Chemical modification of titanium surfaces for covalent attachment of biological molecules

A. Nanci,[1] J. D. Wuest,[2] L. Peru,[1] P. Brunet,[2] V. Sharma,[2] S. Zalzal,[1] M. D. McKee[1]

[1]*Faculty of Dentistry, Université de Montréal, P.O. Box 6128, Station Centre-Ville, Montréal, QC, Canada, H3C 3J7*

[2]*Department of Chemistry, Université de Montréal, P.O. Box 6128, Station Centre-Ville, Montréal, QC, Canada H3C 317*

Received 21 November 1996; accepted 24 March 1997

Abstract: The surface of implantable biomaterials is in direct contact with the host tissue and plays a critical role in determining biocompatibility. In order to improve the integration of implants, it is desirable to control interfacial reactions such that nonspecific adsorption of proteins is minimized and tissue-healing phenomena can be controlled. In this regard, our goal has been do develop a method to functionalize oxidized titanium surfaces by the covalent immobilization of bioactive organic molecules. Titanium first was chemically treated with a mixture of sulfuric acid and hydrogen peroxide to eliminate surface contaminants and to produce a consistent and reproducible titanium oxide surface layer. An intermediary aminoalkylsilane spacer molecule was then covalently linked to the oxide layer, followed by the covalent binding of either alkaline phosphatase or albumin to the free terminal NH_2 groups using glutaraldehyde as a coupling agent. Surface analyses following coating procedures consisted of X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Enzymatic activity of coupled alkaline phosphatase was assayed colorimetrically, and surface coverage by bound albumin was evaluated by SEM visualization of colloidal gold immunolabeling. Our results indicate that the linkage of the aminoalkylsilane to the oxidized surface is stable and that bound proteins such alkaline phosphatase and albumin retain their enzymatic activity and antigenicity, respectively. The density of immunolabeling for albumin suggests that the binding and surface coverage obtained is in excess of what would be expected for inducing biological activity. In conclusion, this method offers the possibility of covalently linking selected molecules with known biological activity to oxidized titanium surfaces in order to guide and promote the tissue healing that occurs during implant integration in bone and soft tissues. © 1998 John Wiley & Sons, Inc. *J Biomed Mater Res,* **40,** 324–335, 1998.

Key words: titanium; bioactive coating; immobilization; silanization; covalent attachment

INTRODUCTION

Implantable biomaterials are designed to replace a part of the body and/or its associated functions. In general, only the surface of an implant is in direct contact with the host tissue, and thus this portion of the biomaterial plays a critical role in determining biocompatibility. The surface of any material can change with time, and it is often distinctly different from the bulk material, predominantly because of oxidation and contamination.[1,2] Therefore, particular attention must be given to the stability of the biomaterial surface because composition and structure generally affect the host response after implantation.[3] For example, the inflammatory response,[4,5] and routine sterilization itself, can modify the surface characteristics of biomaterials.[6–8] Furthermore, contamination resulting from autoclaving or changes in surface energy during plasma cleaning of titanium substrates for cell culture have been shown to affect the expression level of various proteins secreted by osteoblasts grown on these surfaces, notably osteocalcin and alkaline phosphatase.[9]

It is likely that optimization for clinical applications of traditional materials used to manufacture implants will require careful and controlled modification of their surface properties.[3] At present, however, the relationships between the surface of an implant, its reactivity with tissue constituents, and its long-term integrity and clinical efficacy are still poorly understood. The first biological reaction known to occur after implantation of a foreign body is the ad-

Correspondence to: A. Nanci, E-mail: nancia@ere.umontreal.ca

Contact grant sponsor: Theratechnologies

Contact grant sponsor: Medical Research Council of Canada

© 1998 John Wiley & Sons, Inc. CCC 0021-9304/98/020324-12

sorption of tissue fluid proteins onto is surface.[10–12] In order to optimize the integration of implants, these reactions must be controlled so that nonspecific adsorption of proteins is minimized and beneficial molecules are selectively adsorbed onto biomaterials as a result of modifications performed prior to their implantation.[3,13]

A variety of immobilizing techniques have been explored for the covalent binding of proteins onto solid supports for various applications, including recently developed biosensors.[14–17] For example, carboxyl-activated derivatives of various polymers have been coated with growth factors, fibronectin, and collagen via a covalent linkage using a carbodiimide as a coupling agent.[18–22] Exposure of cells *in vitro* to substrates modified by this procedure results in an enhancement of cell growth compared to that observed in the presence of free or simply adsorbed proteins, an effect presumably attributable to the higher local concentration of protein molecules and their essentially permanent attachment.[23] Interestingly, increasing the distance between bound protein and the substrate has been shown to have a further beneficial effect on cell growth.[19] Protein coupling strategies have been used to manufacture antithrombogenic materials by immobilizing thrombomodulin onto hydrolyzed polymer,[24,25] and related biologically active materials also have been obtained by linking heparin to surfaces bearing amino groups.[26]

A particularly active field in implantology has been the development of new biomaterials for the repair or replacement of bones. For example, maxillofacial reconstruction and the installation of hip prostheses now are considered common surgical procedures, and intraosseous dental implants routinely are used as anchoring points for oral prostheses. Bioactive ceramics can be used for implants,[27–29] however, titanium and titanium-based alloys have more suitable bulk properties and lower moduli of elasticity, and thus they are widely used in dental and orthopedic prosthetic applications.[30–34]

The healing of tissues after surgical placement of biomaterials comprises numerous cellular and extracellular events, including those that occur at the implant/tissue interface. In the case of bone, initial tissue repair and eventual osseointegration of the implant are especially complex because this tissue is very dynamic and undergoes continuous modeling and remodeling. Noncollagenous proteins have been shown to play a major role in the formation of mineralized tissues.[35–40] Among these, osteopontin has been identified as a prominent protein concentrated at bone interfaces[41–43] as well as at interfaces of hard tissues with implants and at the cell–substrate interface in osteogenic cell cultures.[44–48] At such sites, osteopontin has been considered to function in cell adhesion and/or in maintaining tissue/implant cohesion.[43,46] This multifunctional protein also is believed to have an inhibitory activity on hydroxyapatite formation while the related protein, bone sialoprotein, appears to be a nucleator of mineralization.[39,49–52]

Some biological moieties, such as growth factors, have been used in conjunction with conventional implants. For example, platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) have been applied to the insertion site of titanium dental implants in dogs, where they have several effects, including increasing bone regeneration.[53] Similarly, bone morphogenetic proteins (BMPs),[54,55] whether adsorbed onto porous hydroxyapatite[56] or titanium implants[57] placed in bone or coupled with other carriers,[58–61] have an osteoinductive effect.[62–65] Disadvantages of these adsorption techniques and of more generalized applications of bioactive products at the implantation site include a necessity for relatively large quantities of protein and the potential for undesirable release and/or diffusion of biological molecules far from the biomaterial/tissue interface where they are required. Alternatively, *coupling techniques* provide an opportunity for immobilization and orientation of biomolecules in order to obtain a more specific and/or rapid physiological response by exposing and aligning the appropriate active sites at the tissue interface. Indeed, previous approaches for immobilizing heparin[66] and cytochrome c[67] have been aimed at orienting the molecules on solid supports.

Two prototypical methods for attaching organic molecules to inorganic surfaces involve 1) the use of silanes to modify the surface of silica[68] and 2) the deposition of thiols as self-assembled monolayers onto gold.[69,70] Although covalent binding of thiols is a conceptually desirable approach because it yields a well defined structure, this procedure has been applied only to compositionally stable metals, such as gold having a high affinity for thiols; however, to the best of our knowledge, it has not been used for Ti. For this reason, and because titanium surfaces usually exist in their oxidized state, we have used silanization, which readily can bind to oxidized titanium and which can yield surfaces with greater stability than those obtained by attachment of thiols.[69] Although previous studies of silanized surfaces have dealt mostly with silica, other oxides, in principle, covalently can be covered by alkylsilanes to form organic films.[68,71–73] The present study describes a method for immobilizing bioactive molecules onto titanium by attaching silane-derivatized spacer arms to its surface as an intermediary for the covalent linkage of molecules with known biological activities.

MATERIALS AND METHODS

Titanium samples were prepared from 99.7% pure (grade I) titanium sheets (0.89 mm thick, Alfa Aesar) by punching 4 mm diameter disks or by cutting 3 × 10 mm rectangular pieces. Samples were rinsed with toluene (toluene was dried over sodium and distilled under N_2 prior to use) and then treated at 25°C for 2 h with a solution consisting of equal volumes of concentrated H_2SO_4 and 30% aqueous H_2O_2. The cleaned, oxidized samples then were rinsed with distilled water and dried under vacuum. Silanization of oxidized samples was performed by heating them for 4 h in refluxing toluene containing 10% (3-aminopropyl)triethoxysilane, after which they were rinsed with toluene and dried under vacuum. The surfaces of selected samples were examined by scanning electron microscopy (SEM) using a JEOL 6300F field emission electron microscope and by atomic force microscopy (AFM) using a Park Scientific Instruments Autoprobe™ CP/LS. To evaluate the stability of the Si-O-Ti linkage, silanized samples were placed in HPLC grade water for 6, 12, 24, 72, and 168 h at 25°C. Prior to coupling with proteins, silanized Ti disks were immersed in a 1% glutaraldehyde solution in 0.1*M* phosphate buffer (pH adjusted to 7.0 with NaOH) for 4 h at 25°C and rinsed with phosphate buffer. The disks then were placed overnight in solutions containing either alkaline phosphatase (Sigma Chemical Co.) or rat albumin (Sigma) dissolved in phosphate buffer. The samples were rinsed and stored in buffer at 4°C.

X-ray photoelectron spectroscopy (XPS) measurements were performed on a VG Escalab 3 Mark II instrument, with nonmonochromatized Mg Kα radiation (1253.6 eV), at a resolution of 0.8 eV at 14 kV and 20 mA, at pressures lower than 10^{-8} Torr and with a perpendicular take-off angle. Dried samples were mounted for spectroscopy on copper stubs using double-sided adhesive copper tape. Elemental concentrations were calculated by measuring peak areas and dividing by sensitivity factors determined in our laboratory. High-resolution Ti, O, N, Si, and C spectra were recorded for each sample. Analysis of variance (ANOVA) was carried out using STATISTICA Ver. 5.1 F for Windows (Statsoft Inc.) Post-hoc comparison of means was first done using the LSD test followed by a more stringent analysis by the Tukey HSD (honest significance difference) test for unequal sample sizes.

The enzymatic activity of alkaline phosphatase-covered disks was assayed by exposing them to p-nitrophenyl phosphate under standard analytical conditions[74] at basic pH. The hydrolysis product, p-nitrophenoxide, was colorimetrically detected by measuring its characteristic absorption peak at 398 cm^{-1} with a UV spectrophotometer (Philips PU 8800).

For immunolabeling of albumin-coated specimens, treated titanium disks first were incubated for 10 min with 0.01*M* phosphate-buffered saline (PBS, pH 7.2) containing 1% ovalbumin to block nonspecific sticking and then for 90 min with rabbit anti-rat albumin antibody (Cappel) diluted 1 in 250. The specimens were rinsed in PBS and again briefly exposed to PBS-ovalbumin as above. The sites of antibody–antigen binding then were revealed by incubating with protein A-gold complex for 30 min. The complex was prepared as previously described[75] using 8 nm of gold particles.[76] Controls consisted of incubating the samples with a nonimmune antibody followed by protein A-gold. The samples were rinsed first with PBS and then with distilled water, dried, and examined by SEM using backscattered electron imaging for visualizing gold particles at the surface of the specimens.

RESULTS AND DISCUSSION

Surface analyses of oxidized titanium

XPS analyses of surfaces of chemically-oxidized samples of titanium are summarized in Table I. After oxidation, no contaminants except for C were found on the surfaces. Even though the samples were immersed in concentrated sulfuric acid, observed surface elements consisted of O, Ti, and C only, without traces of S. The carbon is believed to derive from hydrocarbon contamination, and it was used as an internal reference at 285.0 eV for calibrating peak positions. Indeed, hydrocarbons are considered to represent normal contaminants in XPS analyses and conveniently and routinely are used for calibration.[77] Such surface

TABLE I
Average Concentrations (Atomic Percentage) of Elements Present on Surface of Titanium Samples as Determined by XPS

	Ti	O	C	Si	N	Cl	Na	Zn
Untreated*	9.2	46.8	34.9	0	1.1	0.7	2.8	—
(*n* = 6)	*5.5*	*2.2*	*4.8*		*0.6*	*0.8*	*1.7*	
Oxidized (*n* = 8)	7.5	47.0	30.1	0	0.5	2.2	5.2	—
	6.4	*5.3*	*5.7*		*0.8*	*3.3*	*3.0*	
Oxidized with silane	0.7‡	22.6‡	49.2‡	12.3‡	8.5‡	4.0	1.9	0.4
(*n* = 8)	*1.6*	*4.5*	*4.9*	*1.8*	*1.1*	*3.9*	*1.8*	*0.2*
Nonoxidized† with silane	1.1	24.1‡	48.8‡	12.6‡	7.8‡	3.6	1.5	—
(*n* = 4)	*1.3*	*4.7*	*3.4*	*1.4*	*2.2*	*4.2*	*2.1*	

Italics = standard deviation between measurements + A3; *Depending on samples, traces of Pb, Fe, P, F; †Depending on samples, traces of Pb, P; ‡$p < 0.05$ compared to untreated group using the Tukey honest significant difference test for unequal *N*.

contamination also is observed in other surface analysis methods, like Auger spectroscopy.[8,78] Consistent with previous studies,[79–81] and because low levels of carbon did not interfere with analyses from our studies of surface modification and its biological applications, we did not attempt to eliminate hydrocarbon contaminants. Furthermore, analysis of high-resolution spectra indicates that titanium was totally in the Ti^{IV}-O form, without Ti-C binding. Adsorption of atmospheric oxygen and water on the surface of oxidized titanium presumably explains the discrepancy between the measured (3.4:1) and expected (2:1) ratios of oxygen to titanium.

It is well known that exposed surfaces of titanium spontaneously are covered by a 3–6 nm layer of titanium oxide, mostly as TiO_2.[8,82] This layer is irregular in thickness and composition, noncrystalline, and typically contaminated with various foreign elements taken from the surroundings.[12,82] We intentionally have oxidized titanium with a mixture of sulfuric acid and hydrogen peroxide rather than using the ASTM-F86 passivation procedure, which involves treatment with nitric acid prior to implantation into tissues. It has been shown that such recommended passivations have not been optimized for titanium and its alloys, where they even may be considered to have adverse effects.[83] Unlike many other processes designed to achieve chemical cleaning, the method applied in the present study yields a very 'clean' titanium oxide surface (Table I). Indeed, it is known that previously used standard cleaning procedures for the preparation of implantable materials do not consistently eliminate all elements other than Ti, O, and C. In fact, P and Ca even have been considered as ''normal'' constituents of titanium surfaces prepared for biological applications.[8] We believe that the reproducible manufacture of implants will be facilitated by the high degree of compositional homogeneity achieved by the present method.

Among the various methods available for the oxidation of metals, electrochemical treatment should provide the most control for generating consistent,[84] but not totally pure, oxide layers.[2] However, the time required for electrochemical oxidation, as well as the costs associated with this procedure, makes it less commercially attractive than the simpler operation of chemical oxidation. Furthermore, electrochemical methods may be relatively difficult to apply to implants with complex geometries.

During the course of this study, we also tested other chemical agents and combinations for the oxidation of titanium surfaces (data not shown), but all except the H_2SO_4/H_2O_2 mixture resulted in the deposition of undesirable contaminating ions as assessed by XPS. The combination of sulfuric acid and peroxide prevented such problems, giving a clean, well oxidized surface (Table I). Controlled oxidation of titanium is beneficial because it can produce a clean surface, and it also is important because oxide is the required element for coupling to silanes.[68] Surface analyses of silanized samples of titanium with native oxide layers (see Table I) suggest that their composition is somewhat more variable than those of chemically oxidized silanized samples; larger amounts of contaminants are present in the former case, and a loss of nitrogen with respect to silicon is observed.

SEM analysis of titanium surfaces both before and after oxidation (Fig. 1) did not show microcracks in the oxide layer, as observed by Dunn et al.,[85] where the surfaces were prepared by anodization in sulfuric acid. Our analyses revealed that oxidation reproducibly resulted in an increase in the roughness of the surface, an observation in agreement with other previous studies.[12,84,86] In addition to increasing surface sites available for binding, increased microtexture is believed to have an inherent ability to increase the rate of bone formation.[84,87–89] For this reason, the augmentation of surface area by chemical oxidation with H_2SO_4/H_2O_2 makes our procedure for the treatment of titanium implants a potentially valuable technique, independent of its ability to permit subsequent surface coating with organic molecules.

Silanization

Silane-based strategies for organic moieties recently have been applied to implant metals such as tantalum,[90] Co-Cr-Mo,[71] and titanium oxide.[72,73] In the latter case, self-assembling monolayers (SAMs) terminated with simple functional end groups were used to induce calcium phosphate deposition. The goal of the present study was to develop methods for covalently immobilizing entire organic molecules and to apply them to coat the surface of oxidized Ti implants routinely used in maxillo-facial and orthopedic reconstruction. Indeed, the use of ductile organic spacers, such as silanes, additionally may provide the potential to absorb, at least in part, the stresses normally present at the tissue–implant interface. Intermediary spacers also may allow biological moieties to be appropriately oriented on the exposed surface in an arrangement designed to be most efficacious in stimulating a specific and desired tissue response. ω-Aminoalkylsilanes were selected as the spacer molecules for the present study because they possess two functional groups, one at each extremity. The silane group was used for attachment to the titanium oxide surface while the amino group was used to couple biological molecules at the opposite end. Moreover, it has been shown that an alkyl spacer can increase the stimulatory effect of biological molecules, such as growth factors immobilized on activated polymers.[23] The studies reported

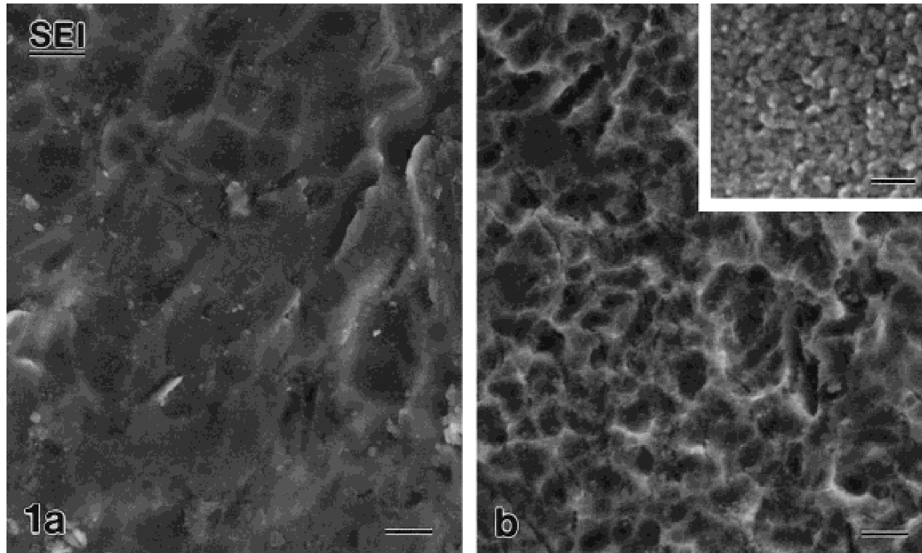

Figure 1. Scanning electron microscopy (SEM) using secondary electron imaging (SEI) of an untreated titanium sample as received directly from the manufacturer (a) and a titanium sample oxidized with H_2SO_4/H_2O_2 (b). Samples oxidized under controlled conditions (b) show uniform and increased surface topography, and at higher magnification (inset), an additional finer texture is apparent. Bars equal A, B, 1 μm; inset, 100 nm.

here have used short-chain ω-aminoalkylsilanes; ongoing studies are directed at determining whether chain length or chain branching may influence the stability of the Ti-O-Si bond, the efficiency of protein coupling, or the activity of the bound biological molecule.

Topographic imaging by SEM and AFM of both silanized and nonsilanized samples revealed that no major differences in surface structure result from silanization (Fig. 2). The microtexture of the specimens, which was created by the initial oxidation step, was retained after silanization.

XPS analysis of titanium disks treated with (3-

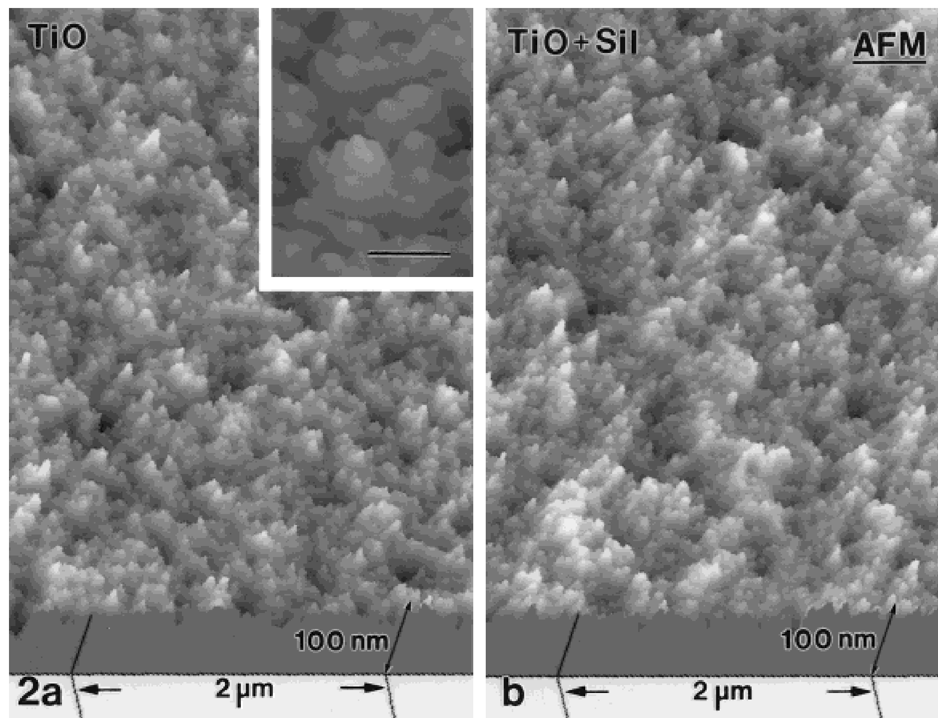

Figure 2. Atomic force microscopy (AFM) of chemically oxidized titanium samples (a and inset) and oxidized and silanized titanium samples (b). The presence of the silane coating does not cause an overt change in surface structure. The fine structure of oxidized samples, as observed by SEM and AFM, is comparable, [compare Fig. 1(b) inset with Fig. 2(a)]. Bar in inset equals 0.1 μm.

aminopropyl)triethoxysilane (Table I) confirmed that silicon and nitrogen could be detected in the presence of other elements (Ti, O, C) normally found at the titanium surface. It has been shown previously that alkylsiloxane SAMs approach a close-packed arrangement on silicon,[91] and extensive coverage of titanium oxide also seems feasible. However, short chain (<10 C) aminosilane films on silicon are highly disordered, yielding films with a thickness less than the length of the carbon chain itself;[92,93] moreover, titanium oxide layers do not present a well defined crystalline face. Theoretical calculations for complete coverage of the silane on TiO_2 by XPS therefore could not be precisely made. It may be expected, however, that the atomic ratio of C/S/N would be 3/1/1, that the ratio of O/Ti would be 2/1, and that the ratio of Ti/S would be greater than 1 as X-rays penetrate deeply into the samples. Our measurements [Ti/Si/C/O/N = 4.8/7.9/50.1/30.5/6.6 (concentrations in Table I normalized to 100%)] indicate somewhat less TiO_2 than expected, suggesting that it is partially masked by surface contaminants, a possibility discussed above. As expected, titanium was found to be in the Ti^{IV}-O form, with no evidence of either Ti-N or Ti-Si. The N concentration was determined to be slightly less than that of Si, although in theory there must be one silicon for each terminal amine. This ratio was calculated by measuring the peak area and dividing by our determined sensitivity factor. Since this factor is lowest for N, the error in measuring this element is correspondingly larger than for the other elements. Although some NH_2-terminal groups may have been lost, the amounts retained appeared sufficient for protein binding, as discussed below.

Various extraneous elements were found in low concentrations on the surface of the treated samples (Table I). Among these, Cl and Na are concluded to derive from the various preparation steps, including impurities in chemical products and the cleaning of glassware. Since sterilization of biomaterials for medical use often results in larger amounts of these and other contaminants, especially after vapor sterilization,[6–8,81] we feel that the small percentage of contamination (less than 2% of the total) is not likely to interfere in a significant way with implantability and linkage of biological molecules.

In order to confirm the stability of the linkage of the aminosilane to the oxidized surface, and to evaluate the amount of noncovalently bound silane, the sample plates were immersed in HPLC-grade water for different time intervals. XPS analysis (Fig. 3) showed very minor fluctuations in the concentrations of the five elements demonstrated previously and discussed above. Because the standard error for quantitation using XPS is about 10%,[94] these data indicate that (3-aminopropyl)triethoxysilane binds in a stable manner to TiO_2, as reported previously for alkoxysilane binding to SiO_2.[95] Silanes therefore appear to be a reliable spacer arm for the attachment of biological molecules to titanium. Titanium oxide surfaces are not stable during implantation, and there is an increase in thickness of the oxide layer and an incorporation of ions, especially phosphate, even after protein adsorption.[84] However, it has been shown previously that alkylsilane monolayers are impermeable to organic species such as peptides and proteins.[96] It is therefore possible that the silane coating could prevent biological moieties from nonspecifically adsorbing onto titanium.[3] In this regard, cell attachment and spreading in the presence of serum occurs preferentially on SAMs terminating in amino groups relative to those having methyl groups.[97]

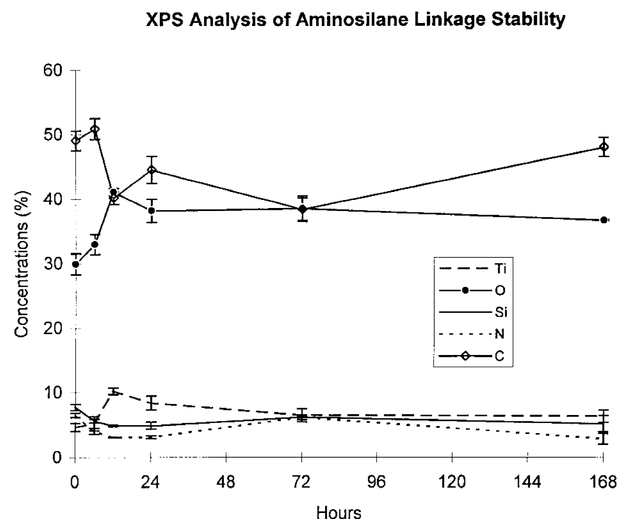

Figure 3. X-ray photoelectron spectroscopic (XPS) profiles of elemental concentrations present at the surface of oxidized and silanized titanium samples after immersion in water. The minimal fluctuations in elemental concentrations indicate the stability of the aminosilane linkage to the oxide surface under aqueous conditions over 7 days.

Silanes have been widely used for covering glass because the surface groups of this substrate are readily available for covalent binding to form Si-O-Si linkages. Royer et al.[98] have suggested that glass treated with (3-aminopropyl)triethoxysilane produces a surface particularly suitable for the covalent binding of enzymes, but the aminosilanized glasses available at the time of that report did not always show high stability. Glass treated with organosilanes also has been shown to be a reliable chromatographic support.[95] Subsequent studies using aminosilanes in dry toluene reliably produced stable monolayers almost instantaneously on acid-etched glass, and multilayers could be obtained under other conditions.[99] Silane-derived spacers also can be used to modify the surfaces of hydrophobic polymers, which often denature proteins when used directly and which therefore need functionalized coatings before they can be used for certain

applications in biotechnology and chromatography. In many instances, amino-terminated organosilanes have been used for these purposes.[90,100,101]

The use of terminal aminosilanes for surface functionalization does not lead to satisfactory results in all cases. For example, loss of N and the amine function from (3-aminopropyl)triethoxysilane was observed by XPS after it was used to coat silicon.[102] However, this study used a nonoxidized surface, which may account for the contradiction with other studies showing the capacity of similar aminosilanes to form monolayers.[92,96,103] Glass coated in this way has been used effectively for the growth and differentiation of cultured cells,[92,96,104] thus indicating that such surfaces are not cytotoxic. Whereas functionalization of glass surfaces or polymers has progressed substantially, with even some attempts to create patterned surfaces,[105–107] studies with metals are relatively few in number.[70,108] However, the presence of a monolayer derived from an aminosilane has been demonstrated on oxidized metal surfaces.[103]

Linkage of proteins

Proteins merely adsorbed onto implant surfaces can be displaced and eventually diffuse away from the site of implantation and, perhaps even more important, may distribute systemically, causing detrimental consequences. In addition, proteins can be conformationally altered or denatured by adsorption,[109–113] so their activity may change.[114,115] Moreover, adsorbed proteins may exchange nonspecifically with others in solution, such as those found in tissue fluids.[109,116] To circumvent these limitations, certain approaches have been devised for covalently immobilizing biological material on substrates bearing functional groups (reviewed in Reference 117). Coupling to terminal amino groups has been used frequently, particularly with carbodiimides and glutaraldehyde as linking agents. Furthermore, the use of glutaraldehyde as a coupling agent has been shown to give satisfactory results for subsequent immunocytochemistry or for *in situ* hybridization and for retaining biological activity of a given molecule *in vitro*.[71,100,114,118] For these reasons, we have opted to link two test molecules, alkaline phosphatase and albumin, to ω-aminoalkylsilane spacers, using glutaraldehyde as the coupling agent according to conventional, commercially employed procedures. Alkaline phosphatase was selected because of its prominent role in bone formation[119,120] and its inductive effect on mineral deposition.[121] Furthermore, the enzymatic activity of alkaline phosphatase is easily detected by a spectrophotometric assay in which basic hydrolysis of p-nitrophenyl phosphate is accompanied by the appearance of a peak at 398 cm^{-1} due to the formation of p-nitrophenoxide. In our experiments, enzymatic activity was retained for the 7-day period examined. No intrinsic hydrolysis was observed in control experiments using similarly cleaned, oxidized, and silanized samples of Ti but without the linked protein. These observations further indicate that neither the covalent binding of alkaline phosphatase nor the presence of a spacer arm between the oxidized surface and the protein significantly modifies the biological activity of the enzyme. They also are consistent with other studies that have shown that silanization and glutaraldehyde coupling are effective techniques for immobilizing immunoglobulins on glass.[101]

We have used a blood serum protein, albumin, to study the distribution and concentration of proteins linked to the surface of silanized titanium. Bound protein was detected by immunogold labeling in which the distribution and density of the labeling were visualized by SEM. Both freshly prepared titanium disks and similar samples stored in buffer for 1 week gave comparable results and showed a generally uniform labeling of the titanium surface (Fig. 4) although occasionally areas of the samples showed a lesser density of gold particles. These data demonstrate that the protein is stably bound and that it retains its antigenicity following the various steps of the linking procedure. Furthermore, the density of labeling obtained suggests an efficient linkage of albumin. As an example, Figure 4(a) shows a representative area of the treated titanium samples with a labeling density of 150 gold particles/μm^2. The control sample illustrated in Figure 4(b) was incubated only with protein A-gold and displays a background labeling of 13 gold particles/μm^2, an amount less than 10% of the experimental value, which reflects, in part, the expected nonspecific adsorption of protein, here protein A, to titanium.[12] Hypothetically, assuming a cell of cuboidal morphology with a 20×20 μm surface (400 μm^2) in contact with the coated titanium, and that each gold particle depicts the location of one molecule of albumin, with the labeling density illustrated in Figure 4(b), a ''face'' of the cell would be exposed to roughly 60,000 linked protein molecules. We consider that such a number of tethered bioactive molecules would be sufficient to participate in biological signaling and would not be a limiting factor for the surface coating of implantable materials. As a relevant example, where receptor sites have been estimated, it has been reported that murine macrophages display over their *entire* surface 10^4 binding sites for osteopontin,[122,123] a noncollagenous extracellular matrix protein with cell and matrix binding properties also found at the surface of osseointegrated biomaterials.[43,46,124–126] If such a density of receptors present on macrophages is ex-

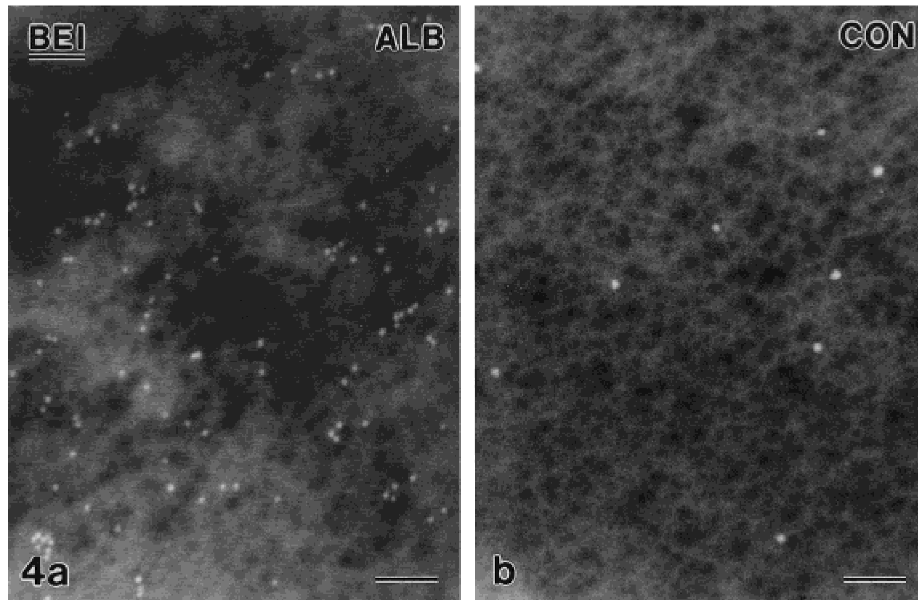

Figure 4. Immunocytochemical preparation viewed by backscattered electron imaging in the SEM of albumin conjugated to silanized titanium (a). The intensity of immunolabeling is substantially greater than that observed under control conditions in which albumin-coated titanium was incubated with nonimmune serum in place of the primary antibody (b). Gold particles generally are homogeneously distributed over the titanium surface although on occasion some areas show a weaker labeling. The relatively high labeling density of the experimental Ti disks (a) is considered to be biologically relevant in circumstances requiring functional ligand–receptor interactions (see text for discussion). Bars equal 100 nm.

trapolated to a $20 \times 20 \times 20$ μm cuboidal bone-forming osteoblast, then fewer than 20,000 molecules of a ligand would be needed to saturate the corresponding cell binding sites in contact with a coated titanium surface. This calculated number of ligands is approximately threefold less than the labeling density obtained for albumin in the present study.

It is not unreasonable to consider that the different steps in the surface treatment procedure may have an effect on the extent of coverage. For example, coupling of aminosilanes could limit subsequent protein binding if too little Si is covalently attached to the TiO_2 surface. However, the measured concentration of Si is high, so we do not consider poor silanization to be a limiting factor for the subsequent linkage of protein. Also, because the measured concentration of N is also high, it is unlikely that amino groups are lost during the silanization of the titanium and that the bound silane chains therefore are unable to bind proteins. In addition, limitations of the protein immobilization step also may be responsible for the low level of coverage. It already has been noted that the concentration of bound proteins can be less when glutaraldehyde is used for coupling (320 ng/cm^2 [101]) than when a carbodiimide is used (1 mg/cm^2 [24,25]). In addition, it is also possible that not all the bound proteins is revealed by the immunocytochemical procedures, thereby leading to an underestimation of the density of surface coverage.

SUMMARY AND CONCLUSIONS

We have devised a chemical method to reproducibly clean and re-oxidize titanium and to covalently bind proteins to its surface using intermediary aminoalkylsilane spacer arms. Bound proteins were linked at a biologically relevant density and retained their enzymatic activity (alkaline phosphatase) and their antigenicity (albumin). This surface modification of implants with appropriate bioactive molecules could be used to advantageously influence the initial stages of biomaterial implantation by activating and/or controlling the early biological events at the implant/host interface. In addition, it is proposed that this procedure could be applied to minimize initial detrimental, nonspecific protein interactions at the implant surface. It also is possible to envisage the orienting and patterning of a variety of molecules at the implant surface according to individual patient needs and anatomical features of the implantation site. This latter scenario is particularly relevant for dental prostheses that are anchored in bone but that also traverse soft connective tissues and the oral epithelium.

The authors are grateful to Park Scientific Instruments for having given them access to the AutoProbe™ CP/LS atomic force microscope, and to Ms. K. Roach for having carried out the analyses. We thank Drs. E. Sacher and O. Savadogo (École Polytechnique, Montréal) for discussions and advice

pertaining to the treatment and analysis of titanium surfaces, Ms. S. Poulin (École Polytechnique, Montréal) for technical help with XPS, and Dr. J. K. Denike (Department of Chemistry, Université de Montréal) for performing preliminary experiments on the silanization of titanium. We also are grateful to Dr. C. E. Smith (Faculty of Dentistry, McGill University) for his advice regarding statistical analyses and for having performed the ANOVA tests of Table I.

References

1. B. D. Ratner, ''Advances in the analysis of surfaces of biomedical interest,'' *Surf. Interf. Anal.,* **23,** 521–528 (1995).
2. J. Lausmaa, B. Kasemo, U. Rolander, L. M. Bjursten, L. E. Ericson, L. Rosander, and P. Thomsen, ''Preparation, surface spectroscopic and electron microscopic characterization of titanium implant materials,'' in *Surface Characterization of Biomaterials,* B. D. Ratner (ed.), Elsevier Science Publishers, Amsterdam, 1988, pp. 161–174.
3. B. D. Ratner, ''New ideas in biomaterials science—A Path to engineered biomaterials,'' *J. Biomed. Mater. Res.,* **27,** 837–850 (1993).
4. C. A. van Blitterswijk, D. Bakker, S. C. Hesseling, and H. K. Koerten, ''Reactions of cells at implant surfaces,'' *Biomaterials,* **12,** 187–193 (1991).
5. P. Tengvall, I. Lundström, L. Sjöqvist, H. Elwing, and L. M. Bjursten, ''Titanium–hydrogen peroxide interaction: Model studies of the influence of the inflammatory response on titanium implants,'' *Biomaterials,* **10,** 166–175 (1989).
6. J. H. Doundoulakis, ''Surface analysis of titanium after sterilization: Role in implant–tissue interface and bioadhesion,'' *J. Prosthet. Dent.,* **58,** 471–478 (1987).
7. R. E. Baier, A. E. Meyer, C. K. Akers, J. R. Natiella, M. Meenaghan, and J. M. Carter, ''Degradation effects of conventional steam sterilization on biomaterial surfaces,'' *Biomaterials,* **3,** 241–245 (1982).
8. N. L. Hernández de Gatica, G. L. Jones, and J. A. J. Gardella, ''Surface characterization of titanium alloys sterilized for biomedical applications,'' *Appl. Surf. Sci.,* **68,** 107–121 (1993).
9. C. M. Stanford, J. C. Keller, and M. Solursh, ''Bone cell expression on titanium surfaces is altered by sterilization treatments,'' *J. Dent. Res.,* **73,** 1061–1071 (1994).
10. T. A. Horbett, ''Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials,'' *Cardiovasc. Pathol.,* **2,** 137S–148S (1993).
11. L. Tang and J. W. Eaton, ''Fibrin(ogen) mediates acute inflammatory responses to biomaterials,'' *J. Exp. Med.,* **178,** 2147–2156 (1993).
12. B. Wälivaara, B.-O. Aronsson, M. Rodahl, J. Lausmaa, and P. Tengvall, ''Titanium with different oxides: *In vitro* studies of protein adsorption and contact activation,'' *Biomaterials,* **15,** 827–834 (1994).
13. K. C. Dee and R. Bizios, ''Mini-review: Proactive biomaterials and bone tissue engineering,'' *Biotechnol. Bioeng.,* **50,** 438–442 (1996).
14. B. Lu, M. R. Smyth, and R. O'Kennedy, ''Oriented immobilization of antibodies and its applications in immunoassays and immunosensors,'' *Analyst,* **121,** 29R–32R (1996).
15. M. D. Ward and D. A. Buttry, ''*In situ* interfacial mass detection with piezoelectric transducers,'' *Science,* **249,** 1000–1007 (1990).
16. L. J. Kepley, R. M. Crooks, and A. J. Ricco, ''Selective surface acoustic waveband organophosphonate chemical sensor employing a self-assembled composite monolayer: A new paradigm for sensor design,'' *Anal. Chem.,* **64,** 3191–3193 (1992).
17. D. Schlatter, R. Barner, C. Fattinger W. Huber, J. Hübscher, J. Hurst, H. Koller, C. Mangold, and F. Müller, ''The difference interferometer: Application as a direct affinity sensor,'' *Biosensors Bioelectron.,* **8,** 109–116 (1993).
18. Y. Ito, S. Q. Liu, M. Nakabayashi, and Y. Imanishi, ''Cell growth on immobilized cell-growth factor. II. Adhesion and growth of fibroblast cells on poly(methyl methacrylate) membrane immobilized with proteins of various kinds,'' *Biomaterials,* **13,** 789–794 (1992).
19. Y. Ito, M. Inoue, S. Q. Liu, and Y. Imanishi, ''Cell growth on immobilized cell growth factor. VI. Enhancement of fibroblast cell growth by immobilized insulin and/or fibronectin,'' *J. Biomed. Mater. Res.,* **27,** 901–907 (1993).
20. S. Q. Liu, Y. Ito, and Y. Imanishi, ''Cell growth on immobilized cell growth factor. IX. Covalent immobilization of insulin, transferrin, and collagen to enhance growth of bovine endothelial cells,'' *J. Biomed. Mater. Res.,* **27,** 909–915 (1993).
21. Y. Ito, ''Cell adhesion factor immobilized materials,'' in *Synthesis of Biocompatible Materials,* Y. Imanishi (ed.), CRC Press, Boca Raton, 1992, pp. 245–284.
22. Y. Ito, S. Q. Liu, Y. Imanishi, ''Enhancement of cell growth on growth factor-immobilized polymer film,'' *Biomaterials,* **2,** 449–453 (1991).
23. Y. Ito, ''Cell growth factor immobilized materials,'' in *Synthesis of Biocomposite Materials. Chemical and Biological Modifications of Natural Polymers,* Y. Imanishi (ed.), CRC Press, Boca Raton, Florida, 1992, pp. 285–305.
24. A. Kishida, Y. Ueno, N. Fukudome, E. Yashima, I. Maruyama, and M. Akashi, ''Immobilization of human thrombomodulin onto poly(ether urethane urea) for developing antithrombogenic blood-contacting materials,'' *Biomaterials,* **15,** 848–852 (1994).
25. A. Kishida, Y. Ueno, I. Maruyama, and M. Akashi, ''Immobilization of human thrombomodulin on biomaterials: Evaluation of the activity of immobilized human thrombomodulin,'' *Biomaterials,* **15,** 1170–1174 (1994).
26. M. Tichá, B. Zelezná, V. Jonáková, and K. Filka, ''Immobilization of heparin on polyacrylamide derivatives,'' *J. Chromatogr.,* **656B,** 423–426 (1994).
27. H. Oguchi, K. Ishikawa, K. Mizoue, K. Seto, and G. Eguchi, ''Long-term histological evaluation of hydroxyapatite ceramics in humans,'' *Biomaterials,* **16,** 33–38 (1995).
28. P. Ducheyne, ''Bioactive ceramics,'' *J. Bone Joint Surg. Am.,* **76B,** 861–862 (1994).
29. L. L. Hench, ''Bioceramics: From concept to clinic,'' *Am. Ceram. Soc. Bull.,* **72,** 93–98 (1993).
30. T. Albrektsson, ''The reactions of bone to non-cemented implants,'' in *Bone, Vol. 5., Fracture Repair and Regeneration,* B. K. Hall (ed.), CRC Press, Boca Raton, Florida, 1992, pp. 153–208.
31. A. Schroeder, E. Van der Zypen, H. Stich, and F. Sutter, ''The reaction of bone, connective tissue and epithelium to endosteal implants with sprayed titanium surfaces,'' *J. Oral Maxillofac. Surg.,* **9,** 15–25 (1981).
32. M. A. Listgarten, ''Soft and hard tissue response to endosseous dental implants,'' *Anat. Rec.,* **245,** 410–425 (1996).
33. T. Albrektsson, P.-I. Brånemark, H.-A. Hansson, and J. Lindström, ''Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man,'' *Acta Odontol. Scand.,* **52,** 155–170 (1981).
34. P.-I. Brånemark, B. O. Hansson, R. Adell, U. Breine, J. Lindström, O. Hallén, and A. Öhman, ''Osseointegrated implants in the treatment of the endentulous jaw. Experience from a 10-year period,'' *Scand. J. Plast. Reconstr. Surg.,* **2,** 1–132 (1977).
35. A. L. Boskey, ''Noncollagenous matrix proteins and their role in mineralization,'' *Bone and Miner.,* **6,** 111–123 (1989).
36. J. P. Gorski, ''Acidic phosphoproteins from bone matrix: A structural rationalization of their role in biomineralization,'' *Calcif. Tissue Int.,* **50,** 391–396 (1992).

37. M. D. McKee and A. Nanci, ''Ultrastructural, cytochemical and immunocytochemical studies on bone and its interfaces,'' *Cells and Mater.,* **3,** 219–243 (1993).
38. H. I. Roach, ''Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption,'' *Cell Biol. Int.,* **18,** 617–628 (1994).
39. G. K. Hunter and H. A. Goldberg, ''Modulation of crystal formation by bone phosphoproteins: Role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein,'' *Biochem. Pharmacol.,* **302,** 175–179 (1994).
40. P. Gehron Robey, ''Bone matrix proteoglycans and glycoproteins,'' in *Principles of Bone Biology,* J. P. Bilezikian, L. R. Raisz, and G. A. Rodan (eds.), Academic Press, Inc., San Diego, 1996, pp. 155–165.
41. M. D. McKee, M. J. Glimcher, and A. Nanci, ''High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia,'' *Anat. Rec.,* **234,** 479–492 (1992).
42. M. D. McKee, and A. Nanci, ''Osteopontin: An interfacial extracellular matrix protein in mineralized tissues.'' *Connect. Tiss. Res.,* **35,** 197–205 (1996).
43. M. D. McKee and A. Nanci, ''Osteopontin at mineralized tissue interfaces in bone, teeth and osseointegrated implants: Ultrastructural distribution and implications for mineralized tissue formation, turnover and repair,'' *Microsc. Res. Tech.,* **33,** 141–164 (1996).
44. H. Kawaguchi, M. D. McKee, H. Okamoto, and A. Nanci, ''Immunocytochemical and lectin–gold characterization of the interface between alveolar bone and implanted hydroxyapatite in the rat,'' *Cells and Mater.,* **3,** 337–350 (1993).
45. X. Shen, E. Roberts, S. A. F. Peel, and J. E. Davies, ''Organic extracellular matrix components at the bone cell/substratum interface,'' *Cells and Mater.,* **3,** 257–272 (1993).
46. A. Nanci, G. F. McCarthy, S. Zalzal, C. M. L. Clokie, H. Warshawsky, and M. D. McKee, ''Tissue response to titanium implants in the rat tibia: Ultrastructural, immunocytochemical and lectin-cytochemical characterization of the bone–titanium interface,'' *Cells and Mater.,* **4,** 1–30 (1994).
47. A. Nanci, S. Zalzal, Y. Gotoh, and M. D. McKee, ''Ultrastructural characterization and immunolocalization of osteopontin in rat calvarial osteoblast primary cultures,'' *Microsc. Res. Tech.,* **33,** 214–231 (1996).
48. J. E. Davies, ''*In vitro* modeling of the bone/implant interface,'' *Anat. Rec.,* **245,** 426–445 (1996).
49. H. A. Goldberg and G. K. Hunter, ''The inhibitory activity of osteopontin on hydroxyapatite formation *in vitro,*'' *Ann. NY Acad. Sci.,* **760,** 305–308 (1995).
50. G. K. Hunter, C. L. Kyle, and H. A. Goldberg, ''Modulation of crystal formation by bone phosphoproteins: Structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation,'' *Biochem. Pharmacol.,* **300,** 723–728 (1994).
51. A. L. Boskey, ''Osteopontin and related phosphorylated sialoproteins: Effects on mineralization,'' *Ann. NY Acad. Sci.,* **760,** 249–256 (1995).
52. A. L. Boskey, M. Maresca, W. Ullrich, S. B. Doty, W. T. Butler, and C. W. Prince, ''Osteopontin–hydroxyapatite interactions *in vitro:* Inhibition of hydroxyapatite formation and growth in a gelatin-gel,'' *Bone Miner.,* **22,** 147–159 (1993).
53. S. E. Lynch, D. Buser, R. A. Hernandez, H. P. Weber, H. Stich, C. H. Fox, and R. C. Williams, ''Effects of the platelet-derived growth factor/insulin-like growth factor-I combination on bone regeneration around titanium dental implants. Results of a pilot study in beagle dogs,'' *J. Periodontal.,* **62,** 710–716 (1991).
54. B. L. M. Hogan, ''Bone morphogenetic proteins: Multifunctional regulators of vertebrate development,'' *Genes Dev.,* **10,** 1580–1594 (1996).
55. J. M. Wozney, ''Bone morphogenetic proteins and their gene expression,'' in *Cellular and Molecular Biology of Bone,* M. Noda (ed.), Academic Press, Inc., San Diego, 1993, pp. 131–167.
56. U. Ripamonti, L. Yeates, and B. Van den Heever, ''Initiation of heterotopic osteogenesis in primates after chromatographic adsorption of osteogenin, a bone morphogenetic protein, onto porous hydroxyapatite,'' *Biochem. Biophys. Res. Commun.,* **193,** 509–517 (1993).
57. T. Kawai, A. Mieki, Y. Ohno, M. Umemura, H. Kataoka, S. Kurita, M. Koie, T. Jinde, J. Hasegawa, and M. R. Urist, ''Osteoinductive activity of composites of bone morphogenetic protein and pure titanium,'' *Clin. Orthop.,* **290,** 296–305 (1993).
58. R. Kenley, L. Marden, T. Turek, L. Jin, E. Ron, and J. O. Hollinger, ''Osseous regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2),'' *J. Biomed. Mater. Res.,* **28,** 1139–1147 (1994).
59. J. O. Hollinger and K. Leong, ''Poly(α-hydroxy acids): Carriers for bone morphogenetic proteins,'' *Biomaterials,* **17,** 187–194 (1996).
60. B. Rutherford, L. Spångberg, M. Tucker, and M. Charette, ''Transdentinal stimulation of reparative dentine formation by osteogenic protein-1 in monkeys,'' *Arch. Oral Biol.,* **40,** 681–683 (1995).
61. Y. Kuboki, A. Yamaguchi, A. Yokoyama, M. Murata, H. Takita, M. Tazaki, M. Mizuno, T. Hasegawa, S. IIda, K. Shigenobu, R. Fujisawa, M. Kawamura, T. Atsuta, A. Matsumoto, H. Kato, H.-Y. Zhou, I. Ono, N. Takeshita, and N. Nagai, ''Osteogenesis induced by BMP-coated biomaterials: Biochemical principles of bone reconstruction in dentistry,'' in *The Bone–Biomaterial Interface,* J. E. Davies (ed.), University of Toronto Press, Toronto, 1991, pp. 127–137.
62. A. H. Reddi and N. S. Cunningham, ''Initiation and promotion of bone differentiation by bone morphogenetic proteins,'' *J. Bone Miner. Res.,* **8,** S499–S502 (1993).
63. U. Ripamonti and A. H. Reddi, ''Periodontal regeneration: Potential role of bone morphogenetic proteins,'' *J. Periodont. Res.,* **29,** 225–235 (1994).
64. E. A. Wang, ''Bone morphogenetic proteins (BMPs): Therapeutic potential in healing bony defects,'' *Trends Biotechnol.,* **11,** 379–383 (1993).
65. J. M. Wozney, ''The potential role of bone morphogenetic proteins in periodontal reconstruction,'' *J. Periodontal,* **66,** 506–510 (1995).
66. V. D. Nadkarni, A. Pervin, and R. J. Linhardt, ''Directional immobilization of heparin onto beaded supports,'' *Anal. Biochem.,* **222,** 59–67 (1994).
67. H.-G. Hong, M. Jiang, S. G. Sligar, and P. W. Bohn, ''Cysteine-specific surface tethering of genetically engineered cytochromes for fabrication of metalloprotein nanostructures,'' *Langmuir,* **10,** 153–158 (1994).
68. B. Arkles, ed., *Silicon Compounds: Register and Review,* Hüls America Inc., Piscataway, New Jersey, 1991.
69. C. D. Bain and S. D. Evans, ''Laying it on thin,'' *Chem. Br.,* **31,** 46–48 (1995).
70. M. Mrksich and G. M. Whitesides, ''Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells,'' *Ann. Rev. Biophys. Biomol. Struct.,* **25,** 55–78 (1996).
71. D. A. Puelo, ''Activity of enzyme immobilized on silanized Co-Cr-Mo,'' *J. Biomed. Mater. Res.,* **29,** 951–957 (1995).
72. B. C. Bunker, P. C. Rieke, B. J. Tarasevich, A. A. Campbell, G. E. Fryxell, G. L. Graff, L. Song, J. Liu, J. W. Virden, and G. L. McVay, ''Ceramic thin-film formation on functionalized interfaces through biomimetic processing,'' *Science,* **264,** 48–55 (1994).
73. A. A. Campbell, G. E. Fryxell, J. C. Linehan, and G. L. Graff, ''Surface-induced mineralization: A new method for produc-

ing calcium phosphate coatings,'' *J. Biomed. Mater. Res.,* **32,** 111–118 (1996).

74. O. A. Bessey, O. H. Lowry, and M. J. Brock, ''A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum,'' *J. Biol. Chem.,* **164,** 321–329 (1946).

75. M. Bendayan, ''Colloidal gold post-embedding immunocytochemistry,'' *Prog. Histochem. Cytochem.,* **29,** 1–163 (1995).

76. G. Frens, ''Controlled nucleation for the regulation of particle size in monodispersed gold suspensions,'' *Nature Phys. Sci.,* **241,** 20–22 (1973).

77. K. S. Robinson and P. M. A. Sherwood, ''X-ray photoelectron spectroscopic studies of the surface of sputter ion plated films,'' *Surf. Interf. Anal.,* **6,** 261–266 (1984).

78. C. Johansson, J. Lausmaa, M. Ask, H.-A. Hansson, and T. Albrektsson, ''Ultrastructural differences of the interface zone between bone and Ti 6Al 4V or commercially pure titanium,'' *J. Biomed. Eng.,* **11,** 3–8 (1989).

79. R. N. S. Sodhi, A. Weninger, and J. E. Davies, ''X-ray photoelectron spectroscopic comparison of sputtered Ti, Ti6A14V, and passivated bulk metals for use in cell culture techniques,'' *J. Vac. Sci. Technol.,* **9,** 1329–1333 (1991).

80. D. S. Sutherland, P. D. Forshaw, G. C. Allen, I. T. Brown, and K. R. Williams, ''Surface analysis of titanium implants,'' *Biomaterials,* **14,** 893–899 (1993).

81. J. A. J. Gardella, T. G. Vargo, T. J. Hook, G. L. Grobe III, L. Salvati, Jr., and J. Hautaniemi, ''Electron and vibrational spectroscopic studies of the surfaces of biocompatible materials,'' in *Surface Characterization of Biomaterials,* B. D. Ratner (ed.), Elsevier Science Publishers, Amsterdam, 1988, pp. 145–160.

82. J. Sundgren, P. Bodö, and I. Lundström, ''Auger electron spectroscopic studies of the interface between human tissue and implants of titanium and stainless steel,'' *J. Colloid Interface Sci.,* **110,** 9–20 (1986).

83. B. W. Callen, B. F. Lowenberg, S. Lugowski, R. N. S. Sodhi, and J. E. Davies, ''Nitric acid passivation of Ti6A14V reduces thickness of surface oxide layer and increases trace element release,'' *J. Biomed. Mater. Res.,* **29,** 279–290 (1995).

84. C. Larsson, P. Thomsen, J. Lausmaa, M. Rodahl, B. Kasemo, and L. E. Ericson, ''Bone response to surface modified titanium implants: Studies on electropolished implants with different oxide thicknesses and morphology,'' *Biomaterials,* **15,** 1062–1074 (1994).

85. D. S. Dunn, S. Raghavan, and R. G. Volz, ''Gentamicin sulfate attachment and release from anodized Ti-6Al-4V orthopedic materials,'' *J. Biomed. Mater. Res.,* **27,** 895–900 (1993).

86. J. Pan, D. Thierry, and C. Leygraf, ''Electrochemical and XPS studies of titanium for biomaterial applications with respect to the effect of hydrogen peroxide,'' *J. Biomed. Mater. Res.,* **28,** 113–122 (1994).

87. K. A. Thomas, J. F. Kay, S. D. Cook, and M. Jarcho, ''The effect of surface macrotexture and hydroxyapatite coating on the mechanical strengths and histologic profiles of titanium implant materials,'' *J. Biomed. Mater. Res.,* **21,** 1395–1414 (1987).

88. J. L. Ricci, J. M. Spivak, N. C. Blumenthal, and H. Alexander, ''Modulation of bone ingrowth by surface chemistry and roughness,'' in *The Bone–Biomaterial Interface,* J. E. Davies (ed.), University of Toronto Press, Toronto, 1991, pp. 334–348.

89. B. Groessner–Schreiber and R. S. Tuan, ''Enhanced extracellular matrix production and mineralization by osteoblasts cultured on titanium surfaces *in vitro,*'' *J. Cell Sci.,* **101,** 209–217 (1992).

90. S. M. Rowland and K. D. Stanley, inventors, Cordis Corporation, assignee, ''Biocompatible metal surfaces,'' U.S. Patent No. 5,356,433, 1994.

91. S. R. Wasserman, G. M. Whitesides, I. M. Tidswell, B. M. Ocko, P. S. Pershan, and J. D. Axe, ''The structure of self-assembled monolayers of alkylsiloxanes on silicon: A comparison of results from ellipsometry and low-angle X-ray reflectivity,'' *J. Am. Chem. Soc.,* **111,** 5852–5861 (1989).

92. D. A. Stenger, C. J. Pike, J. J. Hickman, and C. W. Cotman, ''Surface determinants of neuronal survival and growth on self-assembled monolayers in culture,'' *Brain Res.,* **630,** 136–147 (1993).

93. D. A. Stenger, J. H. Georger, C. S. Dulcey, J. J. Hickman, A. S. Rudolph, T. B. Nielsen, S. M. McCort, and J. M. Calvert, ''Coplanar molecular assemblies of amino- and perfluorinated alkylsilanes: Characterization and geometric definition of mammalian cell adhesion and growth,'' *J. Am. Chem. Soc.,* **14,** 8435–8442 (1992).

94. B. D. Ratner, ''Characterization of biomaterial surfaces,'' *Cardiovasc. Pathol.,* **2 (Suppl.),** 87–100 (1993).

95. E. Grushka and E. J. Kikta, ''Chemically bonded stationary phases in chromatography,'' *Anal. Chem.,* **49,** 1004A–1014A (1977).

96. S. Margel, E. A. Vogler, L. Firment, T. Watt, S. Haynie, and D. Y. Sogah, ''Peptide, protein, and cellular interactions with self-assembled monolayer model surfaces,'' *J. Biomed. Mater. Res.,* **27,** 1463–1476 (1993).

97. D. Kleinfeld, K. H. Kahler, and P. E. Hockberger, ''Controlled outgrowth of dissociated neurons on patterned substrates,'' *J. Neurosci.,* **8,** 4098–4120 (1988).

98. G. P. Royer and F. A. Liberatore, ''The use of silylated derivatives of porous glass as enzyme supports,'' in *Silylated Surfaces,* D. E. Leyden and W. T. Collins (eds.), Gordon and Breach Science Publishers, New York, 1980, pp. 189–199.

99. L. W. Phillips and D. M. Hercules, ''Surface spectroscopic studies of organosilane layers on glass,'' in *Silanes, Surfaces, and Interfaces,* D. E. Leyden (ed.), Gordon and Breach Science Publishers, New York, 1986, pp. 235–365.

100. L.-I. Larsson and D. M. Hougaard, ''Glass slide models for immunocytochemistry and *in situ* hybridization,'' *Histochemistry,* **101,** 325–331 (1994).

101. A. Ahluwalia, D. De Rossi, C. Ristori, A. Schirone, and G. Serra, ''A comparative study of protein immobilization techniques for optical immunosensors,'' *Biosensors and Bioelectronics,* **7,** 207–214 (1991).

102. A. Domingue, K. Piyakis, E. Sacher, M. Di Renzo, S. Dénommée, and T. H. Ellis, ''The structure of the irreversibly bound adhesion promoter–substrate interfacial layer of gamma-aminopropylsilanetriol on crystalline Si, as measured by XPS and FTIR,'' *J. Adhesion,* **40,** 151–162 (1993).

103. R. M. Murray, ''Alkylaminesilanes bound to metal oxide electrodes,'' in *Silylated Surfaces,* D. E. Leyden and W. T. Collins (eds.), Gordon and Breach Science Publishers, New York, 1980, pp. 125–135.

104. C. N. Sukenik, N. Balachander, L. A. Culp, K. Lewandowska, and K. Merritt, ''Modulation of cell adhesion by modification of titanium surfaces with covalently attached self-assembled monolayers,'' *J. Biomed. Mater. Res.,* **24,** 1307–1323 (1990).

105. B. Lom, K. E. Healy, and P. E. Hockberger, ''A versatile technique for patterning biomolecules onto glass coverslips,'' *J. Neurosci. Meth.,* **50,** 385–397 (1993).

106. S. K. Bhatia, J. L. Teixeira, M. Anderson, L. C. Shriver–Lake, J. M. Calvert, J. H. Georger, J. J. Hickman, C. S. Dulcey, P. E. Schoen, and F. S. Ligler, ''Fabrication of surfaces resistant to protein adsorption and application to two-dimensional protein patterning,'' *Anal. Biochem.,* **208,** 197–205 (1993).

107. D. J. Pritchard, H. Morgan, and J. M. Cooper, ''Micron-scale patterning of biological molecules,'' *Angew. Chem. Int. Ed. Engl.,* **34,** 91–93 (1995).

108. R. J. Jackmann, J. L. Wilbur, and G. M. Whitesides, ''Fabrication of submicrometer features on curved substrates by microcontact printing,'' *Science,* **269,** 664–666 (1995).

109. B. Kasemo and J. Lausmaa, ''The biomaterial–tissue interface

and its analogues in surface science and technology,'' in *The Bone–Biomaterial Interface,* J. E. Davies (ed.), University of Toronto Press, Toronto, 1991, pp. 19–32.

110. K. Lewandowska, N. Balachander, C. N. Sukenik, and L. A. Culp, ''Modulation of fibronectin adhesive functions for fibroblasts and neural cells by chemically derivatized substrata,'' *J. Cell Physiol.,* **141,** 334–345 (1989).

111. S.-S. Cheng, K. K. Chittur, C. N. Sukenik, L. Culp, and K. Lewandowska, ''The conformation of fibrnoectin on self-assembled monolayers with different surface composition: An FTIR/ATR study,'' *J. Colloid Interface Sci.,* **162,** 135–143 (1994).

112. E. J. Bekos, J. P. Ranieri, P. Aebischer, J. A. Gardella, and F. V. Bright, ''Structural changes of bovine serum albumin upon adsorption to modified fluoropolymer substrates used for neural cell attachment studies,'' *Langmuir,* **11,** 984–989 (1995).

113. S. A. Dart, C. R. Robertson, and J. A. Berzofsky, ''Adsorption of the protein antigen myoglobin affects the binding of conformation-specific monoclonal antibodies,'' *Biophys. J.,* **53,** 533–539 (1988).

114. D. A. Puleo, ''Biochemical surface modification of Co-Cr-Mo,'' *Biomaterials,* **17,** 217–222 (1996).

115. C.-S. Lee and G. Belfort, ''Changing activity of ribonuclease A during adsorption: A molecular explanation,'' *Proc. Natl. Acad. Sci. USA,* **86,** 8392–8396 (1989).

116. B. Kasemo and J. Lausmaa, ''Material–tissue interfaces: The role of surface properties and processes,'' *Environ. Health Perspect.,* **102 (Suppl. 5),** 41–45 (1994).

117. G. T. Hermanson, K. A. Mallia, and P. K. Smith, eds., *Immobilized Affinity Ligand Techniques,* Academic Press, Inc., San Diego, 1992.

118. Z. Werb, P. Tremble and C. H. Damsky, ''Regulation of extracellular matrix degradation by cell–extracellular matrix interactions,'' *Cell Differ. Dev.,* **32,** 299–306 (1990).

119. C. G. Bellows, J. E. Aubin, and J. N. M. Heersche, ''Initiation and progression of mineralization of bone nodules formed *in vitro*: The role of alkaline phosphatase and organic phosphate,'' *Bone and Miner.,* **14,** 27–40 (1991).

120. P. S. Henthorn, ''Alkaline phosphatase,'' in *Principles of Bone Biology,* J. P. Bilezikian, L. G. Raisz, and G. A. Rodan (eds.), Academic Press, Inc., San Diego, 1996, pp. 197–206.

121. W. Beertsen, T. Van den Bos, and J. Niehof, ''Mineralization of dentinal collagen sheets complexed with alkaline phosphatase and integration with newly formed bone following subperiosteal implantation over osseous defects in rat calvaria,'' *Bone and Miner.,* **20,** 41–55 (1993).

122. R. Patarca, G. J. Freeman, R. P. Singh, F.-Y. Wei, T. Durfee, F. Blattner, D. C. Regnier, C. A. Kozak, B. A. Mock, H. C. Morse III, T. R. Jerrells, and H. Cantor, ''Structural and functional studies of the early T lymphocyte activation 1 (*Eta-1*) gene. Definition of a novel T-cell-dependent response associated with genetic resistance to bacterial infection,'' *J. Exp. Med.,* **170,** 145–161 (1989).

123. R. P. Singh, R. Patarca, J. Schwartz, P. Singh, and H. Cantor, ''Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages *in vitro* and its effect upon macrophages *in vivo,*'' *J. Exp. Med.,* **171,** 1931–1942 (1990).

124. A. Nanci, S. Zalzal, G. F., McCarthy, H. Kawaguchi, S. Sakkal, L. G., Ste-Marie, and M. D. McKee, ''Bone growth at implant surfaces,'' in *Proceedings of the Symposium ''Biomaterials: Cellular Responses to Implanted Materials,''* R. Skalak and S. Chien (eds.), The Institute for Mechanics and Materials and the Institute for Biomedical Engineering, La Jolla, California, 1994.

125. A. Nanci and M. D. McKee, ''The bone–biomaterial interface contains similar components as naturally occurring cement lines in bone,'' in *Electron Microscopy 1994—Applications in Biological Sciences,* Vol. 3B, B. Jouffrey, C. Colliex, D. Hernandez–Verdun, J. Schrevel, and D. Thomas (eds.), Les Éditions de Physique, Les Ulis Cedex A, France, 1994, pp. 885–886.

126. J. E. Davies, B. Lowenberg, and A. Shiga, ''The bone–titanium interface *in vitro,*'' *J. Biomed. Mater. Res.,* **24,** 1289–1306 (1990).