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# The inhibition of *Staphylococcus epidermidis* biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection

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## ABSTRACT

Peri-prosthetic infections are notoriously difficult to treat as the biomaterial implant is ideal for bacterial adhesion and biofilm formation, resulting in decreased antibiotic sensitivity. Previously, we reported that vancomycin covalently attached to a Ti alloy surface (Vanc-Ti) could prevent bacterial colonization. Herein we examine the effect of this Vanc-Ti surface on *Staphylococcus epidermidis*, a Gram-positive organism prevalent in orthopaedic infections. By direct colony counting and fluorescent visualization of live bacteria, *S. epidermidis* colonization was significantly inhibited on Vanc-Ti implants. In contrast, the gram-negative organism *Escherichia coli* readily colonized the Vanc-Ti rod, suggesting retention of antibiotic specificity. By histochemical and SEM analysis, Vanc-Ti prevented *S. epidermidis* biofilm formation, even in the presence of serum. Furthermore, when challenged multiple times with *S. epidermidis*, Vanc-Ti rods resisted bacterial colonization. Finally, when *S. epidermidis* was continuously cultured in the presence of Vanc-Ti, the bacteria maintained a Vanc sensitivity equivalent to the parent strain. These findings indicate that antibiotic derivatization of implants can result in a surface that can resist bacterial colonization. This technology holds great promise for the prevention and treatment of periprosthetic infections.

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## 1. Introduction

Implant surfaces serve as ideal substrates for bacterial colonization. This colonization often results in periprosthetic infection (PPI), a condition that leads to destruction of local tissues, patient disability and morbidity, and on occasion, death [1]. The implant surface accumulates serum proteins which promote bacterial adherence and colonization [2], and serves as a critical first step in the development of PPI. The adherent bacteria synthesize a complex glycocalyx that enmeshes the bacteria and provides

escape for them from immune surveillance or antibiotic treatment [3–7]. Currently, PPI treatment requires removal of the contaminated implant coupled with extensive bone debridement, excision of infected tissues and bone, and prolonged antimicrobial treatment [8].

Various antimicrobial materials have been developed to counter this implant-associated infection. The gold standard for targeted local delivery of antimicrobial agents is antibiotic-impregnated bone cement (polymethylmethacrylate – PMMA); other antibiotic-impregnated biodegradable carriers such as sol–gel coatings [9,10] and hydrogels [11,12] are under development. The use of antibiotic loaded PMMA allows delivery of high concentrations of antibiotics to the affected area without causing systemic toxicity. There are, however, numerous problems with the current local antibiotic delivery systems. First, most local antibiotic delivery systems are, by their nature, targeted to initiate treatment upon implantation,

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with levels of eluted drug remaining high for a well-defined period. Subsequently, the elution of antibiotics falls below the minimal inhibitory concentration for a longer period, a situation that could result in emergence of resistant organisms. Second, the drug delivery is ineffective in penetrating to the bacteria embedded within the biofilm on the implant surface. Finally, addition of many antibiotics to PMMA is not possible as the exothermic reaction caused by curing of the cement results in heat inactivation of the drug [13,14].

Attachment of bacteria to the implant surface appears to be the initial and the most critical step in development of PPI and hence, we and other investigators have invested extensive efforts to prevent bacterial adherence. We have previously reported that covalently bonding antibiotics to a metal implant surface can inhibit bacterial attachment. This antibiotic-derivatized surface is stable in the face of *Staphylococcus aureus* (*S. aureus*) challenges and prevents its colonization [15]. In this report, we examine the effect of this surface on a strain of *Staphylococcus epidermidis* (*S. epidermidis*, ATCC<sup>TM</sup> 155<sup>®</sup>), an organism that is prevalent in orthopaedic infections and notorious for forming biofilms. Furthermore, we evaluate the ability of this surface to prevent bacterial colonization in the presence of serum proteins. Results of the study indicate that derivatization of implant surfaces with antibiotics may provide a practical approach to inhibiting bacterial colonization of implants and thereby minimizing PPI.

## 2. Materials and methods

### 2.1. Experimental design

Colonization of the surface of vancomycin-modified Ti alloy (Ti90Al6V4) rods (Vanc-Ti) by *S. epidermidis* was evaluated. We chose Ti90Al6V4 for these studies as it is commonly used for manufacturing orthopedic implants. Surface colonization was measured by determining the numbers of *S. epidermidis* colony forming units (cfu) adherent to the rods and by treatment of the surface with a vital stain. In parallel, *S. epidermidis* colonization of the surface was visualized using scanning electron microscopy (SEM), currently the gold standard for biofilm detection. The specificity of inhibition was evaluated using the gram negative organism *E. coli*, an organism that is not sensitive to this antibiotic. Finally, we determined if there was emergence of resistant strains of *S. epidermidis* on the Vanc-Ti rods following multiple bacterial challenges.

### 2.2. Antibiotic modification of Ti alloy

One mm diameter Ti90Al6V4 wire (Ti alloy, Goodfellow, Cambridge, UK) was passivated with H<sub>2</sub>O<sub>2</sub>/H<sub>2</sub>SO<sub>4</sub> and aminopropylated under argon using 5% (v/v) aminopropyl-triethoxysilane in anhydrous toluene. It was coupled sequentially with two Fmoc-[2-(2-amino-ethoxy)-ethoxy]-acetic acid (AEEA) linkers and vancomycin in the presence of O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU); between each addition, deprotection was achieved with 20% piperidine in N,N-dimethylformamide [15–17].

### 2.3. Evaluation of surface colonization

Control and Vanc-Ti rods (5–10 mm × 1 mm) were weighed, sterilized with 70% EtOH for 15 min, and washed twice with BBL<sup>TM</sup> Trypticase<sup>TM</sup> Soy Broth (TSB, BD Bioscience). *S. epidermidis* ATCC<sup>TM</sup> 155<sup>®</sup> or *E. coli* DH5 $\alpha$ <sup>®</sup> was cultured in TSB, 250 rpm, 37 °C, 12–16 h (overnight culture) and diluted to 1 × 10<sup>4</sup> cfu/ml using a 0.5 McFarland standard, a turbidity standard which is equivalent to 10<sup>7</sup>–10<sup>8</sup> cfu of *S. aureus* (PML Microbiologicals, Wilsonville, OR). To evaluate surface colonization, 0.5 ml of the diluted culture was incubated with rods for 2, 5, 8, 12, or 30 h at 37 °C under static conditions. At each time period, 5 control and 5 Vanc-Ti rods were removed. Each rod was washed 7 times with phosphate-buffered saline (PBS), to remove non-adherent bacteria. Two rods were used to determine surface bacterial adhesion/viability and 3 were used for measurement of numbers of adherent bacteria. Additionally, 3 control and 3 Vanc-Ti rods per time point were processed for SEM.

### 2.4. Effect of serum on microbiocidal activity

Control and Vanc-Ti rods were incubated with complete fetal bovine serum (FBS, Atlanta Biochemicals) or dH<sub>2</sub>O statically at 37 °C for 24–48 h. Rods were removed and either fixed with 4% paraformaldehyde for 5 min and immunostained for fibronectin, or incubated with 1 × 10<sup>4</sup> cfu/ml *S. epidermidis* for 24 h and evaluated as above.

### 2.5. Determination of biofilm formation

Biofilms were stained with crystal violet using a modification of the method of O'Toole et al. [18]. Specifically, 0.5 ml ( $C_i = 10^4$  cfu/ml) of *S. epidermidis* cultures were statically incubated in 24 well plates containing control or Vanc-Ti rods for 2, 5, or 8 h at 37 °C. The medium containing non-adherent bacteria was carefully removed and Vanc-Ti and control rods moved to fresh wells. These rods were washed 6X with PBS and then stained, in a new well, with 1 ml of PBS containing 100  $\mu$ l of 1% (w:v) crystal violet (Sigma-Aldrich, St. Louis, MO) and incubated for 15 min at room temperature. The rods in the wells were washed 3 times with PBS and crystal violet then solubilized by addition of 2 ml of 95% EtOH, with rocking for 15 min; absorbance was measured ( $\lambda = 570$  nm) using a SPECTRAFluorPlus fluorimeter (Tecan, Research Triangle Park, NC).

### 2.6. Rod activity following repeated bacterial challenges

Control or Vanc-Ti rods that had been incubated with *S. epidermidis* in TSB for 24 h were cleaned by washing with 1% Triton X-100 and 10% sodium lauryl sulfate (Fisher Scientific) in dH<sub>2</sub>O for 24 h. They were then washed 5 times with PBS, sterilized with 70% EtOH for 15 min, and washed twice with TSB. Cleaned rods were incubated with 1 × 10<sup>4</sup> cfu/ml *S. epidermidis* in TSB at 37 °C for 24 h. Following washing 5 times with PBS, the rods were assayed for bacterial adhesion/viability (first re-challenge). Each re-challenge involved an additional round of washing and culturing with *S. epidermidis* as described above.

### 2.7. Evaluation of development of resistance

*S. epidermidis* was cultured ( $C_i = 10^4$  cfu/ml) in TSB and incubated with the Vanc-Ti rods for up to 8 weeks, with TSB replaced every 3–4 days. Weekly, the rods were removed from the medium, sonicated in TSB to suspend adherent bacteria, and 200  $\mu$ l plated on agar plates. These colonies were assessed by the Thomas Jefferson University Clinical Microbiology Labs for resistance using growth on vancomycin plates (6  $\mu$ g/ml) as well as the Kirby-Bauer disk diffusion assay.

### 2.8. Quantitative measure of adherent bacteria

Adherent bacteria were detached by sonication for 10 min in 1 ml TSB, followed by vortexing for 2 min, using a modification of the method of Barth et al. [19]. Total numbers of bacteria were determined by serial dilutions on TSB agar plates; the plates were incubated at 37 °C for 24 h, and colonies counted. For detection of slow growing colonies, those plates exhibiting no growth after 24 h were incubated for an additional 24 h. Numbers of released bacteria were calculated, based on dilution, and expressed as a function of rod weight.

### 2.9. Fluorescent staining of adherent bacteria

To remove non-adherent bacteria, rods were washed 7 times with PBS. These were stained using the Live/Dead<sup>®</sup> BacLight<sup>TM</sup> Viability Kit (Invitrogen) for 15 min to fluorescently label viable bacteria, followed by three PBS washes to remove nonspecific stain. Fluorescent, adherent bacteria were visualized by confocal laser microscopy (Olympus Fluoview 300).

### 2.10. Immunohistological detection of vancomycin and fibronectin

Rods were washed twice with PBS and incubated with a primary antibody against vancomycin (mouse anti-vancomycin IgG, 1:500, US Biologicals) in PBS 4 °C, 2 h. For visualizing fibronectin, after incubation in PBS, rods were fixed with 4% paraformaldehyde, incubated with rabbit anti-bovine fibronectin IgG (1:500, Invitrogen) in PBS, 4 °C, 2 h. They were then washed 3 times with PBS, incubated in PBS for 30 min, and then treated with AlexaFluor 488-coupled donkey anti-rabbit or anti-mouse IgG (1:500, Invitrogen) in PBS, 1 h, followed by 5 PBS washes and incubation in PBS for 30 min. Stain was visualized by confocal laser microscopy (Olympus Fluoview 300).

### 2.11. Scanning electron microscopy

Vanc-Ti rods were incubated with *S. epidermidis* for 24 h in TSB. Samples were washed extensively up to 6 times in PBS and fixed with 2.5% glutaraldehyde in 0.1 mol/L piperazine-1,4-bis(2-ethanesulfonic acid) buffer (pH 7.4) for 5 min. Following fixation, the samples were stained with 1% OsO<sub>4</sub> in 0.1 mol/L piperazine-1,4-bis(2-ethanesulfonic acid) buffer (pH 6.8) for 1 h and then re-stained with 2% uranyl acetate for 1 h. The rods were dehydrated in ethanol and sputter-coated with gold. Surfaces were visualized using an FEI Strata<sup>TM</sup> DB235 focused ion beam SEM (FEI Co, Hillsboro, OR).

### 2.12. Statistics

Experiments were performed a minimum of 3 independent times using at least 3 randomly chosen samples for each repeat. To establish significance, a one-tailed Student's *t* test was used (\**p* < 0.05). The results were tested by the Kolmogorov-Smirnov normality tests.

### 3. Results

#### 3.1. *S. epidermidis* colonization of Vanc-Ti

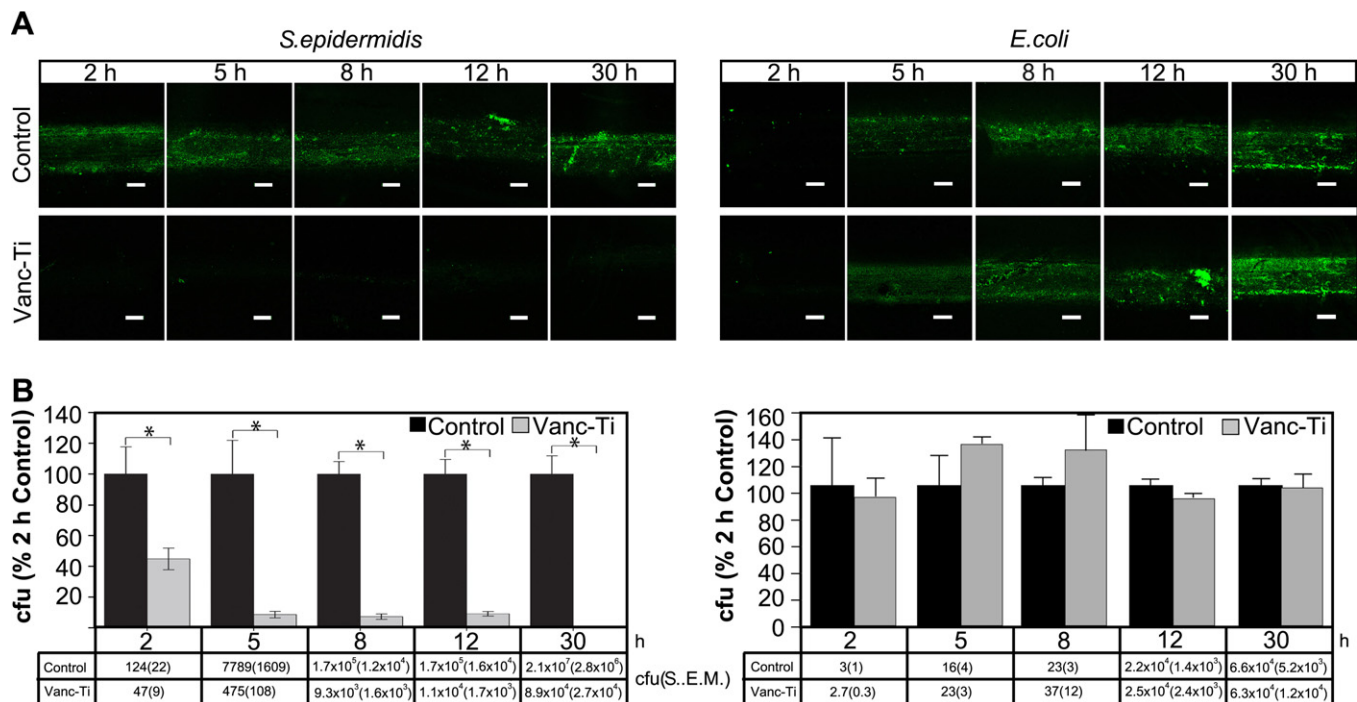
We first determined if the Vanc-Ti surface resists bacterial colonization over a 30 h time course (Fig. 1). To visualize bacterial colonization, we used the Live/Dead Assay in which viable bacteria stain with the membrane permeable Syto9 dye (green) but exclude propidium iodide (red); non-viable bacteria show both green and red staining. After incubation with *S. epidermidis* ATCC™ 155®, the control surface evidences abundant fluorescence, indicating extensive bacterial colonization (Fig. 1A left, top panels). At 8, 12 and 30 h, the intense fluorescence indicates the presence of bacterial foci, characteristic of biofilm formation. In contrast, when the Vanc-Ti rods are incubated with *S. epidermidis*, the fluorescence is very low at 8, 12 and 30 h (bottom panels), indicating that Vanc-Ti rods resisted bacterial colonization. Only the green channel was imaged in these and subsequent pictures as dead cells did not remain adherent to the surfaces. We also examined the specificity of the treated surfaces. Vancomycin is active against Gram-positive, but not Gram-negative organisms. We therefore challenged the Vanc-Ti rod with a Gram negative organism, *E. coli* DH5α®. *E. coli* colonization is abundant on both the control and Vanc-Ti rods and colonization appeared to increase over time (Fig. 1A, right, top and bottom panels, respectively).

The number of *S. epidermidis* adherent to control Ti or Vanc-Ti rods were then quantified by direct plating. Bacterial numbers (colony forming units, cfu) are significantly ( $p < 0.05$ ) lower on the

Vanc-Ti rod than on the control rod at 2 (40% control), 5 (6.2% of control), 8 (5.5% of control), 12 (6.5% of control), and 30 (0.4% of control) h (Fig. 1B, left). With *E. coli*, a Gram negative organism that is not susceptible to the Gram-positive-specific vancomycin, there were increased colony counts for both the control and Vanc-Ti rods with time, with no significant differences in numbers of colonies (Fig. 1B, right). *S. epidermidis* colonization is inhibited by Vanc-Ti where *E. coli* colonization readily occurred, indicating that the antibacterial properties of the surface duplicate the specificity of vancomycin, i.e., gram positive bacteria are susceptible whereas gram negative bacteria are not. Thus, whether evaluated directly using the live/dead fluorescence assay or by counting adherent bacteria, the results of this first study indicate that the Vanc-Ti rods specifically reduce bacterial adherence of gram positive organisms.

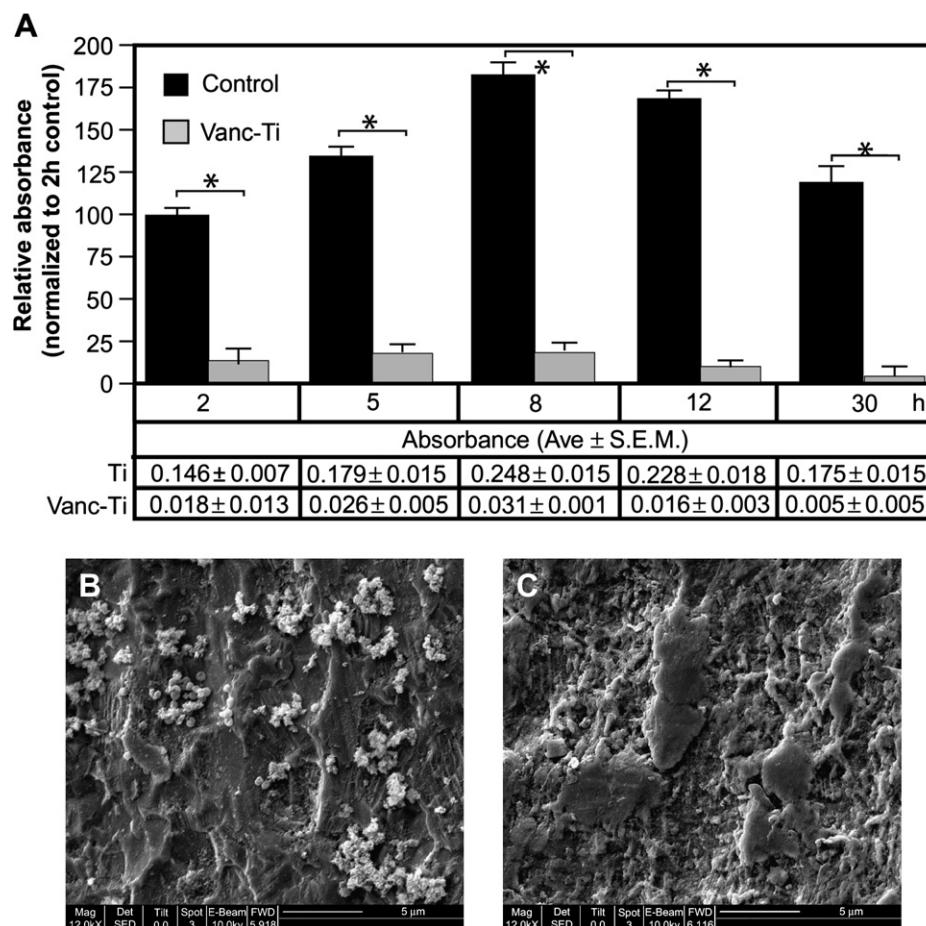
#### 3.2. Biofilm formation on Vanc-Ti

The results described above suggested that Vanc-Ti, at the least, inhibited *S. epidermidis* adhesion. To learn if the antibiotic prevented biofilm formation, the rods were stained with crystal violet (Fig. 2A). Predictably, the stain on the control rods increases with time. These data suggested that the *S. epidermidis* adherent to the control rod have started to secrete a matrix; the Vanc-Ti rods show little evidence of matrix formation. Between 2 and 5 h and between 5 and 8 h, significant increases in crystal violet staining is evident on the control rods. This elevated level of crystal violet incorporation is maintained up to 12 h; after this time, there is a decline, presumably due to normal bacterial death in the now-established



**Fig. 1.** Bacterial colonization of Vanc-Ti surfaces. Control and Vanc-Ti rods were challenged for 30 h with *S. epidermidis*, a gram positive organism that falls within the spectrum of activity of vancomycin, or *E. coli*, a gram negative organism that is not sensitive to vancomycin and hence tests whether the derivatized Vanc-Ti shows the same activity as the parent Vanc. Viable, adherent bacteria were fluorescently stained with the Live/Dead reagent and live cells imaged. (A). *S. epidermidis*: Control surfaces show abundant bacterial colonization, with extensive fluorescence across the surface at all time points. At early time points, the fluorescence is diffuse; compact areas of intense fluorescence are seen after 8 h. In contrast, the vancomycin-modified surface (Vanc-Ti) shows minimal fluorescence at all time intervals, indicating inhibition of bacterial colonization and biofilm formation in keeping with the known activities of Vanc. *E. coli*: On both the Vanc-Ti and the control surface, accumulation of adherent *E. coli* initiated after ~8 h, which gradually increased during the subsequent 30 h, suggesting that the Vanc-Ti surface retained the selectivity of Vanc against Gram positive, but not Gram negative organisms. Magnification: bar = 200 μm. (B). Adherent bacteria were removed from the rods by sonication and plated for cell counts. *S. aureus*: When normalized to the counts from the control rod for each time point, Vanc-Ti rods shows greatly decreased colonization with significant differences at all time points. By 30 h, Vanc-Ti counts are only 0.4% of control compared to 2 h where they were 40% of control. *E. coli*: There were no significant differences in the numbers of bacteria retrieved from the control and the Vanc-Ti rods, with large numbers of adherent bacteria only apparent after 8 h. The actual counts (cfu ± S.E.M.) are shown below each bar of the histogram (\* Significantly different from control,  $p < 0.05$ ). For each test, the Vanc-Ti rod inhibited *S. epidermidis* but not *E. coli* colonization, suggesting that the Vanc-Ti activity was due to the presence of Vanc. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]





**Fig. 2.** Biofilm formation on Vanc-Ti surfaces. (A) Absorption of crystal violet was used as an indicator of biofilm formation by *S. epidermidis*. The relative absorption of the crystal violet value was normalized to the 2 h control. Control surfaces consistently showed significantly more crystal violet incorporation than the Vanc-Ti surfaces. At 8 and 12 h, crystal violet incorporation achieved maximum values on the control surfaces, while the Vanc-Ti surface showed no change in staining intensity with time. The absorbance values of the crystal violet are shown below each bar of the histogram. The absorbance values on the Vanc-Ti rods were near baseline values, indicating no biofilm formation. (\*  $p < 0.05$ ). SEM analysis: Twenty-four hours after a challenge with *S. epidermidis*, Vanc-Ti and control rods were imaged by SEM. Both surfaces were passivated in order to achieve similar levels of surface activation. Control surfaces show extensive colonization by round staphylococci encased in a matrix that suggests biofilm formation (B). In contrast, the Vanc-Ti surface is devoid of such colonization, with a few single bacterial-like particles observed (C). Magnification: bar = 5 µm.

biofilm [3–7]. At all time points, however, crystal violet staining of the Vanc-Ti rods is significantly lower than the control rods. In fact, at the end of the 30 h incubation period, crystal violet incorporation on the Vanc-Ti surface approaches zero, indicating that the Vanc-Ti surface significantly prevents biofilm formation.

SEM was used to further examine biofilm formation on Vanc-Ti rods that had been incubated with *S. epidermidis* for 24 h (Fig. 2B, C). The surface displayed an overall roughness due to the presence of small pits; some smoothening is seen when the vancomycin is covalently attached to the Ti alloy. After colonization with *S. epidermidis*, the control surface displays multiple small, spherical bacteria (diameter  $\approx 0.3\text{--}1\text{ }\mu\text{m}$ ) of a size consistent with the *Staphylococci* and that conglomerate in grapelike clusters that are characteristic of *Staphylococci* (Fig. 2B). Importantly, the clustering and the appearance of multiple, small protrusions on the bacteria are consistent with initiation of slime/glycocalyx formation. In contrast to the control surface, the Vanc-Ti surface (Fig. 2C) displays only isolated individual bacteria; the absence of clustering lends strength to the notion that biofilm is not formed on the Vanc-Ti surface.

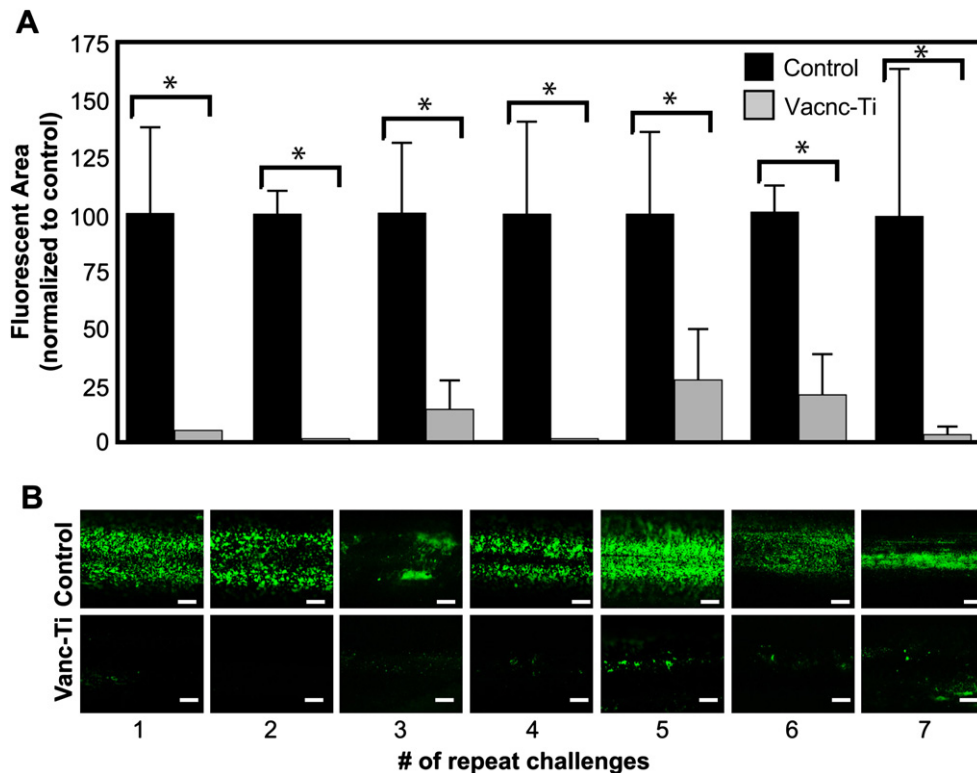
### 3.3. Multiple *S. epidermidis* challenges of Vanc-Ti

Since an implant must maintain anti-bacterial activity in the face of multiple bacterial challenges, we evaluated colonization

following seven separate cycles of a 24 h exposure to *S. epidermidis* (Fig. 3). After each cycle of cleaning and re-exposure to bacteria, the control rods continue to exhibit high live/dead fluorescence as evidenced by measurement of fluorescent area (Fig. 3A), with the direct observation of adherent bacteria shown in Fig. 3B. When the rod fluorescence was quantified by densitometry (Fig. 3A, controls set to 100%), throughout the first 4 cycles of repeat challenges, Vanc-Ti rods retain their ability to resist colonization. After 5 cycles, both the control and Vanc-Ti rods show a low level of variability in the extent of colonization. By observation, the control rods showed abundant fluorescence, indicative of abundant colonization (Fig. 3B, top panel). In contrast, the Vanc-Ti rods show a low level of fluorescence and hence minimal colonization (Fig. 3B, bottom panel). The results support the contention that the Vanc-Ti rods retain their activity in the face of multiple challenge exceptionally large initial numbers ( $>10^7$  cfu) of *S. epidermidis*.

### 3.4. Vanc-Ti activity after serum coverage

We next determined if the antibiotic would remain effective after incubation with serum proteins (Fig. 4). Incubation in FBS causes deposition of fibronectin on the control and Vanc-Ti rods; fibronectin staining is absent from rods that had not been exposed to serum (Fig. 4A, left). When the rods were probed for vancomycin coverage using immunofluorescence, abundant fluorescence is



**Fig. 3.** Antibacterial activity of Vanc-Ti rods following multiple challenges. Control and Vanc-Ti surfaces were incubated with bacteria for 24 h, stripped by detergent lysis, imaged, and re-incubated with bacteria for another 24 h; the cycle was repeated 7 times and bacteria were visualized with the Live/Dead stain. (A) Abundant bacteria (green) colonized the control surfaces. In contrast, the Vanc-Ti showed minimum bacterial staining, indicative of significant inhibition of bacterial colonization after each cycle. Magnification: bar = 200  $\mu$ m. (B) The fluorescent area of the rods was measured and normalized to the control area for each challenge. Subsequent to the first 3 independent challenges, the surfaces show some variability in their response to further cycles of challenge. Values shown are the average  $\pm$  S.E. of 3 independent experiments (\*Significantly different from control,  $p < 0.05$ ). [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

observed over the surface of the Vanc-Ti rods exposed to FBS; control rods show no detectable fluorescence (Fig. 4A, right). Consequently, extensive coating with serum protein does not mask the ability of the covalently-tethered vancomycin to bind antibody. To learn if serum proteins affect the antibacterial activity of the surfaces, the rods were challenged with *S. epidermidis* ( $C_i = 10^4$  cfu/ml) for 24 h. Abundant bacterial colonization is observed on the control rods, regardless of pre-incubation in dH<sub>2</sub>O or FBS (Fig. 4B). Importantly, Vanc-Ti rods show minimum bacterial colonization, independent of surface serum absorption.

### 3.5. Bacterial susceptibility after Vanc-Ti incubation

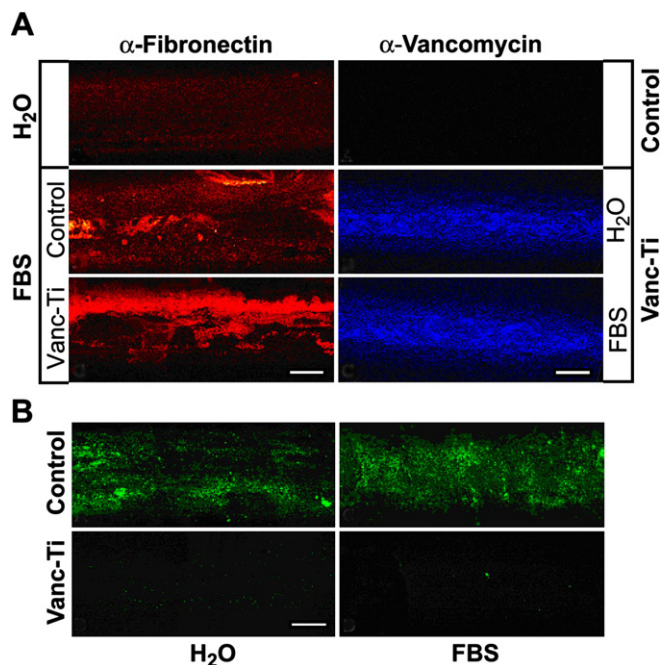
Finally, we determined if continuous exposure of *S. epidermidis* to the Vanc-Ti rods, *in vitro*, would foster emergence of resistant bacteria. After 8 weeks of continuous exposure to control or Vanc-Ti surfaces, both planktonic and adherent bacteria were assessed for vancomycin sensitivity. Bacteria incubated under these conditions fail to grow (24–64 h assessment) on bacterial plates containing 6  $\mu$ g/ml vancomycin, indicating that MIC for vancomycin for these bacteria is below 6  $\mu$ g/ml. These bacterial samples were then further characterized using the Kirby-Bauer disk diffusion assay [20,21]. Strains derived from control rods exhibit a zone of inhibition of  $17.69 \pm 0.26$  mm, whereas those derived from Vanc-Ti rods showed a zone of  $17.59 \pm 0.42$  mm. Importantly, no stray colonies grew in the cleared zones.

## 4. Discussion

Implant-associated, periprosthetic infections pose immense challenges to the medical community and imparts a huge economic

burden on society. The ability of microorganisms to colonize implant surfaces and form biofilms is the critical step in the initiation of PPI. Once encased in a biofilm, bacteria become recalcitrant to immune surveillance and antibiotic therapy is of limited value. Herein, we address this problem by reporting that modifications of the Ti alloy surface with covalently tethered vancomycin is protective against both bacterial adhesion and biofilm formation by *S. epidermidis*. This organism together with *S. aureus* account for over 90% of implant-associated infections, and because of the avidity with which *S. epidermidis* forms biofilms, it is especially difficult to eradicate [22–25]. We show that *S. epidermidis* readily colonizes Ti alloy rods and forms a biofilm. When these rods are covalently derivatized with vancomycin, colonization is inhibited and biofilm formation is minimized. Remarkably, inhibition is maintained in the face of multiple challenges with bacteria in numbers that far exceed those encountered *in vivo*, and adsorbed serum proteins do not alter the activity of the tethered antibiotic. Together these *in vitro* studies suggest that we have prepared a novel surface that exhibits sustained activity against a major pathogen and that activity is maintained without fostering emergence of resistant bacterial strains.

We monitored biocidal activities of the Vanc-Ti surface using a number of different techniques: *S. epidermidis* viability, adhesion, biofilm formation, SEM and microbiological counting. The use of crystal violet, an agent that is retained in the secreted glycocalyx and reacts with peptidoglycans and teichoic acids, provided useful insight into biofilm formation. The observation that there was an elevation in the crystal violet retention with time indicated that *S. epidermidis* was not merely adhering to the Ti surface but synthesizing a protective biofilm. The fact that crystal violet absorption did not appreciably increase after 12 h reflected early



**Fig. 4.** Antimicrobial activity of the Vanc-Ti surface in the presence of serum. (A) *Fibronectin adsorption and vancomycin fluorescence.* (Left) Control or Vanc-Ti rods were incubated with FBS for 24 h and fibronectin adsorption detected by immunofluorescence analysis. Both surfaces showed abundant fibronectin (red stain), compared to the rods incubated in H<sub>2</sub>O. (Right) Following incubation with serum proteins, rods were incubated with an antibody against vancomycin and visualized by immunofluorescence. Despite protein adsorption, vancomycin fluorescence (blue stain) was clearly detectable on the Vanc-Ti rods, with no staining on control surfaces. (B) *Antimicrobial activity.* Vanc-Ti rods that had been treated with serum proteins were challenged with *S. epidermidis* for 24 h and live, adherent bacteria stained with the Live/Dead kit (green). Fluorescence detected from the serum-treated Vanc-Ti rods was very low and similar to the fluorescence detected from the H<sub>2</sub>O-incubated Vanc-Ti rods. In both cases, this fluorescence was much less intense than the fluorescent yield generated by bacteria that had colonized the control rods. Magnification: bar = 200  $\mu$ m.

proliferative activities of the adherent bacteria on the control surface and probably activation of quorum sensing proteins to form the stable biofilm [26–28]. A second important indicator of biofilm formation was microbiological counting. While the latter technique provided a quantitative estimate of the number of viable bacteria, its use was dependent on the strength of adhesion (a variable quantity) to the implant surface and the ability to completely transfer and adequately sample the transported bacteria. Despite these drawbacks, the bacterial number was significantly lower on the Vanc-Ti surface than the controls at all times sampled. Noteworthy, perusal of the actual data indicates that there was considerable variation in bacterial counts, especially at long time intervals. Much of this variation was due to the high bacterial number utilized for the study. Furthermore, the adherent bacteria on the control surfaces tended to be associated with biofilms which would decrease the efficiency of suspension. Any adherent bacteria on the Vanc-Ti surfaces tended to be in small colonies with limited biofilm formation, allowing a more efficient suspension of these bacteria. Therefore, comparisons of the recovered bacteria from control vs. Vanc-Ti surfaces tended to underestimate differences. Nevertheless the very large differences in bacterial numbers between the tethered vancomycin and the control rods lend strong direct support to the contention that the antibiotic retained its biocidal activities and inhibited biofilm formation, two major events in PPI development.

Cognizant of the fact that PPI probably results from one or more episodes of exposure to blood-borne organisms, we evaluated the impact of multiple challenges with *S. epidermidis*. Using numbers of organisms that were many orders of magnitude greater than might

be expected *in vivo*, we found that the Vanc-Ti surface was biocidal for four or possibly more challenges with *S. epidermidis*. The fact that the surface remained active over the 24 h of the challenge and that re-challenges resulted in continued activity raises interesting questions about interactions of the geometrically-constrained antibiotic (i.e., surface-tethered) with the bacterial membrane. As is the case for the antibiotic in solution, we would hypothesize that vancomycin was bound reversibly to the Lys-D-Ala-D-Ala peptidoglycan that cross linked the bacterial cell wall. After bacteriolysis occurred, the reversible vancomycin–peptidoglycan interaction would result in release of any bacterial remnants, leaving the surface-bound vancomycin free to engage other bacteria. While it is also likely that some vancomycin was degraded, there were, based on the data, sufficient numbers of antibiotic molecules tethered to the Ti surface to maintain a high level of activity as the rod was assailed with successive rounds of bacterial challenges. Probably, both the high local concentration of attached antibiotic molecules together with the reversible nature of the drug–bacteria interaction would together serve to maintain the activity of the nano-engineered Ti surface.

It could be argued that while the Vanc-Ti surface inhibited colonization of *S. epidermidis*, a Gram positive organism, inhibition was due to alterations in surface charge rather than the presence of a tethered antibiotic. Indeed, when the surfaces were examined by SEM, some differences in the topography were seen, mainly reflecting a small change in overall roughness. This change could promote bacterial adhesion. To exclude this possibility, we evaluated the effect of the antibiotic on a Gram negative organism, *E. coli*. We noted that unlike the studies with *S. epidermidis*, the Vanc-Ti surface had minimal effect on *E. coli* adhesion and bacterial counts. Thus the Vanc-Ti surface supported *E. coli* adherence, whereas *S. epidermidis* adherence was inhibited. The data strongly suggested that the tethered vancomycin acts as an antibiotic against a specific class of organisms and its biocidal properties did not merely reflect changes in surface structure.

An important final test of the utility of the methodology was the demonstration that the Vanc-Ti rods did not foster the emergence of resistant *S. epidermidis* even after prolonged exposures. Using the conventional Kirby–Bauer diffusion assay with the CLSI vancomycin standards[30], our strain of *S. epidermidis* (ATCC<sup>TM</sup> 155<sup>®</sup>) exhibited a zone of inhibition of ~17.6 mm; this was unchanged after 4 weeks of exposure to the Vanc-Ti rods. Furthermore, the absence of stray colonies in the zones of inhibition indicated that we could detect no subsets of insensitive or resistant bacteria. Thus, unlike controlled release systems where decreasing levels of antibiotic can provide a selective growth advantage, the tethered vancomycin was stably present at high surface concentrations [29] and therefore does not confer such an advantage.

## 5. Conclusions

We have shown that vancomycin covalently tethered to a Ti alloy surface prevents bacterial colonization and biofilm formation. Moreover, the tethered antibiotic is stable, maintains its activity even when challenged multiple times with bacteria and does not foster resistance. Since *S. epidermidis* infection is closely linked to the pathogenesis of PPI, the Vanc-Ti system holds promise of controlling or even preventing this devastating orthopaedic condition.

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