Titanium-Tethered Vancomycin Prevents Resistance to Rifampicin in Staphylococcus Aureus in Vitro

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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 10⁶ 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 (PJIs) [1]. However, PJIs persist and are notoriously difficult to treat with systemic administration of antibiotics.

The management of infected implants often necessitates a highly invasive treatment plan [3,4] such as the two stage revision procedure [5]. The first stage removes the infected implant and surrounding tissue which is followed by six to twelve weeks of antibiotic treatment. After antibiotic treatment, a second procedure is performed to reconstruct the joint with new implants [6]. A two stage procedure can be a long and costly process. One strategy to potentially improve patient outcome and reduce the burden of care, is a single stage revision [3,7]. In a single stage procedure, the infected implant is removed, the wound is debridged and cleaned and a new prosthesis is inserted, all during the same surgical setting. Such single stage procedures were initially performed with antibiotic-releasing bone cement but are now also performed using cementless implants. Single stage procedures have been shown to reduce the cost of PJI revision [6], however concerns about the efficacy of treatment and the potential for the recurrence of infection persist.

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.
Tethering of vancomycin to titanium discs

Preparation of Titanium Discs

Materials and Methods

follows: experimental discs were submerged in a sealed glass
techniques, the titanium discs were polished until they had
other materials were purchased from Sigma Aldrich (St. Louis,
MO) unless otherwise noted. Using standard metallurgical
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
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 (Sa~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, Trenton NJ).
Following cleaning, discs were passivated by treatment in 50%
nitric acid at 30°C with sonication for 1 h and then washed with
denitized H2O and dried in a 100°C oven. Discs were placed in
self-scaling 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 H2O, 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-[2-
(fmoc-amino)ethoxy]ethoxycetic acid (0.3 g, 0.9 mmol), COMU
(0.34 g, 0.8 mmol) and i-Pr2NEt (0.4 ml, 2.2 mmol) in dimethyl-
formamide (50 ml) for 1 h at 22°C. After washing (dimethylfor-
mamide), the fmoc group was removed by treatment with 25%
pyridine in dimethylformamide (50 ml), and the discs were
washed again (dimethylformamide and 2-propanol). The 2-[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 i-Pr2NEt (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 H2O) 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 480 nm, emission 525 nm) was
read on a Biotek H1 synergy plate reader (Biotek, Winooski, 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 (2×10^5 to 2×10^8
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 unattatched 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 560 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
3×10^8 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
3×10^8 CFU/well, then incubating with two-fold serial dilutions

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
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
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Titanium-Tethered Vancomycin
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 ImmunoSorbent 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 specifically detecting vancomycin with a fluorescent secondary antibody, the amount of vancomycin tethered to the disc surface was determined using a competitive Fluorescent Linked ImmunoSorbent Assay. Anti-vancomycin antibody was mixed with 5-fold dilutions of soluble vancomycin and then incubated with the surface-tethered vancomycin. After washing and detecting with a fluorescent secondary antibody, the amount of vancomycin bound to the disc surface was calculated to be 0.20 mg [±/-0.06] (average from a duplicate assay of 3 batches [±/- standard deviation]) or 0.14 nmol. This corresponds to 8.3×10¹⁵ vancomycin molecules per disc face, or one vancomycin molecule per 3.8 nm².

The antimicrobial effect of vancomycin tethered to titanium 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 resorufin fluorescence according to the initial bacterial inoculum used to seed the well. The distinction between measured fluorescence on vancomycin-bound discs and control titanium discs was large (>5-fold) for inocula of 2×10⁴, 2×10⁵ and 2×10⁶ CFU/disc (respective p values of 0.024, 0.047 and 0.022). However, at the highest inoculation of 2×10⁷ 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.
However, control discs inoculated with $2 \times 10^8$ 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 $2 \times 10^8$ CFU.

The surfaces of the discs inoculated with $2 \times 10^8$ CFU were
observed by confocal laser scanning microscopy (CLSM) after
live/dead staining (Figure 3B). The vancomycin-tethered discs
were free of adhering bacteria (data not shown) whereas the
control discs were covered with a nascent biofilm populated with
a mixed population of live as well as dead bacteria incapable of
excluding propidium iodide. In the absence of the tethered
vancomycin, the rifampicin-resistant mutants were capable of
adhering to the surface and colonizing the medium and the disc
surface.

**Discussion**

Implant infection is a significant source of patient morbidity and
is predicted to impact future healthcare costs [29]. Antibiotic
resistance is persistent concern and revision surgery remains
a highly invasive procedure. There will always be a risk of infection
at the revision site since tissue spared to enable reconstruction may
harbor an inoculum. As a result, the development of new
approaches to treat implant related infections is of great interest.
In this study, it was demonstrated that vancomycin tethered
to titanium surfaces was effective for inhibiting the growth of *S. aureus*
and preventing the development of resistance to rifampicin *in vitro*.

Vancomycin was selected for this study because it is used
clinically to treat periprosthetic joint infections in combination
with rifampicin [24]. Tethering vancomycin directly to the
implant surface solves the problem of its poor tissue distribution
relative to rifampicin, since it is present at an effective level *ab initio*.
Vancomycin is suitable for tethering because of its stability, its
amenability to covalent binding [15] and its mode of action.
Because vancomycin acts on the outer bacterial wall it can remain
tethered to the implant surface while interacting with biofilm
forming micro-organisms.

The antibacterial activity of vancomycin tethered to titanium
surfaces 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 aminopropylsilanization of 0.22 nmol/cm$^2$ or 1
peptide per 0.8 nm$^2$ by Spencer et al. [17]. Approximating the
diameter of *S. aureus* as 1 µm, 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
1.5x10$^4$ nm$^2$, or 0.023% of the 6.5x10$^5$ 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 aminopropyltriethoxysilane is a versatile method for co-
valently attaching organic molecules to oxides. The resulting
terminal amino group is amenable to further functionalization
using standard solid-phase organic synthesis techniques. Although
this silane-based chemistry is stable *in vitro* [33], concerns have
been raised about its stability under physiological conditions [19].
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 phosphonate-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 aminooxyethoxyacetic 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.

References

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. 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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