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Covalent bonding of vancomycin to Ti6Al4V alloy pins provides long-term inhibition of *Staphylococcus aureus* colonization

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Abstract—Self-protecting Ti6Al4V alloy pins were prepared by covalent bonding of bis(ethylene glycol) linkers, then vancomycin to the oxidized, aminopropylated Ti6Al4V alloy surface. Fluorescence modification-enabled estimation of yields of free amines on the metallic surface monolayer at each reaction step. The vancomycin-protected Ti6Al4V pins were not colonized by *Staphylococcus aureus*, even after 44 days storage in physiological buffer. These results provide a basis for testing self-protection against *S. aureus* colonization in animal models.

Millions of patients who receive implant devices experience biomaterial-associated infections as one of the most destructive complications. The formation of a biomaterial-associated biofilm often leads to inflammation and degradation of surrounding bone, despite aggressive antibiotic treatment, and the necessity for removal of the affected device or implant.[1–3] As an alternative to antibiotic-impregnated bone cement,[4] which is the current standard of care or antibiotic-eluting biodegradable materials,[5–7] we are studying permanent covalent bonding of antibiotics to implants to achieve self-protection. In our initial studies,[8] we utilized the last resort glycopeptide antibiotic vancomycin (VAN), which acts at the inner leaflet of Gram-positive bacterial cell walls to block peptidoglycan synthesis.[9,10] Previously we reported the coupling of VAN via a silane connector[11,12] and hydrophilic oligoethylene glycol linkers[8] to particles of titanium (Ti), a highly reactive metal with excellent biocompatibility that is commonly used either in its pure state or as an alloy in the fabrication of orthopedic and dental implants.

We then hypothesized that VAN could also be coupled to Ti6Al4V alloy, which is well established as a primary metallic biomaterial used to fabricate orthopedic implants.[13] The primary interaction between Ti6Al4V alloy and the surrounding biological environment depends on the surface properties, not on the bulk properties of the alloy. Ti6Al4V alloy has excellent biocompatibility compared with other metals, and enjoys improved ductility and fracture toughness relative to pure Ti.[14] While pure Ti particles exhibit 6.0 OH/nm^2 on their surfaces,[15] the crowding of the Ti6Al4V alloy surface with excess Al and V in preference to Ti[16] greatly reduces the availability for conjugation reactions of reactive OH groups to 1.5 OH/nm^2 on the Ti6Al4V alloy surface.[17] Indeed, we were unable to bond a detectable amount of VAN onto Ti6Al4V alloy pins under the conditions we used for pure Ti particles.[8] As a result, it was necessary to oxidize the surfaces of the Ti6Al4V alloy pins to produce a Ti-OH layer that could be aminopropylated sufficiently (Scheme 1), as others have found for bonding of alkoxy, amino or chloro moieties to Ti6Al4V alloy surfaces, yielding a surface-bound monolayer of organosiloxane under ideal conditions.[11,18] The coupling of flexible hydrophilic bis(ethylene glycol) spacers

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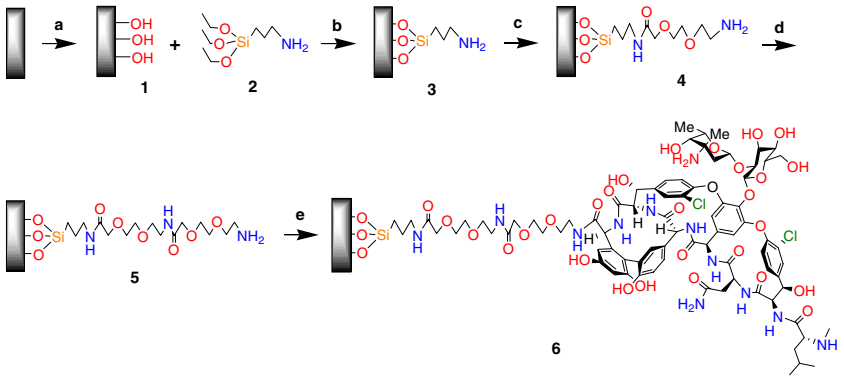

Scheme 1. Reaction scheme for synthesis of VAN bonded to Ti6Al4V. Reagents and conditions: (a) acetone, Alconox, 0.1 M NaOH, MeOH/HCl, H_2O_2/H_2SO_4; (b) 5% $NH_2PrSi(OEt)_3$ in toluene, 100 °C, 5 h; (c) i—Fmoc-AEEA, HATU, Me_2NCHO, *i*-Pr_2EtN; ii—piperidine/Me_2NCHO; (d) i—Fmoc-AEEA, HATU, Me_2NCHO, *i*-Pr_2EtN; ii—piperidine/Me_2NCHO; (e) VAN, HATU, Me_2NCHO, *i*-Pr_2EtN.

and selective N-terminal conjugation of VAN to activated Ti6Al4V alloy pin surfaces (Scheme 1) resulted in a surface layer that was capable of killing *S. aureus* on contact, precluding the possibility of biofilm formation, even after 44 days of exposure to physiological buffer. As shown in Scheme 1, (a) Ti6Al4V wire, diameter 1 mm (#264-155-36, Goodfellow, Oakdale, PA, USA) was cut into 1 cm length pins, then washed twice with acetone (5 mL), and once each with saturated aqueous Alconox (3 mL), 0.1 M NaOH (3 mL),[19] then concentrated HCl/MeOH (1:1, 5 mL) for 30 min at room temperature with 20 s periods of sonication at 0, 5, 12, 15, and 20 min. The Ti6Al4V alloy forms a surface oxide layer much more slowly than the 325 mesh pure Ti particles we studied previously.[8] Therefore, we washed the cleaned pins twice with double-deionized H_2O (5 mL), oxidized them with H_2O_2/H_2SO_4 (1:1, 5 mL) ('piranha' solution)[20] for 4 h, and then washed them with double-deionized H_2O. Finally the oxidized pins were washed four times with anhydrous Me_2NCHO (5 mL), dried overnight under vacuum, and placed in the argon atmosphere chamber of an MO-20M glove box (Vacuum Atmospheres, Hawthorne, CA).

(b) Oxidized pins were refluxed in toluene (10 mL) with azeotropic removal of H_2O (Dean-Stark trap). Then 5% $NH_2PrSi(OEt)_3$ (Aldrich, Milwaukee WI, USA) in anhydrous toluene (v/v, 0.5 mL) was added to the flask under an argon atmosphere to minimize the attack of H_2O on $NH_2PrSi(OEt)_3$. The flask was closed with a septum and the pins were heated at 100 °C for 5 h. After cooling to room temperature, the pins were washed thrice with anhydrous toluene (10 mL), four times with anhydrous Me_2NCHO (10 mL), once with PBS (10 mL) to remove nonbonded adsorbed $NH_2PrSi(OEt)_3$, then dried under vacuum overnight. The pins were then baked at 110 °C for 20 min in a convection oven to establish stable crosslinked attachment of NH_2PrSiO at the Ti6Al4V alloy surface.[21] (c) NH_2PrSiO–Ti6Al4V pins (10 cm length of wire) were washed twice with anhydrous Me_2NCHO (5 mL), then coupled with a fourfold molar excess of 8-fluorenylmethoxycarbonyl-aminoethoxyethoxyacetate, (Fmoc-AEEA) linker (Peptides International, Louisville, KY, USA), activated with a fourfold molar excess of 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetra-methyl-uronium hexa-fluorophosphate (HATU) (Applied Biosystems, Foster City, CA, USA) in anhydrous Me_2NCHO (5 mL), catalyzed by *i*-Pr_2EtN, followed by Fmoc deprotection with 20% piperidine in anhydrous Me_2NCHO (5 mL). (d) A second AEEA linker was coupled to exposed terminal amines and deprotected. All Fmoc deprotection solutions and washes were collected for analysis of coupling yields by measuring the absorbance of released dibenzofulvene at 301 nm ($\varepsilon_{570} = 7.8 \times 10^3$/M cm). (e) Finally, a fourfold excess of VAN (US Biochemicals, Cleveland, OH, USA) was coupled by the same protocol. After the VAN coupling step, the Ti6Al4V pins were washed twice with anhydrous Me_2NCHO (5 mL) and then dried under vacuum overnight. For the determination of the surface monolayer loading value of amino functionalized Ti6Al4V pins at each reaction step (**3**–**6**), we first washed the pins three times with toluene (6 mL), Me_2NCHO (6 mL), and PBS (6 mL) at room temperature with 1-min periods of sonication, in order to avoid multilayers on the surface. The same process was repeated until the Ti6Al4V pins yielded a negative assay with ninhydrin,[8] measuring the absorbance at 570 nm ($\varepsilon_{570} = 1.5 \times 10^4$/M cm).

Washed Ti6Al4V pins at each reaction step (**3**–**6**) were assayed for amino derivatization on their surfaces by conjugation with AlexaFluor 532 succinimidyl ester (Molecular Probes, Eugene OR, USA), freshly dissolved at 1 mg/mL in anhydrous Me_2NCHO (0.6 mL) to maximize the lifetime of the ester, at a fourfold molar excess, stirred at room temperature for 2 h. The AlexaFluor 532 conjugates are highly photostable and remain fluorescent over a broad range of pH from 4 to 10.[22] The Ti6Al4V pins were then washed with Me_2NCHO (1 mL) and then treated with 1.0 M Bu_4F in tetrahydrofuran (0.6 mL) (Aldrich) at room temperature for 4 h to cleave the AlexaFluor 532 conjugation products from the surface. Bu_4F is a convenient source of fluoride ion that cleaves and replaces the Ti–O–Si bond under neutral conditions. The pins were removed from the Bu_4F solutions. From each cleavage solution, 200 μL was pipetted into each well of a 96-well opaque black

microplate (Matrix Technologies, Hudson, NH, USA), used to protect the light sensitive AlexaFluor 532, with minimum background, crosstalk, and scattered light. The fluorescence intensities in each well were measured in an FL 600 microplate reader (Biotek Instruments, Winooski, VT, USA) running KC_4 software, with filters for excitation at 485 ± 25 nm, and emission at 530 ± 25 nm. Quantitation of fluorescence intensity data was based on a calibration curve (Fig. 1) prepared with solutions of AlexaFluor 532 succinimidyl ester (0.2–10 μg) in Me_2NCHO. Each measurement was carried out in triplicate; the average and standard deviation of the linear regression slope and intercept were calculated, with $p < 0.05$. By AlexaFluor 532 conjugation we identified the loading value of amino groups on the Ti6Al4V pins at each stage, first after $NH_2PrSi(OEt)_3$ conjugation to the Ti oxide surface (0.131 ± 0.042 nmol/cm^2), second after the first AEEA linker (0.139 ± 0.01 nmol/cm^2), third after the second AEEA linker (0.163 ± 0.041 nmol/cm^2), and finally after VAN coupling (0.195 ± 0.046 nmol/cm^2), indistinguishable from the initial bonding of $NH_2PrSi(OEt)_3$ onto the surface, implying a monolayer. Hence, quantification of AlexaFluor 532-conjugated surface amines after cleavage showed that loading of VAN was indistinguishable from the initial bonding of $NH_2PrSi(OEt)_3$ onto the surface. Our results agree with a previous report of $NH_2PrSi(OEt)_3$ derivatization measured by $H^{14}CHO$ radiolabeling (0.22 nmol/cm^2). [23] The AlexaFluor 532-conjugated surface showed unchanged fluorescence intensity after storage for 2–3 days at room temperature. MALDI-TOF mass spectroscopy on a SELDI spectrometer (Ciphergen, Fremont. CA, USA) was used to test for covalent linkage of VAN to the Ti6Al4V surface. Analysis of material stripped from the pins with Bu_4F (Fig. 2) showed a peak corresponding to VAN-AEEA-AEEA-NH-Pr-SiF_3 + 3 Na at 1934 Da. Other cleavage products were identified at 1796 Da (VAN-AEEA-AEEA-NH- Pr-OH), 1640 Da (VAN-AEEA-COOH + 2 Na), 1561 Da (VAN-AEEA-AEEA-NH-Pr-SiF_3, VAN lacking one sugar), 1499 Da (VAN-AEEA-AEEA-COOH + 3 Na, VAN lacking two sugars). Some of the fragments showed VAN without one or two sugars because the pure VAN starting material included some VAN without one sugar at 1305.1 Da, and VAN without two sugars at 1143.2 Da, along with the completely glycosylated VAN at 1450.1 Da.

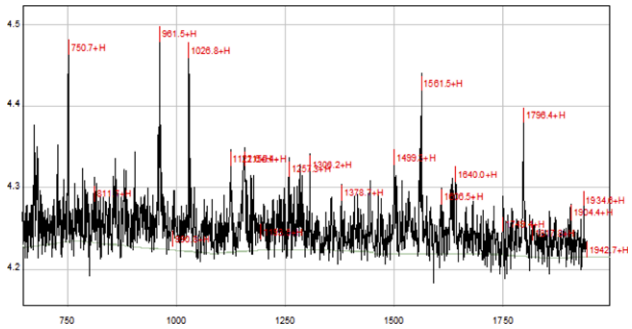

Figure 2. MALDI-TOF mass spectrum in positive mode showing the products cleaved from VAN-AEEA-AEEA-Ti6Al4V pins by 1.0 M Bu_4NF in tetrahydrofuran. The fragments were desorbed and ionized from a matrix of α-cyano-4-hydroxycinnamic acid.

To determine the distribution of VAN on the modified Ti6Al4V pins, they were exposed to anti-VAN antibodies, mouse monoclonal anti-VAN IgM (1:300) or anti-fibronectin IgG (1:300) (US Biochemicals), followed by a fluorescent secondary antibody, AlexaFluor 488-coupled donkey anti-mouse IgG (1:300), then examined by confocal fluorescence microscopy on a Fluoview 300 (Olympus, Center Valley, PA, USA) (Fig. 3). After the

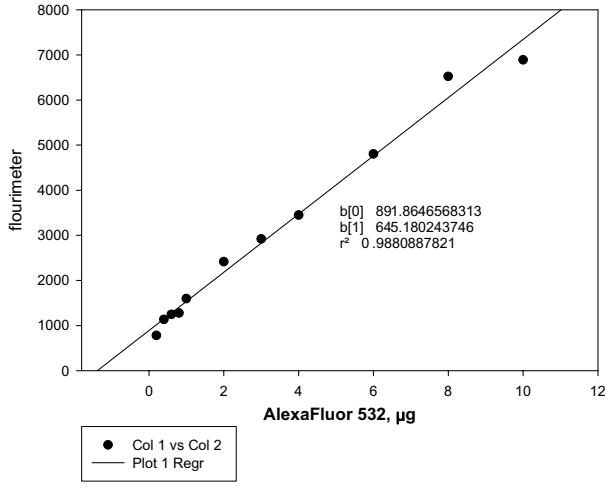

Figure 1. Calibration curve obtained from fluorescence measurements of AlexaFluor 532 succinic ester (0.2–10 μg) in Me_2NCHO upon excitation at 485 ± 25 nm and emission at 530 ± 25 nm. Based on the linear regression fit of the data to the linear equation $y = mx + b$, the number of amines on the surface of Ti6Al4V was quantified by AlexaFluor 532 conjugation.

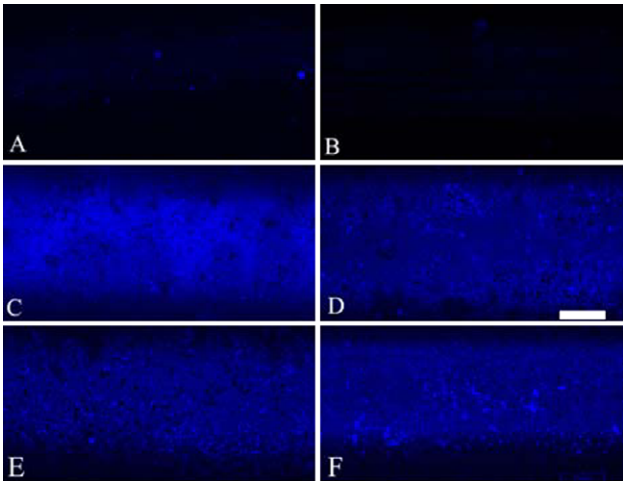

Figure 3. Immunofluorescent detection of VAN bonded to Ti6Al4V pins. (A) Background staining with mouse anti-VAN mAb followed by AlexaFluor 488-coupled donkey anti-mouse IgG on an underivatized, control pin. (B) Nonspecific staining by anti-fibronectin antibody on VAN-Ti6Al4V pins (no FBS coating). Specific anti-VAN staining was observed on VAN-Ti6Al4V pins after (C) no incubation, (D) 24 h incubation in PBS, (E) 24 h incubation in *S. aureus*, or (F) 5 weeks incubation in *S. aureus*.

Me_2NCHO washes, VAN-specific immunofluorescence was intense over the Ti6Al4V pin surface. After 24 h of incubation in PBS, the immunofluorescence decreased, presumably due to elution of adsorbed noncovalent VAN. Importantly, no further decline in fluorescence was observed, even after 5 weeks of continuous incubation with *S. aureus* subspecies Rosenbach (#25923, American Type Culture Collection, Germantown, MD, USA) in Luria–Bertani Miller broth (Becton–Dickinson, Sparks, MD, USA) at 37 °C. In a simple baseline test of long-term stability, control Ti6Al4V pins and VAN-Ti6Al4V pins were incubated in PBS at room temperature for up to 44 days. On selected days, pins were removed from PBS, weighed, disinfected with 70% EtOH, and washed five times with PBS. The washed pins were cultured with 1×10^4 cfu/mL of *S. aureus* in Luria–Bertani Miller broth at 37 °C for 24 h, washed three times with PBS to remove loosely adherent bacteria, and assessed for bacterial/adhesion viability with the BacLight™ Live/Dead® viability assay (Molecular Probes, Eugene, OR, USA), which fluorescently labels viable bacteria green and dead bacteria red. After labeling, the Ti6Al4V pins were washed three times with PBS to remove nonspecific stain and visualized by confocal laser fluorescence microscopy on a Fluoview 300.

Only sparse green fluorescence was observed on VAN-Ti6Al4V pins over 4 days (Fig. 4) indicating minimal colonization. At 5 days, colonization of the control pin was abundant with some punctate staining apparent in the background aura. Punctate staining could have been caused by surface reflectivity or incomplete coverage by VAN. Importantly, after 44 days in PBS, 24 h of incubation with *S. aureus* yielded abundant colonization of the control pin, while the VAN-Ti6Al4V pin showed only background fluorescence. Future tests of stability will be carried out at 37 °C in PBS and a pH 5 buffer, to reflect physiological conditions, and in animal models. The RSi–O–Ti bond has been found stable in a wide variety of applications,[24] including dental implants in the oral cavity, typically pH 5. Furthermore, we recently reported that VAN-Ti6Al4V pins prevented bacterial colonization in vitro (1) after exposure to supra-physiological doses of *S. aureus* over time, (2) after extended incubation in physiological buffers, and (3) after repeated bacterial challenges.[25] In addition, we have observed that when murine MC3T3 pre-osteoblastic cells were seeded on the VAN-Ti6Al4V surface, the cells exhibited no change in viability, indicating that the VAN-Ti6Al4V surface supports bone cell adhesion.[26]

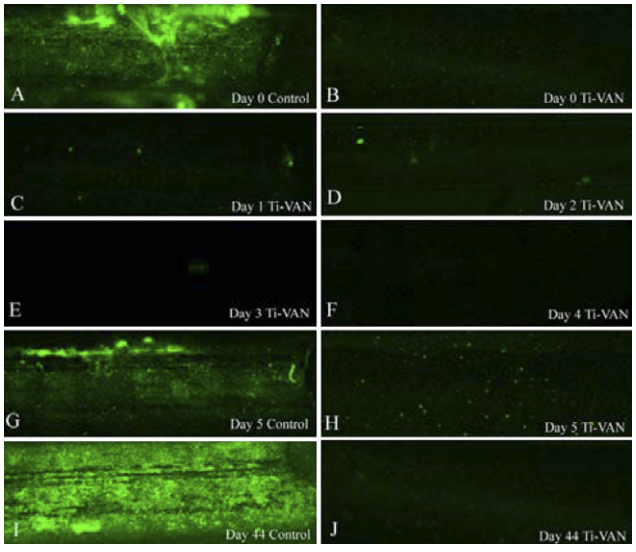

Figure 4. Evaluation of VAN-Ti6Al4V bactericidal activity after 44 days of exposure to PBS at room temperature over 44 days. A control Ti6Al4V pin (A) was incubated with *S. aureus* for 24 h, followed by staining with the Live/Dead® BacLight™ viability kit, and visualization of live bacteria by confocal fluorescence microscopy. Similarly, VAN-Ti6Al4V pins were incubated for 24 h with *S. aureus* after 0 days (B), 1 day (C), 2 days (D), 3 days (E) or 4 days (F) of incubation in PBS. For longer experiments, control (G, I) or VAN-Ti6Al4V pins (H, J) were incubated in PBS for 5 (G, H) or 44 (I, J) days, followed by detection of live bacteria by confocal fluorescence microscopy.

As we discussed previously,[8] selection for VAN-resistant mutant bacteria requires high bacterial titers, as in the mouth and intestines. In contrast, peri-prosthetic infections start with very few bacteria. Furthermore, only those bacteria that actually land on the VAN-implant surface will be subject to selection for resistance. This situation enormously reduces the probability of a mutation that confers resistance, because the bacteria will not encounter a subclinical VAN concentration that permits continued growth of a few resistant cells.[27] On a solid phase surface covered with 0.2 nmol VAN/cm^2 or $1.2. \times 10^6$ VAN residues/μm^2, each landing bacterium ($\approx 1 \times 2$ μm) will dock simultaneously and cooperatively with $0.5–1 \times 10^6$ VAN residues. Even in the case of a mutant peptidoglycan that binds weakly to VAN,[5] cooperative binding will be lethal.

In conclusion, we have demonstrated covalent bonding of a monolayer of VAN to the surface of Ti6Al4V alloy pins, quantitated by fluorescence modification of reactive amines. Mass spectroscopy indicated that AEEA linkers and VAN had indeed been coupled to each other and to the Ti6Al4V surface. Immunofluorescence assays illustrated long-term resistance of covalently bound VAN to aqueous hydrolysis at neutral pH, necessary for retention of an active biofilm-resistant implant surface. Bacterial live/dead fluorescence assays revealed long-term bactericidal activity of the VAN-Ti6Al4V pin surfaces for up to 44 days storage in physiological buffer. These results are consistent with our hypothesis, and support progress into animal studies. This covalent bonding strategy might prevent Gram-positive bacterial infections of orthopedic implants, increasing their useful lifetime.

Acknowledgments

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References and notes

1. Garvin, K. L.; Hanssen, A. D. *J. Bone Joint Surg.* **1995**, *77A*, 1576.
2. Duggan, J. M.; Georgiadis, G. M.; Kleshinski, J. F. *Infect. Med.* **2001**, *18*, 534.
3. Zimmerli, W.; Trampuz, A.; Ochsner, P. E. *N. Engl. J. Med.* **2004**, *351*, 1645.
4. Haddad, F. S.; Masri, B. A.; Campbell, D.; McGraw, R. W.; Beauchamp, C. P.; Duncan, C. P. *J. Bone Joint Surg. Br.* **2000**, *82*, 807.
5. Loll, P. J.; Axelsen, P. H. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 265.
6. van de Belt, H.; Neut, D.; Schenk, W.; van Horn, J. R.; van der Mei, H. C.; Busscher, H. J. *Acta Orthopaed. Scand.* **2001**, *72*, 557.
7. Frazier, D. D.; Lathi, V. K.; Gerhart, T. N.; Hayes, W. C. *J. Biomed. Mater. Res.* **1997**, *35*, 383.
8. Jose, B.; Antoci, V., Jr.; Zeiger, A. R.; Wickstrom, E.; Hickok, N. J. *Chem. Biol.* **2005**, *12*, 1041.
9. Walsh, C. *Nature* **2000**, *406*, 775.
10. Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. *Chem. Rev.* **2005**, *105*, 425.
11. Puleo, D. A.; Kissling, R. A.; Sheu, M. S. *Biomaterials* **2002**, *23*, 2079.
12. Jennissen, H. P. *Ann. N. Y. Acad. Sci.* **2002**, *961*, 139.
13. Zreiqat, H.; Akin, F. A.; Howlett, C. R.; Markovic, B.; Haynes, D.; Lateef, S.; Hanley, L. *J. Biomed. Mater. Res. A* **2003**, *64*, 105.
14. Robinson, J. M.; van Brussel, B. A.; de Hosson, J. T. M.; Reed, R. C. *Mater. Sci. Eng. A* **1996**, *208*, 143.
15. Baier, R. E. In *Adhesion in Biological Systems*; Manly, R. S., Ed.; Academic Press: New York, 1970; pp 15–48.
16. Milosev, I.; Metikos-Hukovic, M.; Strehblow, H. H. *Biomaterials* **2000**, *21*, 2103.
17. Mikulec, L. J.; Puleo, D. A. *J. Biomed. Mater Res.* **1996**, *32*, 203.
18. Puleo, D. A. *J. Biomed. Mater. Res.* **1997**, *37*, 222.
19. Lu, X.; Leng, Y.; Zhang, X.; Xu, J.; Qin, L.; Chan, C. W. *Biomaterials* **2005**, *26*, 1793.
20. Kern, W. *Handbook of Semiconductor Wafer Cleaning Technology*; Noyes Publications: Park Ridge, NJ, 1993.
21. Crampton, N.; Bonass, W. A.; Kirkham, J.; Thomson, N. H. *Langmuir* **2005**, *21*, 7884.
22. Panchuk-Voloshina, N.; Haugland, R. P.; Bishop-Stewart, J.; Bhalgat, M. K.; Millard, P. J.; Mao, F.; Leung, W. Y.; Haugland, R. P. *J. Histochem. Cytochem.* **1999**, *47*, 1179.
23. Xiao, S. J.; Textor, M.; Spencer, N. D.; Wieland, M.; Keller, B.; Sigrist, H. *J. Mater. Sci. Mater. Med.* **1997**, *8*, 867.
24. Williams, D. F. In *Titanium in Medicine*; Brunette, D. M., Tengvall, P., Textor, M., Thomsen, P., Eds.; Springer: Berlin, 2001; pp 13–24.
25. Antoci, V., Jr.; King, S. B.; Jose, B.; Parvizi, J.; Zeiger, A. R.; Wickstrom, E.; Freeman, T. A.; Composto, R. J.; Ducheyne, P.; Shapiro, I. M.; Hickok, N. J.; Adams, C. S. *Journal of Orthopaedic Research* **2007**, *25*, in press.
26. Antoci, V., Jr.; Adams, C. S.; Parvizi, J.; Wickstrom, E.; Zeiger, A. R.; Composto, R. J.; Ducheyne, P.; Shapiro, I. M.; Hickok, N. J. *Clinical Orthopaedics and Related Research* **2007**, submitted for publication.
27. Neu, H. C.; Gootz, T. D. *Bacterial Resistance*; University of Texas Medical Branch Press: Galveston, TX, 1996.