Chemistry & Biology, Vol. 12, 1041–1048, September, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.06.013

Vancomycin Covalently Bonded to Titanium Beads Kills *Staphylococcus aureus*

Binoy Jose,[1,4] Valentin Antoci, Jr.,[2] Allen R. Zeiger,[1] Eric Wickstrom,[1,3,5,*] and Noreen J. Hickok[1,2]
[1]Department of Biochemistry and Molecular Biology
[2]Department of Orthopaedic Surgery
[3]Department of Microbiology and Immunology
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

Summary

Periprosthetic infections are life-threatening complications that occur in about 6% of medical device insertions. Stringent sterile techniques have reduced the incidence of infections, but many implant patients are at high risk for infection, especially the elderly, diabetic, and immune compromised. Moreover, because of low vascularity at the site of the new implant, antibiotic prophylaxis is often not effective. To address this problem, we designed a covalent modification to titanium implant surfaces to render them bactericidal. Specifically, we aminopropylated titanium, a widely used implant material and extended a tether by solid phase coupling of ethylene glycol linkers, followed by solid phase coupling of vancomycin. Vancomycin covalently attached to titanium still bound soluble bacterial peptidoglycan, reduced *Staphylococcus aureus* colony-forming units by 88% ± 16% over 2 hr, and retained antibacterial activity upon a repeated challenge.

Introduction

Implant-associated infections are serious complications of medical device insertions [1–3]. An estimated 11 million people in the United States alone have received at least one implanted medical device [4]. Suffering and additional health costs due to implant infections are a major public health problem. Such infections are difficult to treat for two principal reasons. The first of these is that the implant is surrounded by a fibrous coating, as is typical in the response to a foreign body. This encapsulation provides a protected environment for bacterial growth that is largely inaccessible to the host immune system. The second reason is that bacteria such as *Staphylococcus aureus* (*S. aureus*) adhere readily to implant surfaces. Thus the implant surface, which is coated with a host of extracellular matrix serum proteins, provides an ideal environment to facilitate bacterial adhesion and proliferation to ultimately result in a bacterial population encased in a polysaccharide glycocalyx, termed a biofilm. At this point, the biofilm-coated surface is largely resistant to pharmacological agents as well as host defenses [5, 6]. Implant-associated infections can arise in the weeks immediately following surgery due to contamination; at later times, infections can arise from hematogenous sources, such as urinary tract infections and periodontal disease. In joint replacements, treatment entails aggressive systemic and local antibiotic treatment, debridement, and, in many cases, implant removal and reimplantation. Despite aggressive antibiotic treatment, eradication of established implant-associated infections often fails [3, 7–9]. Difficult cases lead to arthrodesis, amputation, or death.

Current clinical practice relies on insertion of antibiotic-impregnated bone cement, usually in the form of spacer blocks [10]. Such blocks suffer from poor elution profiles, material properties, and the requirement for surgical removal. Controlled-release, antibiotic-impregnated biodegradable materials have been developed for this application [11–16]. However, they suffer from fragility and/or undesirable modification of the implant environment during their degradation. Importantly, these materials deliver active prophylaxis only from the time of implantation and last at most only through the immediate postoperative period [17]. Thus, delivery of local antibiotic prophylaxis is less than ideal with the materials described above. A new paradigm to interdict deep-seated bone infections both in the immediate postoperative period and throughout the life of the implant would have great clinical importance for implant surgery.

We hypothesized that permanent covalent bonding of drugs to implants would provide the desired protection. For the first test of the permanent covalently bonded strategy, we examined titanium (Ti), a metal with excellent biocompatibility that is commonly used either in its pure state or as an alloy in the fabrication of orthopaedic and dental implants. Ti biocompatibility appears to depend not on its bulk properties but on the Ti-OH surface layer, which displays a dielectric constant of 80 or more [18], very close to that of water. Importantly, Ti and its alloys osseointegrate, that is, bone and its matrix come into stable association with the metal implant, thus allowing stable fixation of the implant.

Osseointegration of Ti implants has been enhanced through altering surface topography, as well as through surface adsorption or surface modification with bioactive molecules to enhance osseointegration [19–21], for example, fabrication of an interconnected porous Ti surface, or adsorption of calcium phosphate [22]. The Ti-OH surface layer is sufficiently reactive to allow silanization [23]. Because the $3d^2$ outer shell orbitals of titanium are similar in reactivity to the $3p^2$ orbitals of silicon, the Ti-OH outer layer of Ti metal can be aminopropylated just like Si-OH to permit permanent attachment of interesting molecules. This approach has been used to attach bioactive peptides, enzymes, and proteins to glass, titanium, quartz, and silicone aminopropylated surfaces [23–30].

Although antibiotics have not yet been covalently bonded to Ti implant devices, coupling to Ti through aminopropylation seemed conducive to antibiotic at-

*Correspondence: eric@tesla.jci.tju.edu
[4]Present address: SK Biopharmaceuticals, Fairfield, NJ 07004.
[5]Lab address: http://tesla.jci.tju.edu

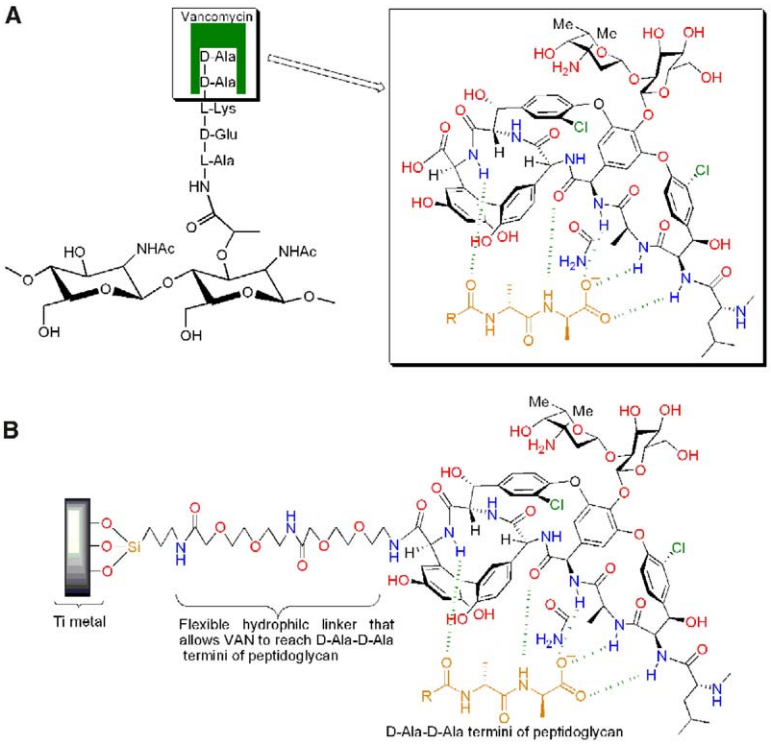

Figure 1. Mechanism and Structure of VAN Bonded to Ti

(A) Inhibition of transpeptidation and transglycosylation steps of cell wall biosynthesis by VAN through the binding of the D-Ala-D-Ala termini of peptidoglycan with VAN by five hydrogen bonds.

(B) Schematic of the proposed VAN-tethered Ti implant material.

tachment. We hypothesized that the antibiotic implant products would be bacteriostatic or bactericidal, creating a stable microenvironment with a similar spectrum as the soluble antibiotics. Such self-protecting implants represent the beginning of a new generation of prosthetic devices that could lower the incidence of implant-associated infections, prevent bacterial biofilm formation, and ultimately decrease revision surgeries due to infection-associated implant loosening. In this model, antibiotic protection of implant surfaces would not be compromised by bone implant cement because bacterial colonization of the metal surface beneath the cement constitutes the central problem.

We chose the well-studied antibiotic vancomycin (VAN) (Figure 1) to bond to Ti because VAN provides potent treatment for a large spectrum of Gram-positive bacterial infections, the most common causes of nosocomial and hematogenous periprosthetic infections [31–33]. VAN blocks steps in the biosynthesis of the peptidoglycan layer of the cell wall of Gram-positive bacteria by binding to L-Lys-D-Ala-D-Ala termini of the nascent peptidoglycan (Figure 1) [34, 35], thereby preventing the crosslinking that is necessary for osmotic stability. In fact, membrane-localized VAN shows enhanced activity against VAN-resistant *S. aureus* [36]. As the C-terminal carboxylic acid of VAN is not necessary for antibiotic activity [37–39], it is suitable for covalent coupling of VAN to the aminopropylated Ti surface. Thus, it is reasonable to hypothesize that VAN could be tethered to Ti beads via linkers and could kill susceptible bacteria by acting at the inner leaflet of the bacterial cell wall. Here we report the synthesis, characterization, ligand binding activity, and infection control of *S. aureus* with VAN-tethered Ti beads.

Results and Discussion

Design of Antibiotic-Tethered Metal Implant

Implant-associated infection can be recalcitrant to treatment, suggesting that prevention is the best strategy. One way to prevent the establishment of infection is to render the implant surface bactericidal via covalent modification. Specifically, we planned to produce a bactericidal surface by coupling VAN to a Ti surface derivatized with 3-aminopropyltriethoxysilane (APTS; $NH_2PrSi(OEt)_3$) (Figure 2). We were particularly interested in using Ti as the metal in our initial studies because of its demonstrated superiority over other orthopaedic implant materials.

Therefore, we hypothesized that an antibiotic could be bonded to aminopropylated Ti ($NH_2PrSiOTi$) by solid phase peptide synthesis (Figure 2). We tested this hypothesis by measuring two key properties: (1) binding of Ti-coupled VAN to its macromolecular target; and (2) bactericidal or bacteriostatic action of Ti-coupled VAN against adherent bacteria.

To extend VAN away from the silanized Ti surface, we chose 8-amino-3,6-dioxaoctanoate (aminoethoxyethoxyacetate; AEEA), a flexible linker that is hydrophilic due to the presence of ether linkages in the molecule and amide linkages to the Ti particles and to VAN. We hypothesized that extending VAN away from the Ti surface by the length of two AEEA linkers and the NH_2PrSi modification (24 bonds, $\approx$40 Å) would be sufficient to

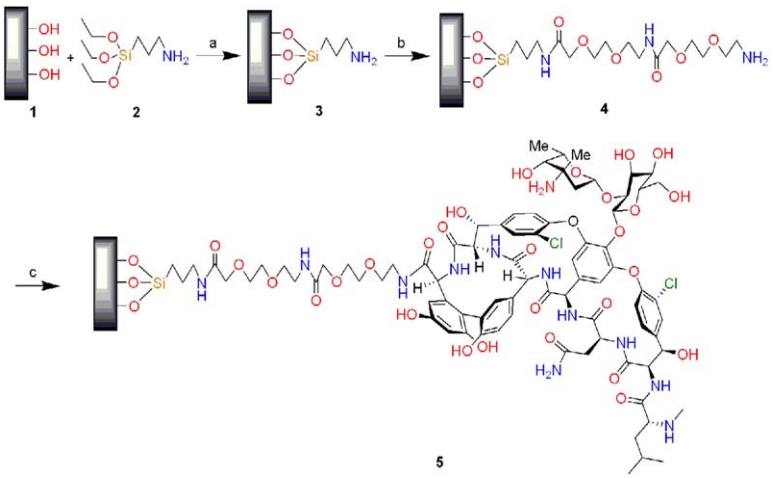

Figure 2. Reaction Scheme for Synthesis of VAN Bonded to Ti

(a) Toluene, room temperature. (b) (i) Fmoc-AEEA, HATU, Me_2NCHO, N-methylmorpholine, room temperature; (ii) Piperidine/Me_2NCHO; (iii) Fmoc-AEEA, HATU, Me_2NCHO, N-methylmorpholine, room temperature; (iv) Piperidine/Me_2NCHO. (c) VAN, HATU, Me_2NCHO, N-methylmorpholine, room temperature.

allow VAN to enter the bacterial cell wall to bind to L-Lys-D-Ala-D-Ala termini of the peptidoglycan precursors. A schematic of the proposed implant material is shown in Figure 1, illustrating the interaction of VAN with the L-Lys-D-Ala-D-Ala terminus of the peptidoglycan precursors.

Synthesis and Characterization of VAN-Tethered Ti Implant

We tethered VAN to the Ti-OH surface via $NH_2PrSi(OEt)_3$ and AEEA linkers (Figure 2). We derivatized Ti particles (1) with 5% (v/v) $NH_2PrSi(OEt)_3$ (2) in toluene under argon to generate $NH_2PrSiOTi$ beads (3; a structure reflecting low NH_2PrSi modification). Ninhydrin assays of $NH_2PrSiOTi$ particles gave yields of 8–12 μmol of amine per gram of beads. Two Fmoc-AEEA linker groups (4) and VAN were then coupled sequentially to the $NH_2PrSiOTi$ beads (3) by solid phase peptide synthesis using N-[(dimethylamino)-1-*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (O-(7-azabenzo-triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HATU) as the coupling reagent. The Fmoc groups were cleaved with 20% piperidine in anhydrous Me_2NCHO; quantitation of the absorbance spectra of the Fmoc deprotection solutions showed stoichiometric addition of each of the two AEEA linkers to the $NH_2PrSiOTi$ support. Linker coupling was also quantitated by ninhydrin assays of amine functional groups on the Ti beads following Fmoc deprotection.

Covalent coupling of VAN to the AEEA linkers and attachment to the $NH_2PrSiOTi$ particles was tested by cleavage of Si-O-Ti bonds with Bu_4NF, a mild deprotection reagent for silyl protection groups [40]. The cleaved product was analyzed by MALDI-TOF mass spectroscopy (Figure 3). A peak corresponding to the cleaved VAN-AEEA-AEEA-$NHPrSiF_3$ was observed at 1863 Da. Other cleavage products were observed at 1616 Da (VAN-AEEA-COOH + Na), 1576 Da (VAN-AEEA-OH), 1549 Da (VAN-AEEA-COO^-), and 1514 Da (VAN-CO-NH-CH_2-CH_2-OH + Na). Based on the assignments of these fragments, we concluded that the Ti beads were derivatized by $NH_2PrSi(OEt)_3$, coupled to two AEEA linker groups, and covalently bound to VAN.

Analysis of VAN Distribution on the Ti Surface

To further analyze the presence and distribution of VAN bonded to the Ti surface, VAN coverage was visualized with a specific anti-VAN antibody using indirect immunofluorescence (Figure 4D). Thus, VAN derivatization was apparent by immunofluorescence and appeared to cover the surface evenly, with areas of focal concentrations. When control Ti particles were stained, little fluorescence was observed (Figure 4A). The control Ti beads covered ~50% of the well (Figure 4B). When the VAN-AEEA-AEEA-NHPrSiOTi particles were stained, diffuse fluorescence was present (Figure 4C) that corresponded to the distribution of Ti beads (Figure 4D). Thus, VAN derivatization was apparent by immunofluorescence and appeared to cover the surface evenly, with areas of focal concentrations. To begin to address the question of long-term stability, the VAN distribution was also examined in a different batch of particles that had been incubated in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum for

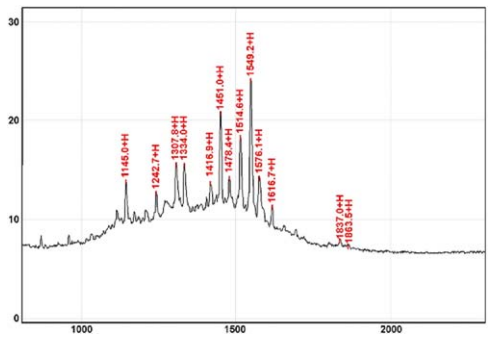

Figure 3. MALDI-TOF Mass Spectrum of Cleaved VAN-AEEA-AEEA-NHPrSiOH

Ti-O-Si bonds were cleaved with 1 M Bu_4NF in tetrahydrofuran for 2 hr at room temperature.

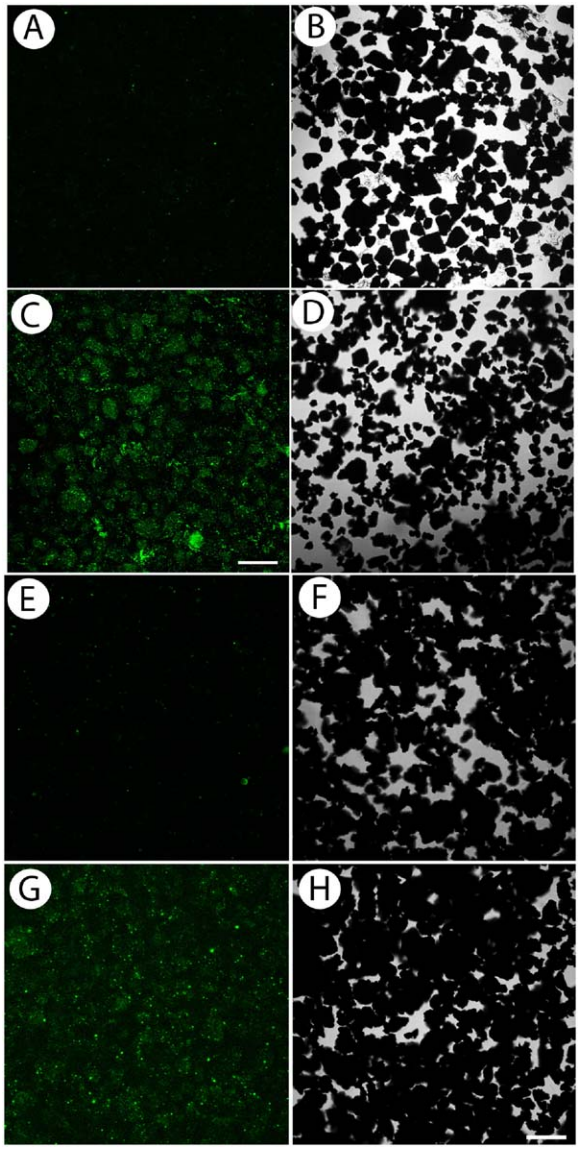

Figure 4. Immunofluorescent Detection of VAN on Ti Particles

Ti or VAN-AEEA-AEEA-NHPrSiOTi beads were incubated with anti-VAN antibody for 24 hr, followed by incubation with an AlexaFluor 488-coupled secondary antibody. Left: fluorescence; right: bright field. Results are shown for newly synthesized particles (A–D) and for particles that had been incubated with DMEM containing 10% fetal bovine serum, 25°C for 3 weeks (E–H). Specific binding of the anti-VAN antibody was detected by confocal laser scanning microscopy (new: [A] and [C]; 3 weeks: [E] and [G]) and particle distribution was determined by a bright field image (new: [B] and [D]; 3 weeks: [F] and [H]). Control Ti beads (new: [A]; 3 weeks: [E]) showed little specific signal associated with binding of the anti-VAN antibody, whereas VAN-AEEA-AEEA-NHPrSiOTi particles showed diffuse staining (new: [C]; 3 weeks: [G]) that corresponded to the distribution of the beads in the well (new: [D]; 3 weeks: [H]). The scale bar represents 50 μm.

3 weeks at room temperature (Figures 4E–4H). As in the newly synthesized particles, binding of the VAN antibody to the control Ti beads after incubation was minimal (Figure 4E), whereas significant binding was observed with the VAN-AEEA-AEEA-NHPrSiOTi particles (Figure 4G) assessed under the same conditions. Taken together, these images imply stability of VAN on the Ti surface for at least 3 weeks.

Analysis of Biochemical Activity of VAN by Ligand Binding

The biochemical activity of covalently bound VAN was tested with a substrate binding assay. Washed Ti, NH_2PrSiOTi, and VAN-AEEA-AEEA-NHPrSiOTi were incubated in physiological buffer (0.15 M NaCl, 0.01 M Na_2HPO_4 [pH 7.4]; PBS) in the presence of soluble peptidoglycans containing multiple L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala termini [41, 42]. Specific peptidoglycan binding was determined by liquid scintillation counting of unbound radioactivity. VAN-AEEA-AEEA-NHPrSiOTi particles bound 50%–60% of the labeled peptidoglycan, while NH_2PrSiOTi or unmodified Ti beads bound only ~15% of the labeled peptidoglycan. To reduce nonspecific binding, particles were also incubated with 0.1% (w/v) bovine serum albumin in 0.15 M NaCl, 0.01 M $(HOCH_2)_3CNH_2$-HCl (pH 7.4), 0.1% Tween-20 (TBST) prior to addition of labeled soluble peptidoglycan. L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala soluble peptidoglycan binding was reduced overall, with VAN-AEEA-AEEA-NHPrSiOTi particles binding ~25% of the label compared with 5% or lower for NH_2PrSiOTi or unmodified Ti beads. These data imply that VAN tethered to Ti beads can still bind its peptidoglycan ligand specifically.

Antibacterial Activity Assay on VAN-Tethered Ti Particles

Staphylococci account for the majority of orthopaedic implant device-related infections; *S. aureus* is the most common pathogen identified [2, 8, 43, 44]. The ability of *S. aureus* to adhere to the extracellular matrix and plasma proteins deposited on biomaterials is a significant factor in the pathogenesis of medical device-related infections [45, 46]. For these reasons, we chose *S. aureus* to study the antibacterial activity of the VAN-AEEA-AEEA-NHPrSiOTi beads.

The antibacterial activity of VAN-AEEA-AEEA-NHPrSiOTi particles was determined by static incubation with ≈1 × 10^4 cfu active *S. aureus* at 37°C with Ti or VAN-AEEA-AEEA-NHPrSiOTi particles for 2 hr. *S. aureus* incubation with VAN-AEEA-AEEA-NHPrSiOTi showed significant antibacterial effects at each time point compared to the incubation with Ti particles (Figure 5). In fact, VAN-AEEA-AEEA-NHPrSiOTi surfaces reduced the colony formation by 76% ± 15% at 1 hr and 88% ± 16% at 2 hr when compared to controls. Thus, based on bacterial proliferation assays, the VAN-AEEA-AEEA-NHPrSiOTi beads markedly reduced colony counts, suggesting a potent bactericidal effect.

Based on these results, we examined the appearance of the particles after incubation with ≈1 × 10^4 cfu active *S. aureus* for 1 hr at 37°C. Control Ti or VAN-AEEA-AEEA-NHPrSiOTi samples were stained with Live/Dead BacLight kit (Molecular Probes, Eugene, OR) that differentiates viability based on differential dye permeability (Figure 6). When *S. aureus* cells were incubated with Ti beads, most cells remained alive, as evidenced by predominantly green staining (Figure 6A

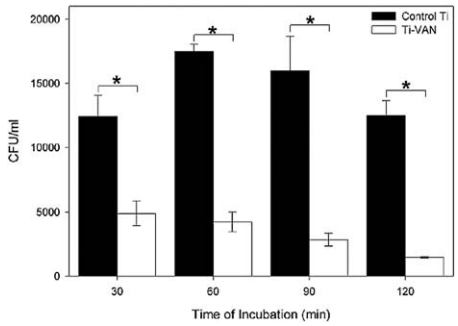

Figure 5. Ti-Tethered VAN Inhibits Proliferation of *S. aureus*

Control Ti (solid bars) or VAN-AEEA-AEEA-NHPrSiOTi (Ti-VAN, open bars) particles were incubated with *S. aureus* under static conditions for up to 120 min. Colony counts were determined by direct counting after serial dilution plating. Incubation of *S. aureus* with VAN-AEEA-AEEA-NHPrSiOTi resulted in significantly decreased colony counts at all times sampled. *S. aureus* in control cultures maintained colony counts without robust proliferation as would be expected in static cultures. Normalized data are shown as a percentage of Ti control at 30 min. The asterisks imply statistical significance in reference to the corresponding time control at $p < 0.05$.

versus 6B; bright field image of the particles is shown in Figure 6C). In contrast, *S. aureus* incubated with VAN-AEEA-AEEA-NHPrSiOTi beads were predominantly dead, as indicated by red staining (Figure 6D versus 6E; bright field in Figure 6F). When beads that had been exposed the day before to *S. aureus* were washed, rechallenged, and stained, VAN-AEEA-AEEA-NHPrSiOTi retained its ability to kill *S. aureus* preferentially (not shown). These staining and proliferation assay results imply that the VAN-AEEA-AEEA-NHPrSiOTi surface actively killed *S. aureus*.

Antibiotic Resistance

Before introducing a new and stable form of VAN in vivo, one must consider the possibility of selection for VAN-resistant mutant bacteria. An environment that is permissive for the selection of resistant organisms relies on (1) the presence of millions of bacteria, characteristically encountered in the gut and the oral cavity and (2) prolonged exposure to concentrations of antibiotics sufficient to suppress growth of sensitive organisms but insufficient to suppress insensitive organisms [47]. In the first case, the number of bacteria that initiate periprosthetic infections is small and as the antibiotic is constrained on the Ti surface, the number of bacteria exposed to the antibiotic is smaller still. Under these circumstances, the probability of a mutation that confers a selective growth advantage to a low number of bacteria is vanishingly small. With respect to the second possibility, the dissociation constant of VAN from L-Lys-D-Ala-D-Ala in solution is 1×10^{-6} M [48]; thus, at limiting concentrations of soluble VAN monomers, enough peptidoglycan precursors are unoccupied at any moment for some bacteria to survive and grow slowly, providing an ideal situation to select for a mutant peptidoglycan with a terminal D-Ala-D-Lac that binds weakly to VAN [11].

That situation is much less likely for a solid phase material bearing multiple VAN residues. In suspension culture, a single bacterium presents thousands of L-Lys-D-Ala-D-Ala precursor peptidoglycan binding sites for soluble VAN over the surface of its cell wall. For a bacterium sitting on a bed of multiple solid phase VAN moieties, bound by each of thousands of accessible peptidoglycans to thousands of VAN residues on the Ti surface, dissociation of that bacterium would require simultaneous cooperative undocking of all peptidoglycans from all VAN-AEEA-AEEA-NHPrSiOTi sites at the same instant. The dissociation constant for multiple peptidoglycan-VAN complexes is the product of the multiple dissociation constants. That is, the K_D for two peptidoglycans from two solid phase VAN moieties is $(10^{-6} \times 10^{-6}\ \text{M}) = 10^{-12}$ M. For three peptidoglycans simultaneously escaping from three VAN residues, one calculates similarly $K_D = 10^{-18}$ M. This analysis was validated by a specific test of 1,3,5-tris(CONH(N-acetyl)-L-Lys-D-Ala-D-Ala) benzene binding to tris(VAN carboxamide), yielding a measured K_D of $(4 \pm 1) \times 10^{-17}$ M [49].

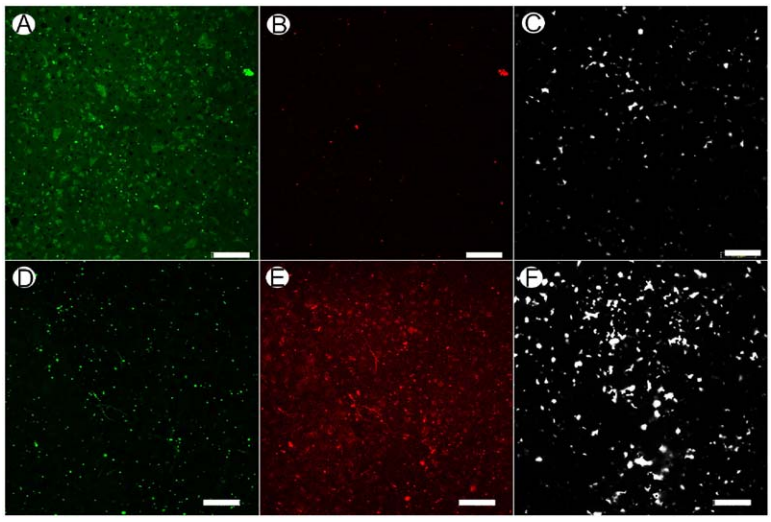

Figure 6. Ti-Tethered VAN Kills Adherent *S. aureus*

Ti (A–C) or VAN-AEEA-AEEA-NHPrSiOTi (D–F) particles were incubated with *S. aureus* for 1 hr. In the control Ti sample, green staining of live cells (A) was more intense than the parallel red signal for dead cells (B). In contrast, the VAN-AEEA-AEEA-NHPrSiOTi beads displayed only scattered green staining for live cells (D) with the greatest red staining for dead cells (E). (C) and (F) show the corresponding bright field images of the particle distribution in each assay. The scale bar represents 100 μm.

For as few as 100 peptidoglycan precursors on a bacterium binding to 100 VAN on a Ti surface, K_D rises to 10^{-600} M, implying no detectable escape of any bound bacterium until the dead fragments dissociate one by one. In other words, simultaneous binding to thousands of solid phase VAN moieties has an effect equivalent to an enormous solution concentration of free VAN. This phenomenon potentiates the bactericidal action of VAN.

The most common VAN resistance mutation is a change of peptidoglycan target from D-Ala-D-Ala to D-Ala-D-Lac. The dissociation constant of VAN from the mutant peptidoglycan will increase to 10^{-3} M [50]. In that situation, we could extrapolate from the hypothetical example of 100 VAN binding to 100 mutant peptidoglycan precursor sites a K_D of 10^{-300} M, but this remains hypothetical in the absence of experiment. Thus, both because the surface will be exposed to relatively small numbers of microorganisms in vivo, and because VAN will show an increased potency because of its surface immobilization, occurrence of bacterial resistance should be extremely unlikely.

Implications for Management of Implant Infections

The covalently tethered antibiotic approach is uniquely suited to the eradication of a nascent infection and thus could reduce the risk of implant-associated infections. Bacterial infection in surrounding tissue and bone cannot be controlled entirely by an antibiotic covalently bonded to an implant surface, but might be managed by a secondary route of release of soluble antibiotic on demand. Moreover, this new paradigm could be easily extended to other agents and materials. Applications of such surfaces extend beyond orthopaedics. Indwelling devices tend to serve as ideal surfaces for establishment of infections. Thus, surface modifications that render devices antibacterial for the long term offer great promise for ameliorating the complications inherent in implant-associated infections and their treatment. Direct chemical modification of implant surfaces with bactericidal agents could provide a new generation of implants that are able to combat implant-associated infection and its devastating consequences.

Significance

Bacterial adhesion to implanted biomaterial surfaces is a critical step in the pathogenesis of infection. The formation of a complex biofilm at the interface between a biomaterial and the biological environment is thought to be responsible for sustaining and exacerbating inflammation and for the ultimate failure of biomedical devices. In this report, we have taken the first steps toward the goal of producing a bacteria-resistant implant. First, these studies demonstrate for the first time, to our knowledge, that an antibiotic can be covalently attached to a biocompatible implant material. The synthesis was validated by step-by-step analysis and by MALDI-TOF mass spectra of the final product. Second, the solid phase antibiotic not only retained the activity of the soluble antibiotic, but may improve on the latter activity by preventing biofilm formation at the site of the implant. Activity of the covalently bound antibiotic was shown at the biochemical level by specificity against the secreted peptidoglycan target of the antibiotic. The tethered antibiotic was also demonstrated to be active at the microbiological level by inhibition of bacterial proliferation and biofilm formation. Finally, the covalently bound antibiotic maintained its stability as demonstrated by activity after a repeat challenge, and by its ability to bind anti-VAN antibody after 3 weeks in culture medium at room temperature. The titanium surface was thus empowered to protect itself from colonization by bacteria. These experiments demonstrate the potential of covalent implant modification for infection control. Unlike a noncovalent coating that slowly releases a free antibiotic, Ti-bonded VAN was not lost from the surface during incubation with the bacteria. Thus, covalent binding can extend the microbiological activity of VAN in precisely the microenvironment in which it is most needed.

Experimental Procedures

Chemicals

All solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents for coupling reactions were purchased from Sigma-Aldrich (St. Louis, MO). Ti particles (350 mesh) were from Alfa Aesar (Ward Hill, MA).

Derivatization of Ti Surfaces with $NH_2PrSi(OEt)_3$

Ti beads (5–10 g of 350 mesh) were cleaned with MeOH/concentrated HCl (1:1, 5 ml) for 20 min at room temperature with 20 sec periods of sonication at 0, 5, 12, 15, and 20 min. The cleaned particles were washed twice with double-deionized water (5 ml), then four times with anhydrous Me_2NCHO (5 ml). Particles were dried overnight under vacuum and placed in the argon atmosphere chamber of an MO-20M glove box (Vacuum Atmospheres, Hawthorne, CA). Particles were washed twice with anhydrous toluene (10 ml) by suspension with a stainless steel spatula, then reacted with 5% $NH_2PrSi(OEt)_3$ in anhydrous toluene (v/v, 5 ml) for 60 min with resuspension every 10 min. The beads were washed twice with anhydrous toluene (10 ml) as above, washed twice with anhydrous Me_2NCHO (10 ml) as above, and dried under vacuum overnight. The beads were then baked at 100°C for 15 min in a convection oven. Aliquots of ≈100 mg were assayed for amine content by suspension in 0.35 mM $SnCl_2$, 0.1 M Na citrate (pH 5; 1 ml), followed by the addition of 4% ninhydrin in EtOH (w/v, 1 ml). After immersion in boiling water for 15 min., the solution was cooled for 2 min, diluted with EtOH/H_2O (3:2, v/v, 5 ml), and amines were quantitated by measuring the absorbance, A_{570}, using $\epsilon_{570} = 1.5 \times 10^4$/M·cm.

Solid Phase Coupling of VAN to the Ti Surface

$NH_2PrSiOTi$ beads (1–2 g, 10–20 μmol amine) were washed twice with anhydrous Me_2NCHO (5 ml), then coupled with a 4-fold molar excess of Fmoc-8-amino-3,6-dioxaoctanoate (aminoethoxyethoxyacetate; AEEA) linker, activated with a 4-fold molar excess of HATU in anhydrous Me_2NCHO (5 ml), followed by Fmoc deprotection with 20% piperidine in anhydrous Me_2NCHO (5 ml). The completeness of deprotection was determined by Kaiser's test. Similarly, a second AEEA linker group was subsequently coupled to the beads. All Fmoc deprotection solutions and washes were collected for analysis. Finally, a 4-fold excess of VAN was coupled by the same protocol. After the VAN coupling step, the Ti beads were washed twice with anhydrous Me_2NCHO (5 ml), and then dried under vacuum overnight. Absorbance spectra of the deprotection solutions were recorded and yields were calculated using A_{570} and $\epsilon_{301} = 7.78 \times 10^3$/M·cm.

Cleavage of Si-O Bond by Bu_4NF Reaction and MALDI-TOF MS

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) was used to characterize the molecular

species covalently attached to the Ti surface. Specifically, the VAN-AEEA-AEEA-NHPrSiOTi particles (≈100 mg) were suspended in Me_2NCHO (0.5 ml), to which was added 1 M Bu_4NF in tetrahydrofuran (4 ml) with stirring for 2 hr at room temperature to cleave the Si-O bonds. After filtering to remove Ti particles, the filtrate solution was analyzed by MALDI-TOF mass spectrometry.

VAN Antibody Binding

Ti and VAN-AEEA-AEEA-NHPrSiOTi particles were washed twice with PBS. The material was blocked for 30 min using 10% fetal bovine serum in distilled, deionized water (dH_2O, blocking buffer). The beads were incubated with mouse anti-VAN IgG (1:300; U.S. Biologicals, Swampscott, MA) in blocking buffer at 4°C for 12 hr, washed with PBS, and incubated with AlexaFluor 488-coupled donkey anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:300 in blocking buffer at room temperature for 1 hr. Both Ti and VAN-AEEA-AEEA-NHPrSiOTi particles were washed three times with PBS, followed by two 15 min incubations in PBS. The particles were then visualized using a Fluoview 300 confocal laser microscope (Olympus, Melville, NY). For long-term experiments, the beads were incubated in DMEM/10% fetal bovine serum for 3 weeks at room temperature, washed four times with PBS, followed by incubation with blocking buffer for 15 min and staining as above.

Soluble Peptidoglycan Binding Assay for VAN

To determine the biological activity of VAN covalently bound to the Ti particles, specific binding of VAN-AEEA-AEEA-NHPrSiOTi to the VAN ligand L-Lys-D-Ala-D-Ala present on soluble peptidoglycan was determined [41, 42]. Samples of Ti, NH_2PrSiOTi, and VAN-AEEA-AEEA-NHPrSiOTi beads (100 mg each) were washed by suspension in PBS (1 ml) in a polypropylene vial, with rocking for 1 hr at room temperature. The wash supernatant was removed, and the particles were incubated with 2.5 nCi of L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala soluble peptidoglycan, ≈10 Ci/mol, prepared as previously described [41, 42] for 2 hr with rocking. Unbound soluble peptidoglycans in the supernatants were collected, and quantitated by liquid scintillation counting in Aquasol II (PerkinElmer Life Sciences, Shelton, CT; 10 ml) in an LS 6000SC scintillation spectrometer (Beckman Instruments, Fullerton, CA). To control for nonspecific binding, the above beads were also analyzed using 0.1% (w/v) bovine serum albumin in TBST in the washing step with samples rocked for 4 hr at room temperature, followed by incubation with 2.5 nCi of L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala soluble peptidoglycan with rocking overnight at 4°C and quantitation as above.

Bacterial Assays

The control Ti and VAN-AEEA-AEEA-NHPrSiOTi particles were sterilized by incubation in 70% ethanol, followed by five PBS washes. The beads were resuspended in 1% dextrose/PBS, and 30 μl of the suspension, corresponding to ≈40 mg of particles, were dispensed into each well of an eight-well chamber slide (Nunc, Rochester, NY). *S. aureus* subspecies aureus Rosenbach ATCC strain 25923 was cultured in trypticase soy broth for 12 hr. The culture was pelleted by centrifugation at 3200 × g for 5 min, and resuspended in 1% dextrose/PBS. Using the McFarland standard, an early log-phase aliquot of *S. aureus* containing ≈1×10^4 cfu in 170 μl was inoculated in each well. The cultures were incubated at 37°C for 30, 60, 90, and 120 min under static conditions.

Quantitative culture was performed at 30, 60, 90, and 120 min by serially diluting 200 μl of sample in 1.8 ml PBS, then plating 200 μl of the relevant dilutions in triplicate on Todd-Hewitt agar plates. The number of cfu per ml was determined by counting the numbers of colonies on plates after 24 hr, determining the mean per sample, and adjusting for the dilution. Plates showing no growth after the initial 24 hr incubation were kept for an additional 24 hr to confirm the absence of growth.

For microscopy, the samples were washed twice with PBS to remove planktonic bacteria. Viability of bacteria was determined at 1 hr by confocal laser microscopy (Olympus Fluoview 300) after staining using the Live/Dead BacLight Viability kit (Molecular Probes) that functions based on the differential membrane permeability to Cyto9 and propidium iodide. After staining, upon excitation, live bacteria show an intense green fluorescence, while dead bacteria fluoresce red.

Acknowledgments

We thank Dr. Christopher Adams and Dr. Irving Shapiro for critical reading of the manuscript and valuable discussions. We thank Dr. Michele Marcolongo, Drexel University, for the gift of the Ti particles. A preliminary account of this work was presented at the American Academy of Orthopaedic Surgeons, 71st Annual Meeting, San Francisco, CA, March 10–14, 2004 [51]. This work was supported in part by DoD grant DAMD-17-03-1-0713.

Received: April 29, 2005
Revised: June 28, 2005
Accepted: June 30, 2005
Published: September 23, 2005

References

1. Garvin, K.L. (1994). Two-stage reimplantation of the infected hip. Semin. Arthroplasty *5*, 142–146.
2. An, Y.H., and Friedman, R.J. (1996). Prevention of sepsis in total joint arthroplasty. J. Hosp. Infect. *33*, 93–108.
3. Garvin, K.L., and Hanssen, A.D. (1995). Infection after total hip arthroplasty: past, present, and future. J. Bone Joint Surg. *77A*, 1576–1588.
4. Moss, A.J., Hamburger, S., Moore, R.M., Jr., Jeng, L.L., and Howie, L.J. (1991). Use of selected medical device implants in the United States, 1988. Adv. Data *77A*, 1–24.
5. Stewart, P.S., and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. Lancet *358*, 135–138.
6. Dunne, W.M., Jr. (2002). Bacterial adhesion: seen any good biofilms lately? Clin. Microbiol. Rev. *15*, 155–166.
7. Duggan, J.M., Georgiadis, G.M., and Kleshinski, J.F. (2001). Management of prosthetic joint infections. Infect. Med. *18*, 534–541.
8. Zimmerli, W., Trampuz, A., and Ochsner, P.E. (2004). Prosthetic-joint infections. N. Engl. J. Med. *351*, 1645–1654.
9. Haddad, F.S., Masri, B.A., Garbuz, D.S., and Duncan, C.P. (1999). The treatment of the infected hip replacement. The complex case. Clin. Orthop. *369*, 144–156.
10. Haddad, F.S., Masri, B.A., Campbell, D., McGraw, R.W., Beauchamp, C.P., and Duncan, C.P. (2000). The PROSTALAC functional spacer in two-stage revision for infected knee replacements. Prosthesis of antibiotic-loaded acrylic cement. J. Bone Joint Surg. Br. *82*, 807–812.
11. Loll, P.J., and Axelsen, P.H. (2000). The structural biology of molecular recognition by vancomycin. Annu. Rev. Biophys. Biomol. Struct. *29*, 265–289.
12. van de Belt, H., Neut, D., Schenk, W., van Horn, J.R., van der Mei, H.C., and Busscher, H.J. (2001). Infection of orthopedic implants and the use of antibiotic-loaded bone cements. A review. Acta Orthop. Scand. *72*, 557–571.
13. Frazier, D.D., Lathi, V.K., Gerhart, T.N., and Hayes, W.C. (1997). Ex vivo degradation of a poly(propylene glycol-fumarate) biodegradable particulate composite bone cement. J. Biomed. Mater. Res. *35*, 383–389.
14. Otsuka, M., Matsuda, Y., Suwa, Y., Fox, J.L., and Higuchi, W.I. (1994). A novel skeletal drug delivery system using a self-setting calcium phosphate cement. 5. Drug release behavior from a heterogeneous drug-loaded cement containing an anticancer drug. J. Pharm. Sci. *83*, 1565–1568.
15. Wang, C.J., Huang, T.W., Wang, J.W., and Chen, H.S. (2002). The often poor clinical outcome of infected total knee arthroplasty. J. Arthroplasty *17*, 608–614.
16. Wright, T.M., Sullivan, D.J., and Arnoczky, S.P. (1984). The effect of antibiotic additions on the fracture properties of bone cements. Acta Orthop. Scand. *55*, 414–418.
17. Lucke, M., Schmidmaier, G., Sadoni, S., Wildemann, B., Schiller, R., Haas, N.P., and Raschke, M. (2003). Gentamicin coating of metallic implants reduces implant-related osteomyelitis in rats. Bone *32*, 521–531.

18. Fulton, C.C., Lucovsky, G., and Nemanich, R.J. (2002). Electronic states at the interface of Ti-Si oxide on Si(100). J. Vac. Sci. Technol. B *20*, 1726–1731.
19. Mikulec, L.J., and Puleo, D.A. (1996). Use of p-nitrophenyl chloroformate chemistry to immobilize protein on orthopedic biomaterials. J. Biomed. Mater. Res. *32*, 203–208.
20. Puleo, D.A., Kissling, R.A., and Sheu, M.S. (2002). A technique to immobilize bioactive proteins, including bone morphogenetic protein-4 (BMP-4), on titanium alloy. Biomaterials *23*, 2079–2087.
21. Morra, M., Cassinelli, C., Cascardo, G., Cahalan, P., Cahalan, L., Fini, M., and Giardino, R. (2003). Surface engineering of titanium by collagen immobilization. Surface characterization and in vitro and in vivo studies. Biomaterials *24*, 4639–4654.
22. Kikuchi, L., Park, J.Y., Victor, C., and Davies, J.E. (2005). Platelet interactions with calcium-phosphate-coated surfaces. Biomaterials *26*, 5285–5295.
23. Wojcik, S.M., and Puleo, D.A. (1997). Biochemical surface modification of Ti-6Al-4V for the delivery of protein to the cell-biomaterial interface. Biomed. Sci. Instrum. *33*, 166–171.
24. Massia, S.P., and Hubbell, J.A. (1990). Covalent surface immobilization of Arg-Gly-Asp- and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates. Anal. Biochem. *187*, 292–301.
25. Dee, K.C., Rueger, D.C., Andersen, T.T., and Bizios, R. (1996). Conditions which promote mineralization at the bone-implant interface: a model in vitro study. Biomaterials *17*, 209–215.
26. Nanci, A., Wuest, J.D., Peru, L., Brunet, P., Sharma, V., Zalzal, S., and McKee, M.D. (1998). Chemical modification of titanium surfaces for covalent attachment of biological molecules. J. Biomed. Mater. Res. *40*, 324–335.
27. Puleo, D.A., and Nanci, A. (1999). Understanding and controlling the bone-implant interface. Biomaterials *20*, 2311–2321.
28. Ferris, D.M., Moodie, G.D., Dimond, P.M., Gioranni, C.W., Ehrlich, M.G., and Valentini, R.F. (1999). RGD-coated titanium implants stimulate increased bone formation in vivo. Biomaterials *20*, 2323–2331.
29. Rezania, A., and Healy, K.E. (2000). The effect of peptide surface density on mineralization of a matrix deposited by osteogenic cells. J. Biomed. Mater. Res. *52*, 595–600.
30. Cavalcant-Adam, E.A., Shapiro, I.M., Composto, R.J., Macarak, E.J., and Adams, C.S. (2002). RGD peptides immobilized on a mechanically deformable surface promote osteoblast differentiation. J. Bone Miner. Res. *17*, 2130–2140.
31. National Nosocomial Infections Surveillance System. (1999). National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1990-May 1999, issued June 1999. Am. J. Infect. Control *27*, 520–532.
32. de Lalla, F. (2001). Antibiotic prophylaxis in orthopedic prosthetic surgery. J Chemother *13 Spec No 1*, 48–53.
33. National Nosocomial Infections Surveillance System. (2004). National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. Am. J. Infect. Control *32*, 470–485.
34. Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. Nature *406*, 775–781.
35. Kahne, D., Leimkuhler, C., Lu, W., and Walsh, C. (2005). Glycopeptide and lipoglycopeptide antibiotics. Chem. Rev. *105*, 425–448.
36. Nicolaou, K.C., Hughes, R., Cho, S.Y., Winssinger, N., Labischinski, H., and Endermann, R. (2001). Synthesis and biological evaluation of vancomycin dimers with potent activity against vancomycin-resistant bacteria: target-accelerated combinatorial synthesis. Chemistry *7*, 3824–3843.
37. Sundram, U.M., and Griffin, J.H. (1995). General and efficient method for the solution- and solid-phase synthesis of vancomycin carboxamide derivatives. J. Org. Chem. *60*, 1102–1103.
38. Walsh, C.T., Fisher, S.L., Park, I.S., Prahalad, M., and Wu, Z. (1996). Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. Chem. Biol. *3*, 21–28.
39. Boger, D.L. (2001). Vancomycin, teicoplanin, and ramoplanin: synthetic and mechanistic studies. Med. Res. Rev. *21*, 356–381.
40. Pei, T., and Widenhoefer, R.A. (2001). Palladium-catalyzed asymmetric diene cyclization/hydrosilylation employing functionalized silanes and disiloxanes. J. Org. Chem. *66*, 7639–7645.
41. Zeiger, A.R., Eaton, S.M., and Mirelman, D. (1978). Antibodies against a synthetic peptidoglycan-precursor pentapeptide cross-react with at least two distinct populations of uncross-linked soluble peptidoglycan secreted by *Micrococcus luteus* cells. Eur. J. Biochem. *86*, 235–240.
42. Zeiger, A.R., Wong, W., Chatterjee, A.N., Young, F.E., and Tuazon, C.U. (1982). Evidence for the secretion of soluble peptidoglycans by clinical isolates of *Staphylococcus aureus*. Infect. Immun. *37*, 1112–1118.
43. Baddour, L.M., Bettmann, M.A., Bolger, A.F., Epstein, A.E., Ferrieri, P., Gerber, M.A., Gewitz, M.H., Jacobs, A.K., Levison, M.E., Newburger, J.W., et al. (2003). Nonvalvular cardiovascular device-related infections. Circulation *108*, 2015–2031.
44. Darley, E.S., and MacGowan, A.P. (2004). Antibiotic treatment of gram-positive bone and joint infections. J. Antimicrob. Chemother. *53*, 928–935.
45. Stickler, D.J., and McLean, R.J.C. (1995). Biomaterials associated infections: the scale of the problem. Cell. Mater. *5*, 167–182.
46. Francois, P., Vaudaux, P., Foster, T.J., and Lew, D.P. (1996). Host-bacteria interactions in foreign body infections. Infect. Control Hosp. Epidemiol. *17*, 514–520.
47. Neu, H.C., and Gootz, T.D. (1996). Bacterial Resistance (Galveston, TX: University of Texas Medical Branch Press).
48. Nieto, M., and Perkins, H.R. (1971). Physicochemical properties of vancomycin and iodovancomycin and their complexes with diacetyl-L-lysyl-D-alanyl-D-alanine. Biochem. J. *123*, 773–787.
49. Rao, J., Lahiri, J., Isaacs, L., Weis, R.M., and Whitesides, G.M. (1998). A trivalent system from vancomycin.D-Ala-D-Ala with higher affinity than avidin.biotin. Science *280*, 708–711.
50. Bugg, T.D., Wright, G.D., Dutka-Malen, S., Arthur, M., Courvalin, P., and Walsh, C.T. (1991). Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry *30*, 10408–10415.
51. Parvizi, J., Wickstrom, E., Zeiger, A.R., Adams, C.S., Shapiro, I.M., Purtill, J.J., Sharkey, P.F., Hozack, W.J., Rothman, R.H., and Hickok, N.J. (2004). Titanium surface with biologic activity against infection. Clin. Orthop. *429*, 33–38.