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Accessibility
Titanium-Tethered Vancomycin Prevents Resistance to Rifampicin in *Staphylococcus aureus in vitro*

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**Abstract**

Rifampicin is currently recognized as the most potent drug against Gram positive implant related infections. The use of rifampicin is limited by the emergence of bacterial resistance, which is often managed by coadministration of a second antibiotic. The purpose of this study was to determine the effectiveness of soluble rifampicin in combination with vancomycin tethered to titanium metal as a means to control bacterial growth and resistance in vitro. Bacterial growth was inhibited when the vancomycin-tethered titanium discs were treated with *Staphylococcus aureus* inocula of ≤2 × 10⁶ CFU, however inocula greater than 2 × 10⁶ CFU/disc adhered and survived. The combination of surface-tethered vancomycin with soluble rifampicin enhanced the inhibitory effect of rifampicin for an inoculum of 10⁷ CFU/cm² by one dilution (combination MIC of 0.008 mg/L versus 0.015 mg/L for rifampicin alone). Moreover, surface tethered vancomycin prevented the emergence of a rifampicin resistant population in an inoculum of 2 × 10⁶ CFU.

**Introduction**

Infection is a persistent complication with a number of implanted devices. In total joint replacement (TJR), implant related infections are debilitating, costly and difficult to treat [1]. Patients with infected implants require prolonged hospitalization and consume a disproportionate amount of healthcare resources [1]. Improved operative techniques and short-term administration of peri-operative antibiotics have proven successful in reducing the overall incidence of prosthetic joint infection (PJI) (1.6% post-op [1]). Improved operative techniques and short-term administration of peri-operative antibiotics have proven successful in reducing the overall incidence of prosthetic joint infection (PJI) (1.6% post-op [1]).

For TJRs performed without cement, titanium alloys are often the preferred implant materials because of their biocompatibility and elastic modulus [9]. The inherent biocompatibility of titanium is attributed to a heterogeneous layer rich in oxygen that spontaneously forms on the titanium surface. Strategies have been developed to tether various molecules of biological interest such as antibiotics to the titanium surface. Antibiotics such as ampicillin [10], daptomycin [11] and vancomycin [12–16] maintain bioactivity when covalently linked (tethered) to a solid surface. Several techniques have been described for the covalent linkage to a titanium oxide surface, where the organic-metal connection is made through silane [17], phosphate [18], phosphonate [19] or catechol [20] linkers. One strategy involving vancomycin is the covalent bonding of the antibiotic to a titanium alloy surface through a short polyethylene (PEG) tether using a silane linker [14].

Vancomycin inhibits cell wall biosynthesis by binding to terminal D-alanyl-D-alanine residues of NAG/NAM peptides, preventing their cross-linking [21]. Surface bound vancomycin can prevent biofilm formation on titanium surfaces in *vitro*, and in *vivo* [22]. The tethering of vancomycin to the titanium surface is an advantageous method of antibiotic prophylaxis since it precludes exposure of the recipient’s flora to antimicrobial agents and the need for intravenous delivery. Since vancomycin is active against 80% of microorganisms causing PJIs [23] it is also a relevant choice for tethering to implant surfaces for the treatment of infection.

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The coadministration of vancomycin and rifampicin is the mainstay in the treatment of methicillin-resistant Staphylococcus aureus implant infection [24] and this combination is often used for treatment durations exceeding six to twelve weeks. Rifampicin is a drug of choice since it has excellent bioavailability, tissue diffusion, can be administered orally once daily, and remains active on biofilms [25]. Rifampicin is commonly coadministered with other antibiotics since the emergence of resistant bacteria is a common problem [26]. While vancomycin is an effective antibiotic for the treatment of methicillin-resistant Staphylococcus aureus, it has a poor tissue diffusion profile and can only be administered intravenously with required serum level monitoring. To ensure adequate bioavailability, administration of vancomycin often precedes rifampicin and delays the beneficial effects of rifampicin therapy.

To overcome these issues, the combination of implant-tethered vancomycin with an oral regimen of rifampicin may be a practical method to achieve an optimal antimicrobial environment immediately following the implantation of a cementless implant for revision surgery. In a clinical setting, intravenous vancomycin could be coadministered to guard against resistance from rifampicin monotherapy outside the joint space. The purpose of this study was to determine if the combination of surface-tethered vancomycin and soluble rifampicin would prevent the emergence of resistance to rifampicin in S. aureus in vitro.

Materials and Methods

Bacterial strain and culture

All microbial studies were conducted using Staphylococcus aureus ATCC strain 25923. Lyophilized stock was subcultured on Tryptic Soy agar plates with 5% sheep blood (BD Diagnostics, Sparks, MD) and a 10 ml preculture performed in Tryptic Soy Broth (TSB) (DIFCO, BD Diagnostics). TSB (1 L) was seeded with the ATCC strain 25923. Lyophilized stock was subcultured on Tryptic Soy agar plates with 5% sheep blood (BD Diagnostics, Sparks, MD) and a 10 ml preculture performed in Tryptic Soy Broth (TSB) (DIFCO, BD Diagnostics). TSB (1 L) was seeded with the preculture and grown to an optical density of 0.5 at 600 nm as measured on a Nanodrop 2000c spectrophotometer (Thermo) and then cooled on ice. The culture was pelleted and the pellet washed with PBS (pH 7.2). Following a second centrifugation the pellet was resuspended in PBS with 10% glycerol and 1 ml aliquots frozen and stored at –80°C. Thawed aliquots were titrated by plating serial dilutions on agar medium.

Preparation of Titanium Discs

Grade 5-ELI titanium alloy (TiAl6V4) discs (21 mm diameter × 1 mm thickness) were fabricated in-house from rod stock. All other materials were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Using standard metallurgical techniques, the titanium discs were polished until they had a mirror finish (S<sub>r</sub> ~0.02 μm). After polishing, discs were cleaned for 30 minutes at 80°C in a 2% solution of Liquinox (White Plains, NY) in an ultrasonic bath (Crest Ultrasonics, Tinton NJ). Following cleaning, discs were passivated by treatment in 50% nitric acid at 30°C with sonication for 1 h and then washed with deionized H<sub>2</sub>O and dried in a 100°C oven. Discs were placed in self-sealing sterilization pouches (Defend, Hauppauge NY) and steam sterilized at 135°C for 20 minutes. At this point the sterilized discs were split into 2 groups, control and experimental.

Tethering of vancomycin to titanium discs

The experimental titanium discs were identical to control discs in every respect except that vancomycin was tethered to the disc surface following the procedure of Jose et al. [14]. Briefly, the tethering of vancomycin to the titanium surface was achieved as follows: experimental discs were submerged in a sealed glass chamber containing a solution of aminopropyltriethoxysilane (1.2 ml, 3 mmol) in anhydrous toluene (50 ml) and heated to 120°C for 3 h. The discs were cooled, washed sequentially with ethyl acetate, ethanol and deionized H<sub>2</sub>O, and dried under vacuum. A short polyethylene glycol (PEG) based tether was then linked to the exposed amino groups by treating the discs with 2-(Fmoc-amino)ethoxy)ethoxycetic acid (0.3 g, 0.8 mmol), COMU (0.3 g, 0.8 mmol) and r-Pr<sub>2</sub>NNEt (0.3 ml, 1.6 mmol) in dimethylformamide (40 ml) for 1 h at 22°C. After washing (dimethylformamide), the fmo group was removed by treatment with 25% piperidine in dimethylformamide (50 ml), and the discs were washed again (dimethylformamide and 2-propanol). The 2-(Fmoc-amino)ethoxy)ethoxycetic acid coupling and deprotection protocol was repeated to double the length of the PEG linker. The antibiotic was then covalently linked by treating the discs with vancomycin hydrochloride (Hospira, Inc., Lake Forest, IL) (0.4 g, 0.27 mmol), COMU (0.12 g, 0.3 mmol) and r-Pr<sub>2</sub>NNEt (0.4 ml, 2.2 mmol) in dimethylformamide (50 ml) for 16 h at 22°C. The antibiotic-coupled discs were then washed (sequentially with dimethylformamide, 2-propanol and deionized H<sub>2</sub>O) and dried and stored under vacuum at ambient temperature until ready for use.

Competitive Fluorescent Linked ImmunoSorbent Assay (FLISA)

Control and experimental discs were placed in 12-well plates (BD Falcon), blocked with 1% BSA in PBS (3 h at 37°C) and washed with 0.05% PBS-Tween 20 (3×15 min under orbital agitation). Solutions of competing soluble vancomycin (0.25 ml, 0–25 mg/well) were added to each well followed by 0.25 ml of 1:5,000 polyclonal rabbit anti-vancomycin antibody (Pierce Thermo). After 45 min at 37°C, each well was washed (3×0.05% PBS-Tween 20), treated with 0.5 ml 1:5000 Alexa 488-labeled goat anti-rabbit antibody (Invitrogen) and incubated in the dark at 37°C for 45 min. The discs were washed (3×0.05% PBS-Tween 20), and transferred to new plates with PBS (1 ml) in each well. Fluorescence (excitation 488 nm, emission 525 nm) was read on a Biotek H1 synergy plate reader (Biotek, Winnooski, VT, USA). All assays were performed in triplicate.

Resazurin biofilm assay and measurement of surface antimicrobial activity

Control and experimental discs were placed in 12-well plates, containing Mueller Hinton broth (MHB) (1 ml) and seeded with ten-fold serial dilutions of thawed stock bacteria (2×10<sup>6</sup> to 2×10<sup>10</sup> CFU/well). The plates were centrifuged (10 min at 1000 G) and incubated at 36°C for 18 h. The discs were washed gently to remove untethered bacteria (3×PBS) and transferred to a fresh plate. Fresh MHB supplemented with 200 μM resazurin (Sigma) (resazurin MHB) was added and incubated for 1 h. The fluorescence of 100 μl aliquots of the resazurin MHB was then measured (excitation 506 nm, emission 594 nm) to assess the reduction of resazurin into the fluorescent resorufin compound, a measure of metabolic activity of the disc-associated biofilm [27,28]. All assays were performed in triplicate.

Synergy assay

A classic checkerboard synergy assay was performed according to CLSI recommendations in resazurin MHB with an inoculum of 3×10<sup>4</sup> CFU/well under a final volume of 200 μl. The synergy between vancomycin tethered to titanium and soluble rifampicin was evaluated by inoculating control and experimental discs with 5×10<sup>6</sup> CFU/well, then incubating with two-fold serial dilutions...
of rifampicin in resazurin MHB (2 ml/well). The synergy was
determined by comparing the lowest rifampicin concentration
capable of inhibiting growth on vancomycin treated surface to
the lowest concentration capable of inhibiting growth on a control
titanium surface. Inhibition of growth was determined by
colorimetric evaluation of the lack of resazurin reduction.

Confocal laser scanning microscopy of control and
experimental discs
Bacterial inocula suspended in MHB were added to control or
experimental discs placed in 12 well plates and centrifuged
10 minutes at 1000 G. Discs were gently washed three times in
PBS and stained with a live/dead BacLight bacterial viability kit
(Invitrogen) according to the manufacturer’s recommendations,
fixed in 4% paraformaldehyde containing PBS and imaged using
an upright Zeiss LSM 710 confocal microscope. Live bacteria
were stained with Syto9 (green) and dead bacteria stained with
both Syto9 and propidium iodide (yellow).

Statistical analysis
The significance of quantitative data was analyzed using
Student’s t test and a p value <0.05 was considered to be
significant.

Results
Vancomycin tethered to the titanium surface can be
quantified by a competitive Fluorescent-Linked
ImmunoSorbent Assay
The amount of vancomycin tethered to the titanium surface was
determined using a competitive Fluorescent Linked ImmunoSor-
gent Assay. (Figure 1) Anti-vancomycin antibody was mixed with
varying amounts of soluble drug and then allowed to equilibrate
with the surface-tethered vancomycin. After washing and specif-
ically detecting vancomycin with a fluorescent secondary antibody,
the amount of vancomycin tethered to the disc surface was
established by determining the concentration of soluble competitor
that reduced the measured fluorescence by 50%. From this result
it was determined that 0.20 mg ±0.06 of tethered vancomycin
(average from a duplicate assay of 3 batches) was attached per disk
face which also corresponded to 0.14 nmol or 8.3x10^13
vancomycin molecules per disc face or one vancomycin molecule
per 3.8 nm^2.

The antimicrobial effect of vancomycin tethered to
the titanium surface was inoculum dependent
Experimental discs inoculated with increasing amounts of S.
aureus were incubated overnight and the survival of metabolically
active bacteria adhering to the disc surface was measured using
resazurin reduction after thorough washing. Figure 2 shows resoru-
fin fluorescence according to the initial bacterial inoculum
used to seed the well. The distinction between measured
fluorescence on vancomycin-bound discs and control titanium
disks was large (>5-fold) for inocula of 2x10^3, 2x10^4 and 2x10^5
CFU/disc (respective p values of 0.024, 0.047 and 0.022).
However, at the highest inoculation of 2x10^6 CFU/disc,
fluorescence readings on the experimental discs approached levels
of the control disks (1.5 fold ratio, p = 0.1), indicating that bacteria
survived on the antibiotic tethered surface.

The antimicrobial activity of soluble rifampicin was
additive to vancomycin tethered to titanium discs
A checkerboard experiment performed on control discs showed
that S. aureus ATCC 25923 had MICs of 0.5 mg/L to vancomycin
and 0.015 mg/L to rifampicin using the standard 5x10^4 CFU
inoculum defined by CLSI. However, when applied in combina-
tion, sub-inhibitory concentrations of 0.25 mg/L of vancomycin
and 0.008 mg/L of rifampicin were sufficient to inhibit growth,
showing additivity of the two antibiotics. The possibility of
synergism between vancomycin tethered to the titanium surface
and soluble rifampicin was evaluated by determining the
concentration of rifampicin capable of inhibiting the growth of
S. aureus on experimental discs from an inoculum (5x10^6 CFU)
high enough to overload the intrinsic antibacterial properties
of the vancomycin-tethered surface (Figure 2). A sub-inhibitory
concentration of 0.008 mg/L of rifampicin was found to be
sufficient to inhibit the growth on the experimental discs, whereas
0.015 mg/L rifampicin was required to inhibit the growth of
5x10^6 CFU on control discs. Importantly, the MIC of rifampicin
was not modified by the 100-fold increase of inoculum. These
results show that like soluble vancomycin, titanium tethered
vancomycin was additive with soluble rifampicin.

Vancomycin tethered to titanium prevented the
emergence of resistance to rifampicin
The ability of surface-tethered vancomycin to prevent the
emergence of resistance to rifampicin was evaluated by measuring
the metabolic activity of adherent bacteria from increasingly dense
inoculations of S. aureus in the presence of 0.03 mg/L rifampicin
(Figure 3A). Control discs inoculated with 2x10^6 and 2x10^7 CFU
were sterile, as expected from their susceptibility to rifampicin.
However at an inoculum 2.10^6 CFU, the vancomycin-tethered discs did not significantly differ from controls. Vancomycin tethered or titanium was inoculum dependent. The surfaces of the discs inoculated with 2.10^8 CFU were observed by confocal laser scanning microscopy (CLSM) after live/dead staining (Figure 3B). The vancomycin-tethered discs were not significantly different from controls.

However, control discs inoculated with 2.10^6 CFU were colonized with live bacteria that were determined to be rifampicin resistant with an MIC >4 mg/L. In contrast, vancomycin-tethered discs and the overlying medium were sterile when inoculated with 2.10^6 CFU.

The antibacterial activity of vancomycin tethered to titanium has been previously demonstrated [32], however the mechanism of action is not completely understood. Soluble vancomycin inhibits the cross-linking of D-Ala-D-Ala interpeptidic bridges in the peptidoglycan all around the bacterial cell wall whereas vancomycin tethered to titanium has limited cellular contact. The quantity of tethered vancomycin required to inhibit the activity of S. aureus is not understood either. In this study, the coverage of one vancomycin molecule per 3.8 nm^2 is consistent with the reported aminopropylsilanation of 0.22 nmol/cm^2 or 1 peptide per 0.8 nm^2 by Spencer et al. [17]. Approximating the surface area of S. aureus as 1 nm, and that the titanium-tethered vancomycin has a limited reach of <5 nm from the metal surface, the bacterial wall area exposed to antibiotic is less than 1.5 x 10^3 nm^2, or 0.023% of the 6.5 x 10^7 nm^2 surface area (assuming a spherical cell and a compressible organic layer at the bacterium-implant interface). Furthermore, a coverage of one vancomycin molecule per 3.8 nm^2 would allow <4000 molecules to interact with the cell wall of a given bacterium. The results of this study suggest that in vitro, 4000 vancomycin molecules or one molecule per 3.8 nm^2 provide a sustained antibacterial effect.

This study determined that soluble rifampicin worked in conjunction with surface-tethered vancomycin in vitro. The results serve as an important proof of concept for more sophisticated experiments which could address pharmacokinetic questions. While promising, the effectiveness of this technique remains to be evaluated in vivo where stability of the tethered antibiotic may be an important issue. Silanization of the oxidized titanium surface with aminopropytriethoxysilane is a versatile method for covalently attaching organic molecules to oxides. The resulting surface has been raised about its stability under physiological conditions [19].

vancomycin has a limited reach of 5 nm from the metal surface, the bacterial wall area exposed to antibiotic is less than 1.5 x 10^3 nm^2, or 0.023% of the 6.5 x 10^7 nm^2 surface area (assuming a spherical cell and a compressible organic layer at the bacterium-implant interface). Furthermore, a coverage of one vancomycin molecule per 3.8 nm^2 would allow <4000 molecules to interact with the cell wall of a given bacterium. The results of this study suggest that in vitro, 4000 vancomycin molecules or one molecule per 3.8 nm^2 provide a sustained antibacterial effect.

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of the control discs inoculated with 2 × 10^6CFU prevented biofilm formation when inoculated with the same inoculum. Microscopy. Control discs inoculated with lower inocula were free of live cells, however, _S. aureus_ grew in wells inoculated with 2 × 10^8 CFU. A Resazurin assay: biofilm-forming bacteria reduce resazurin to resorufin on control discs inoculated with 2 × 10^8 CFU/well, whereas vancomycin tethered discs prevented biofilm formation when inoculated with the same inoculum. B Confocal laser scanning microscopy shows that the surface of the control discs inoculated with 2 × 10^8 CFU/well are colonized with a mixture of live (green, labeled by Syto9) and dead (yellow, co-labeled by Syto9 and propidium iodide) bacteria.

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**Figure 3. Vancomycin tethered titanium discs prevented the emergence of resistance to rifampicin.** Vancomycin tethered and control discs were seeded with 2 × 10^6, 2 × 10^7 or 2 × 10^8 CFU in presence of a suprainhibitory concentration of rifampicin (0.03 mg/L). Following centrifugation and incubation for 18 h, adhering bacteria were detected by resazurin assay and the discs were imaged by confocal laser scanning microscopy. Control discs inoculated with lower inocula were free of live cells, however, _S. aureus_ grew in wells inoculated with 2 × 10^8 CFU.

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**In vivo** studies of implants coated with silane-linked antibiotic [22] have demonstrated the potential for this chemistry to be sufficiently stable for use in medical devices. Furthermore, the general strategy employed in our current study could readily be adapted if necessary to potentially more robust phosphate-based linking chemistries [19]. Another area for optimization would be to increase the access of the antibiotic to the bacterium. A flexible linker connecting vancomycin to the aminopropylsilane anchor was used to allow the vancomycin molecule to adopt an effective position. Longer linkers may be advantageous by allowing a greater number of antibiotic-bacteria interactions (vide supra), but may suffer from lower yields in the chemical coupling reactions. In this study, a previously validated aminoxyethoxysuccinic acid dimer [14], which has a length <5 nm, was employed, but alternative linkers are under current investigation.

The use of implant-tethered vancomycin may provide a promising adjunct for the immediate postoperative use of rifampicin in the treatment of periprosthetic joint infections. The results of this study suggest that further validation of this approach is warranted. For example, the impact of the vancomycin tethering surface treatments on osseointegration should be determined, and evaluation in an animal model of PJI would provide additional, valuable information. While this approach has been developed to facilitate single stage revision for PJI, it could be applied to any titanium implant vulnerable to infection, from external pin fixation to dental implants or endovascular stents.

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**Author Contributions**

Conceived and designed the experiments: MR JG SAH. Performed the experiments: MR JG. Analyzed the data: MR JG SAH. Contributed reagents/materials/analysis tools: SAH. Wrote the paper: MR JG SAH.

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