm diameter) was cut into 1-mm-thick disks by an electric discharge machining (EDM) (New Jersey Precision). The disks were polished using 240 and 500 grit sandpaper and then cleaned by sonication in methylene chloride, followed by rinses with hot methyl ethyl ketone (one rinse) and methanol (three rinses). Samples were then dried and stored in air at 200 °C for at least 1 h. 11-Phosphonoundecanol was prepared as previously described.[13] Diphosphonic acids were characterized by NMR. Surface-modified samples were analyzed using a Midac M2510C Interferometer equipped with a Surface Optics SOC4000 SH specular reflectance head attachment. Fluorimetry experiments were done using a Photon Technology International Fluorescence Spectrometer. A Zeiss LSM 510 confocal fluorescence microscope was used for fluorescence imaging.

Synthesis of α,ω-Diphosphonic Acids. 1,4-Diphosphonobutane (**1a**; Acros), 1,12-diphosphonododecane (**1b**),[20] 1,4-diphosphono-2-butene (**1c**),[21] and 1,4-diphosphonoxylene (**1d**)[22] were prepared by reaction of triethyl phosphite with the corresponding dibromide starting materials followed by hydrolysis. The diphosphonic acids were purified by recrystallization from methanol.

Preparation of Diphosphonic Acid Films on Ti-6Al-4V. Polished disks of Ti-6Al-4V were suspended vertically in dilute solutions of the diphosphonic acids (**1**; 0.1 mM in methanol) in a wide-mouth flask. The solvent was allowed to evaporate so that the meniscus slowly traversed the disk surface to give the SAM of the diphosphonic acid (**2**).[17] Coated disks were then heated in a tube furnace at 140 °C for 48 h to convert this film to the SAM of the diphosphonate (**3**). The disks were then rinsed and sonicated extensively in methanol and Millipore water and characterized by IR.

Quartz Crystal Microbalance (QCM) Determination of Alkylphosphonate Surface Loadings on Ti. Diphosphonate film loadings on Ti surfaces were determined for **3a**−**d** by Ti electrode-equipped QCM measurements, as previously described.[13]

Determination of Mechanical Shear Strength. Mechanical shear strengths of the interfaces for **3a**−**d** and **10** on Ti-6Al-4V were measured according to ASTM test F-1066, as previously described.[15] Coupons of polished Ti-6Al-4V were coated pairwise with each of **3a**−**d** and **10**, and coupon pairs were joined using Cytec Fiberite FM 1000 epoxy to achieve an overlap of 2.84 cm^2. The alloy−epoxy−alloy sandwiches were then pressed together and heated in an oven that was ramped from room temperature to 170 °C at 2 °C/min and then held at 170 °C for 90 min. The oven was then cooled to room temperature at 2 °C/min to avoid any crack propagation or weakening of the epoxy layer. The sandwiches were then stressed in an Instron model 1331 load cell that was programmed to increase load at 0.5 kN/s and to end testing when the sandwich failed. Stress/strain factors and the point of maximum stress, where the interface failed, were recorded.

Zirconium Alkoxide Complex-Modified Surface 5b. Ti alloy disks coated with **3b** were placed in a deposition chamber which could be externally cooled and which was equipped with two stopcocks for exposure either to vacuum or to vapor of tetra(*tert*-butoxy) zirconium (**4**) (Lancaster). The chamber was then evacuated at 10^{-3} Torr for 30 min. Samples were exposed to vapor of **4** at 10^{-3} Torr for 15 min with external evacuation followed by 30 min of exposure without external evacuation. Samples were then evacuated at 10^{-3} Torr for 16−24 h to ensure removal of excess **4** and were then analyzed by IR spectroscopy (ν_{C-H} = 2973 cm^{-1}).

Spatially Controlled Modification of 3b by 4. Neat **4** was applied to disks of **3b** using a nonreactive applicator, such as a thin wire, under an inert atmosphere. Excess **4** was removed by rinsing with dry THF and the disks were then immediately immersed in a 5 mM solution of *N*-(5-(dimethylamino)-1-naphthylsulfonyl)-cysteine (**11**; Research Plus) in dry acetonitrile for 10 min. The disks were then rinsed and sonicated in acetonitrile, and the region of surface derivatization (**9b**) was imaged using confocal fluorescence microscopy.

Surface-Immobilized RGD (8b). A 5 mM solution of 6-maleimidohexanoic acid (**6**) (Fluka) was prepared in dry THF and disks of **5b** were exposed to this solution for 15 min while it was stirred under argon. Surface-modified disks (**7b**) were rinsed with sonication successively with THF, methanol, and Millipore water, and were then dried under vacuum and analyzed by IR (ν_{CO} = 1705 cm^{-1}). A 2 mM solution of RGDC (American Peptide) was prepared in doubly distilled Millipore water. The pH of the solution was adjusted to 6.5 using 0.1 mM NaOH in doubly distilled water. Disks of **7b** were kept in the RGDC solution at room temperature for 24 h, then removed from solution, rinsed, and sonicated for 15 min in Millipore water to give **8b**. Evacuation at 0.01 Torr and analysis by IR (ν = 1650−1690 cm^{-1}) followed each step of rinsing and sonication.

(20) Putvinski, T. M.; Schilling, M. L.; Katz, H. E.; Chidsey, C. E. D.; Mujsce, A. M.; Emerson, A. B. *Langmuir* **1990**, *6*, 1567.
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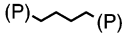
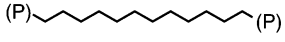
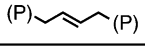
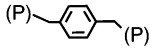
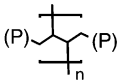
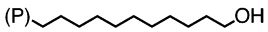
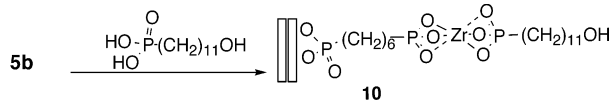
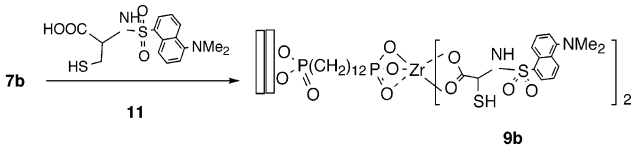
Table 1. Surface Loadings and Film−Surface Interfacial Shear Strengths (Average of Three Samples) Depend on Chain Structure and Tail Group

Surface SAM	Surface loading (nmol/cm^2)	Cross-sectional area (Å^2/molecule)	Interfacial shear strength (MPa)
3a 	0.40 ± 0.03	41.5	40.0 ± 4.0
3b 	0.52 ± 0.02	31.9	51.5 ± 3.1
3c 	0.77 ± 0.01	21.6	71.2 ± 7.4
3d 	0.25 ± 0.01	66.4	41.1 ± 2.6
3e 	0.77 ± 0.01	21.6	50.3 ± 2.4
11-hydroxyundecylphosphonate 	1.00 ± 0.09	16.6	52.1 ±2.1

Scheme 3



Scheme 4. Derivatization of 3



Determination of Hydrolytic Stability of 9b via Fluorescence Spectroscopy. Solutions of *N*-(5-(dimethylamino)-1-naphthylsulfonyl)-RGDC (DANSYL-RGDC, **12**) (Fisher) (0.4−51 μM in doubly distilled H_2O; pH 7.5) were prepared, and a calibration curve of fluorescence intensity vs concentration was measured. A second calibration curve was measured for **12** (0.4−51 μM) in aqueous solution at pH 12. Disks of DANSYLated surface **13b** were prepared as described for **8b** (Scheme 2) and were placed in a cuvette of doubly distilled H_2O adjusted to pH 7.5 with 0.1 mM NaOH. The fluorescence intensity of the supernatant was measured between 1 min and 5 days of immersion. The disks were then removed from solution, dried, and immersed in a cuvette of doubly distilled H_2O adjusted to pH 12 using 1 M NaOH for 3 h. The fluorescence intensity of this second supernatant was then measured.

Interactions of Human Osteoblasts with Modified Surfaces. Human fetal osteoblasts (HFOB 1.19; ATCC) were maintained as previously described.[13,15] Cells were released from tissue culture dishes using 0.1 mg/mL trypsin and 0.2 mg/mL EDTA in PBS, washed with 0.5 mg/mL soybean trypsin inhibitor (Sigma), and resuspended in a serum-free medium at 1×10^5/mL. Five hundred microliters 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 and 24 h (34 °C). Samples taken at each time period were washed with PBS, fixed, and stained to visualize the actin cytoskeleton and vinculin-containing focal adhesions, as described previously.[23] Immunofluorescent staining was visualized using a Nikon Optiphot-2 microscope as previously described.[13] A quantitative assessment of cell adhesion was made by counting the number of cells from three random fields per substrate (0.52 mm^2). Values were expressed as the mean number of adherent cells.

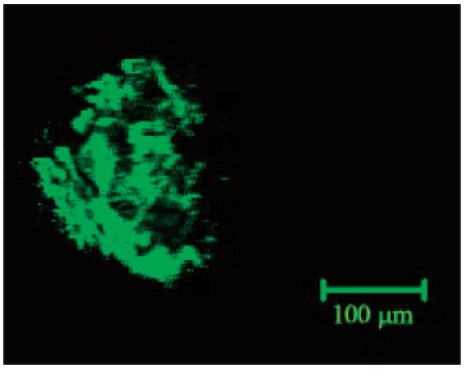


Figure 1. A fluorescent microdot of **9b** "written" on **3b** and "developed" with **11**.

Results and Discussion

Diphosphonic acids form self-assembled monolayers (SAMs) on the native oxide surfaces of Ti and Ti-6Al-4V using the "T-BAG" procedure,[17] in which the meniscus of a dilute solution of the diacid is allowed to traverse the surface by slow evaporation. Heating yields the covalently bound monolayer. After rinsing and sonication in methanol and water, IR analysis indicated that the film was bound directly to the titanium surface by one phosphonate unit ($\nu_{P-O(bound)} = 1093\ cm^{-1}$; c.f. 1093 cm^{-1} for Ti oxide surface-bound octadecylphosphonate;[14] $\nu_{P-O(free)} = 1253\ cm^{-1}$). X-ray photoelectron spectroscopy (XPS) showed a peak for P(2s) at 192.0 eV (c.f. 192.0 for **1b**) with a shoulder at 193.3 eV, assigned to surface-bound phosphonate. Contact angle measurements using water ($\Theta = 45 \pm 2°$) were also consistent with a phosphonic acid-terminated film. Films of **3b** showed typical absorbances in the aliphatic region ($\nu_{CH2,asymm} = 2920\ cm^{-1}$ and $\nu_{CH2,symm} = 2850\ cm^{-1}$), but comparable signals were too weak for **3a**. A quartz crystal microbalance (QCM) experiment[13] measured the surface loading of **3b** to be 0.52 ± 0.02 nmol/cm^2 (cross-sectional area 31.9 Å^2/molecule). This loading is only slightly more than half that measured for octadecylphosphonate/Ti (cross-sectional area 16.7 Å^2/molecule).[13] We had previously measured the surface loading of 11-hydroxyundecylphosphonate/Ti also to be less than that for the simple aliphatic case (1.00 ± 0.09 nmol/cm^2; cross-sectional area 16.6 Å^2/molecule). As for thiols on Au,[24] it is seems

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(24) Schreiber, F. *Prog. Surf. Sci.* **2000**, *65*, 151.

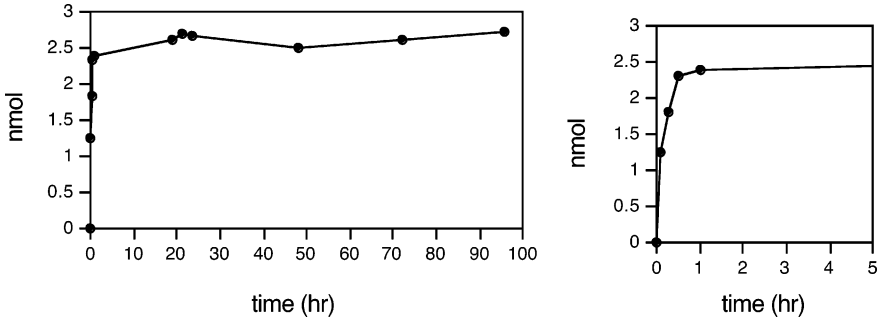


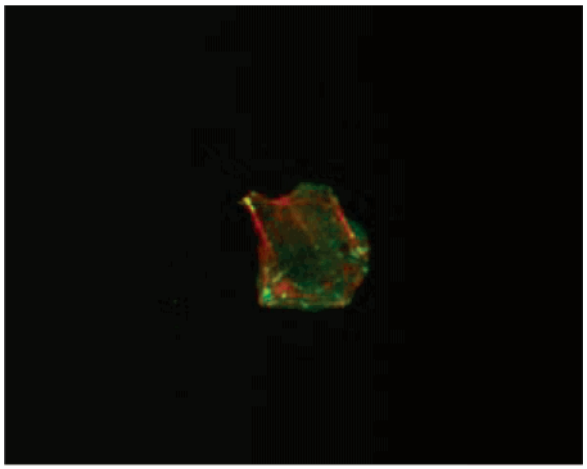
Figure 2. Hydrolysis of DANSYL-RGDC from **13b** measured for a disk of area 1 cm^2. No increase in dissolved fluorescent material was observed at pH 7.5 after 90 h (a) and after initial removal of residual material (b). The amount of such material released at pH 12.5 is a measure of amino acid that remains surface-bound at pH 7.5.

that these functionalized "tail" groups affect chain spacings, perhaps through creating hydrogen-bonded networks. It is not surprising that, at least by the IR criterion,[25,26] the alkyl chain portions of **3b** are not ordered; the cross-sectional area per molecule is notably larger than that in crystalline Zr phosphonates (24 Å^2/molecule),[27] so the chains may be too far apart on average to enforce an all-trans conformation in the film. Surface loading of **3a**, **3c**, and **3d** were also measured. As shown in Table 1, each also had a lighter loading than octadecylphosphonate/Ti; the more rigid, yet small, **3c** chain has the highest level of coverage of the diphosphonate series; the very low loading of **3d** is likely due to the large size of the aromatic ring. Thus, as has also been discussed for substituted thiols on gold, chain structure, too, affects film structure.[24,28]

Shear strengths of the interfaces formed by **3a**−**d** were measured mechanically,[15] and it was found that they do not correlate simply with surface film coverage densities. In particular, as shown in the table, film stiffening by the incorporation of olefinic or aromatic groups apparently leads to higher film−surface interface shear strengths than would be expected solely based on surface loading. In this regard, it is especially interesting that **3c**, when exposed to laboratory fluorescent lighting, underwent cross-linking to give **3e**, which had a measured shear strength considerably less than its olefinic precursor, even though there is no change in surface loading effected by this step (as confirmed by QCM).

The creation of molecular hierarchies using Zr^{4+} species as diphosphonate group linkers has been developed elegantly in which aqueous zirconyl solutions are used.[20,27,29] We were surprised, therefore, to observe that similar procedures have thus far failed to deposit Zr^{4+} onto our diphosphonate SAMs. In contrast, Zr alkoxide **4** reacts rapidly with these films, either from the vapor or liquid phases, to give surface-bound Zr alkoxide complexes, **5b**. That the complex−SAM interface is mechanically strong was demonstrated by derivatizing **5b** as an 11-hydroxyundecylphosphonate adduct **10** (Scheme 3).[30] Shear testing of **10** showed its strength to be 55 MPa, which is comparable to that of the parent **3b**. Thus, the shear strength of the complex linker is at least as strong as the surface−phosphonate interface itself.

(a) diphosphonate **3b** control



(b) RGD modified surface **8b** (90 min)

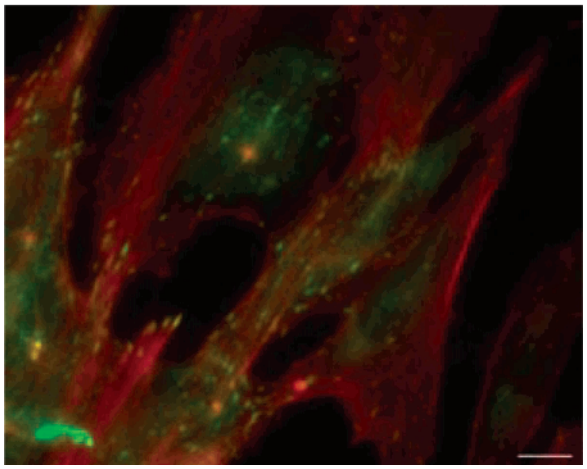


Figure 3. Osteoblast cell spreading after 24 h on (a) diphosphonate **3b** control and (b) RGD-modified surface **8b**. In these images, red staining shows actin, green staining shows vinculin, and yellow shows the merged overlap of actin and vinculin; the scale bar shown is 10 μm, and both images are at the same magnification.

Because Zr alkoxide **4** reacts quickly with the free phosphonic acid tail groups of **3**, spatially controlled derivatization is possible. For example, **4** was "dotted" on disks coated with **3b** using a thin wire. Nonchemisorbed alkoxide was removed, and surface-bound material (**7b**) was "developed" using fluorescent label-tagged DANSYLcysteine **11** to give **9b** (Scheme 4); rinsing removed any nonbound **11**. As shown by fluorescence microscopy, the labeled cysteine species adhered to the

(25) Sondag, A. H. M.; Raas, M. C. *J. Chem. Phys.* **1989**, *91*, 4926.
(26) Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. *J. Am. Chem. Soc.* **1989**, *111*, 321.
(27) Cao, G.; Hong, H.-G.; Mallouk, T. E. *Acc. Chem. Res.* **1992**, *25*, 420.
(28) Yang, G.; Qian, Y.; Engtrakul, C.; Sita, L. R.; Liu, G.-Y. *J. Phys. Chem. B* **2000**, *104*, 9059.
(29) Lee, H.; Kepley, L. J.; Hong, H.-G.; Mallouk, T. E. *J. Am. Chem. Soc.* **1988**, *110*, 618.
(30) Gawalt, E. S.; Lu, G.; Bernasek, S. L.; Schwartz, J. *Langmuir* **1999**, *15*, 8929.

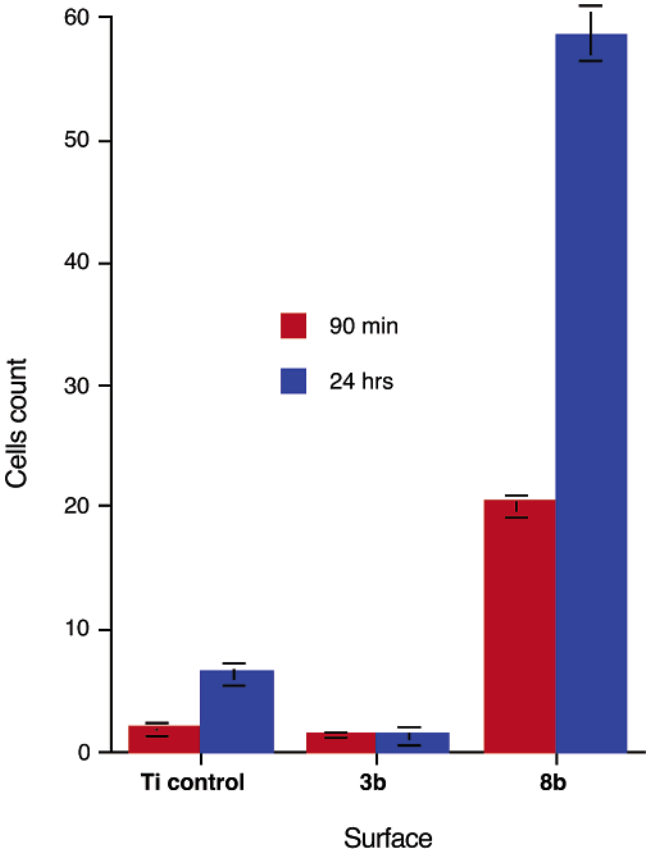


Figure 4. Cell counting (average of three random fields, 0.52 mm^2 each) for osteoblasts on (a) untreated Ti control, (b) diphosphonate **3b** control, and (c) RGD-modified surface **8b**.

surface only where **4** was "dotted" (Figure 1); phosphonic acid tail groups in regions "undotted" with **4** remained available for reaction with other species. Thus, with use of liquid **4**, a simple way exists in principle to pattern the Ti surface.

The use of surface-bound diphosphonic acids to immobilize biomolecules has been described in which a priming step is involved, usually involving a siloxane,[29,31,32] but surface-siloxane linkages can be labile under physiological conditions.[12,13] Our procedure does not involve such primers. To test the stability to hydrolysis of complex interfaces such as **8b**, fluorescently labeled analogue **13b** was prepared using DANSYLated RGDC (**12**; Scheme 2). Disks comprehensively covered with **13b** were immersed in water at pH 7.5 for 5 days at room temperature, and the supernatant was analyzed by fluorescence spectroscopy. As is shown in Figure 2, there was essentially no release of surface-bound fluorescent material into solution following initial removal of residues of synthesis. The disks were then removed from this solution, dried, and immersed in doubly distilled H_2O at pH 12 for 3 h; this treatment forcibly cleaves the Zr ion from the surface as ZrO_2. Analysis of this supernatant by fluorescence spectroscopy gives the total amount of amino acid in **13b**, which was measured to be 1.54 nmol. Since the area of the disk used is 2.65 cm^2, the surface loading of amino acid stable at pH 7.5 is 0.58 nmol/cm^2, corresponding to about 55 mol % coverage of the parent film (based on an ideal stoichiometry of two cysteines per diphosphonate; Scheme 4), comparable to amino acid loadings formed by covalent attachment to 11-hydroxyphosphonate/Ti (24 mol %, based on an ideal stoichiometry of one cysteine per hydroxyphosphonate).[13]

The complex interface **8b** is noncytotoxic, stable under physiological conditions, and promotes cell adhesion and proliferation. Compared to **3b,** disks coated homogeneously with **8b** supported extensive osteoblast attachment at 90 min and spreading over a 24-h period (Figures 3 and 4). Spread cells on **8b** had organized actin filaments and vinculin-positive focal adhesions, while cells on **3b** remained rounded (Figure 3). Osteoblasts were also able to proliferate once attached to RGD-modified **8b**, with significantly higher cell counts after 24 h on this surface (Figure 4).

Conclusions

Bonding phosphonic acids to a variety of oxide-coated surfaces starting from self-assembled monolayers is easily done using our simple deposition "T-BAG" protocol. Given the high reactivity of terminal phosphonic acid groups in the films we describe herein, it should be possible to accomplish true patterning of a wide range of such oxide-coated metals using an appropriate metal alkoxide as a linker complex precursor. Through such patterning of an implant surface, uncoated phosphonic acid sites might remain accessible, for example, for metallic ion binding.[4] In this regard, stabilizing biomineralized calcium phosphates on metallic surfaces might be possible in the context of implant osteointegration, and to that end, studies of patterned surface mineralization are now in progress.

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