Bactericidal activity of extended 9-glycyl-amido-minocyclines

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Abstract—The need for self-protecting polymer or alloy implants resistant to a broad spectrum of bacterial challenges led us to investigate covalent bonding of minocycline (MIN), a tetracycline derivative, to polystyrene beads and to titanium alloy foils by oligoethylene glycol spacers. 9-Hydrazino-acetyl-amido-MIN, and simpler glycylcycline derivatives, retained minimum inhibitory concentration (MIC) against *Staphylococcus aureus* comparable to MIN. However, PEG-glycyl-amido-MIN showed very low activity. Hence, we coupled 9-hydrazino-acetyl-amido-MIN to the aldehyde termini of oligoethylene glycol spacers bonded to polystyrene and titanium alloy surfaces to form acid-releasable hydrazone linkages. 9-Hydrazino-acetyl-amido-MIN was released from the monolayers more rapidly at pH 5.0 than at pH 7.4.

Infections are a devastating complication of medical implant devices that cause significant morbidity. The management of infected implants has serious economic implications. For example, in the United States, treatment of 3500–4000 cases of knee and hip infections every year costs an estimated 150–200 million dollars.[1] Because of poor vascularity and biofilm formation at the implant sites, such infections have proven difficult to prevent or treat. The local application of antibiotics is a well-known and accepted therapy for such kind of implications.[2–4] Current practice includes antibiotic-impregnated bone cement[5] and antibiotic-containing coating systems.[6–8] These methods are intended to release antibiotics rapidly and irreversibly, and require a carrier material other than the orthopaedic implant itself to transport the antibiotic materials. Some non-resorbable materials used in the methods even need to be removed after the surgery.[9]

Because of the combination of their many biocompatible properties, titanium and titanium alloys are frequently applied in a wide variety of biomedical devices.[10] In our initial studies[11] using fine titanium (Ti) particles which had been aminopropylated,[12,13] we utilized the last resort glycopeptide antibiotic vancomycin (VAN), which acts at the surface of the Gram-positive bacterial cell wall to block peptidoglycan synthesis.[14,15] Hydrophilic bis(ethylene glycol) spacers were coupled to the solid phase NH_2PrSiO–Ti particles, followed by VAN.[11] The biological results showed that the VAN-modified Ti particles effectively inhibited the growth of *S. aureus* compared with control Ti particles on which the bacteria grew abundantly.[11]

The strategy was then extended to Ti6Al4V alloy pins, widely used for orthopaedic implants.[16] We found that VAN could be covalently linked to the Ti6Al4V alloy surface on orthopaedic implants, following oxidation.[17] Covalently bound VAN was retained on the surface for an extended period of time and retained significant antibiotic activity.[18]

Most implant infections are caused by Gram-positive bacteria,[19] but some are caused by Gram-negative bacteria, which are susceptible to broad-spectrum antibacterial agents such as tetracyclines. Bacterial resistance to tetracyclines has curtailed their clinical effectiveness. A minimum of semisynthesis and analoging has been carried out with tetracyclines, compared to other classes of antibiotics, perhaps due to their complex chemistry, chemical liability and lack of reactivity of the intact naphthacene nucleus.

Glycylcyclines, such as tigecycline, recently approved by the FDA,[20] are active against a broad range of Gram-positive and Gram-negative bacterial strains resistant to tetracycline or doxycycline. Tigecycline, however,

lacks functional groups suitable for connecting to a solid support. Minocycline (MIN), on the other hand, allows exclusive 9-nitration, the starting point for glycylcycline extension.

MIN is effective against a broad range of Gram-positive and Gram-negative bacteria,[21] displaying an MIC of 0.12 μg/mL against *S. aureus*.[22] Against *E. faecalis* and *E. faecium*, by comparison, MIN was found ~8-fold more active than linezolid and 32-fold more than quinu-pristin–dalfopristin.[23] Bulky groups at the 9-position of glycylcyclines usually contribute to activity,[24] supporting our strategy of coupling moieties to the 9-position of MIN to create new glycylcyclines. We therefore chose MIN as the basis for glycylcycline tethering to solid surfaces.

Here we report the design and synthesis of several MIN C9 derivatives and the determination of their minimum inhibitory concentration (MIC). Attachment of the MIN derivatives to polystyrene beads and Ti6Al4V alloy foils via acid-labile linkers was carried out to characterize the bioactivity of the tethered MIN derivatives.

We synthesized [4S-(4α,12aα)]-9-H$_2$N-4,7-bis(Me$_2$N)-1,4,4a,5,5a,6,11,12a-octohydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide (9-H$_2$N-MIN) as described.[22] Briefly, to an ice-cold solution of MIN·HCl (1 g, 2.03 mmol) in 9 mL of concentrated H$_2$SO$_4$ was added KNO$_3$ (224 mg, 2.4 mmol). The reaction mixture was stirred at 0 °C for about 1.5 h and monitored by HPLC on a 150 × 4.6 mm Phenomenex$^{\circledR}$ Luna phenyl–hexyl 5 μm column, eluted with 25% CH$_3$CN in 35 mM Na$_2$HPO$_4$, pH 7.5, on a Waters 600 liquid chromatograph (Milford, MA, USA). When the reaction came to completion, the mixture was slowly poured into 240 mL of ice-cold Et$_2$O. The precipitate was collected by filtration and washed with Et$_2$O three times. The solid was dried overnight under vacuum to give 9-O$_2$N-MIN·(SO$_4$)$_2$. The product was characterized by HPLC, showing a prominent product peak, and MS of the main peak (SELDI-TOF mass spectrometer Ciphergen Fremont, CA), calcd 502.47 Da, found 502.5 Da. The dried 9-O$_2$N-MIN·(SO$_4$)$_2$ (1.29 g, 1.8 mmol), 200 mg of 10% palladium on charcoal and 4.5 mL of 2 N H$_2$SO$_4$ in 6 mL of 2-MeOEtOH were mixed and hydrogenated in a Parr apparatus at 40 psi for 1.5 h. The catalyst was filtered and the filtrate was added dropwise to a mixture of 2-PrOH (210 mL) and Et$_2$O (150 mL). The yellow solid was collected by filtration, washed several times with Et$_2$O and dried under vacuum at room temperature overnight to yield 1.16 g of 9-H$_2$N-MIN·(SO$_4$)$_2$ (90% yield). The product was characterized by HPLC and MS: calcd 472.2 Da, found 472.0 Da.

We then converted 9-H$_2$N-MIN into [4S-(4α,12aα)]-9-[(HNAc)NH]-4,7-bis(Me$_2$N)-1,4,4a,5,5a,6,11,12a-octohydro-3,10,12,12a–tetrahydroxy-1,11-dioxo-2-naphtha-cenecarboxamide (9-Gly-NH-MIN). To a solution of Boc-Gly (458 mg, 1.2 mmol) in 3 mL Me$_2$NCHO was added 412 mg of 2-(7-*aza*-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexa-fluorophosphate (HATU) (Applied Biosystems, Foster City, CA, USA)

(1.1 equiv) and 311 μL of *i*Pr$_2$EtN. The reaction mixture was stirred at room temperature for 30 min, then mixed with 9-H$_2$N-MIN·(SO$_4$)$_2$ (575 mg, 0.95 mmol) in 4 mL Me$_2$NCHO. After 1 h at room temperature, the product was precipitated by addition of two volumes of ice-cold Et$_2$O. After sedimentation and removal of supernatant, the precipitate was dissolved in 4 N HCl/dioxane and incubated for 10 min at room temperature to remove the Boc protecting group. The final product, 9-Gly-H$_2$N-MIN·(cl)$_3$, was precipitated twice from Et$_2$O. MS: calcd 529.54 Da, found 530.1 Da.

Next, we prepared [4S-(4α,12aα)]-4,7-Bis(Me$_2$N)-9-[(HNAc)NH]-4,7-bis(Me$_2$N)-1,4,4a,5,5a,6,11, 12a-octohydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-2-naphtha-cenecarboxamide (9-hydrazino-Ac-H$_2$N-MIN) (Scheme 1). To a solution of *N,N,N′*-Boc$_3$-hydrazinoacetic acid (369 mg, 0.95 mmol) in 3 mL Me$_2$NCHO were added 397 mg of HATU (1.1 equiv) and 264 μl of *i*Pr$_2$EtN. The reaction mixture was stirred at room temperature for 30 min, then mixed with 9-H$_2$N-MIN·(cl)$_3$ (600 mg, 0.9 mmol) in 4 mL Me$_2$NCHO and 1 g of Na$_2$CO$_3$, and stirred for 1 h at room temperature. It was found that the yellow solution changed to brown. The solid was removed by filtration and the filtrate was applied to a 150 × 15 mm C$_{18}$ column (WAT 020594, Waters Associates). The column was washed with H$_2$O to remove Me$_2$NCHO. Because of the strong hydrophobicity of 9-Boc$_3$-hydrazino-Ac-NH-MIN, the impurities in the reaction mixture could be eluted from the C$_{18}$ column by 20% CH$_3$CN in aqueous 0.1% CF$_3$CO$_2$H. The product was eluted with pure CH$_3$CN, concentrated by rotatory evaporation and lyophilized overnight. The dried sample was dissolved in 4 N HCl in dioxane to deprotect the Boc groups. After 10 min, a precipitate was formed of shaking at room temperature. The precipitate was collected by filtration and washed with Et$_2$O three times. The solid product was dried under vacuum to give 510 mg of 9-hydrazino-Ac-H$_2$N-MIN·(Cl)$_2$ as a yellow powder (92% yield). The product was characterized by HPLC and MS: calcd 544.56 Da, found 544.6 Da.

To PEGylate 9-Gly-NH-MIN, we added 1 equiv of HATU and 1.2 equiv of *i*Pr$_2$EtN to 1 mL Me$_2$NCHO containing 100 mg of *O*-(N-Fmoc-2-aminoethyl)-*O*′-(2-carboxyethyl)-undecaethyleneglycol (PEG$_{12}$carboxylate) (Merck, Darmstadt, Germany), stirred at room temperature for 30 min, was added 1.1 equiv of 9-Gly-NH-MIN. The reaction mixture was kept at room temperature for 2 h under stirring. The reaction was stopped by addition of H$_2$O. Taking the molecular mass (1351 Da) of 9-PEG$_{12}$-Gly-NH-MIN into consideration, we employed a Sephadex G-25 column (1.7 × 30 cm) eluted with aqueous 0.5% HOAc to separate the conjugate from the impurities. The eluent corresponding to the first peak was collected, concentrated and freeze-dried. The dried sample was redissolved in H$_2$O, then characterized by HPLC and MS: calcd 1351.49 Da, found 1351.6 Da. 9-PEG$_{26}$-Gly-NH-MIN was prepared similarly. The product was characterized by HPLC and MS: calcd 2056.33 Da, found 2056.0 Da.
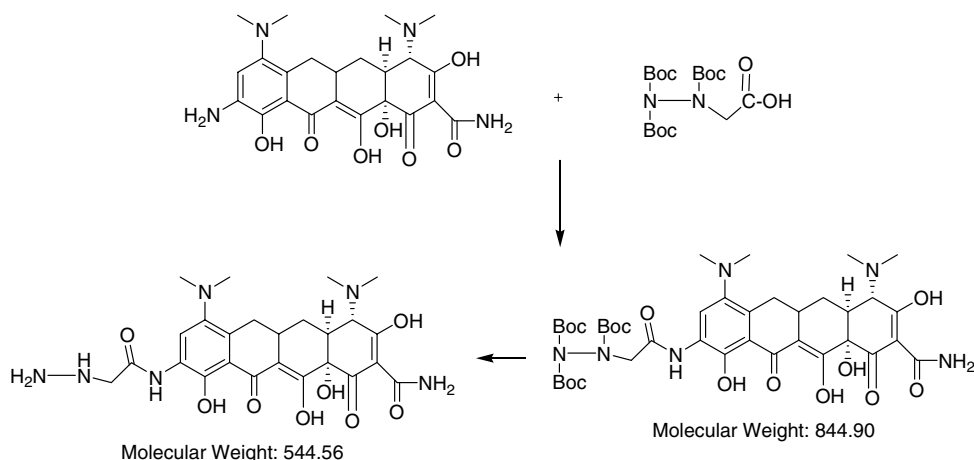
Scheme 1. Reaction scheme for synthesis of 9-hydrazino-Ac-NH-MIN.

Measurements of MIC[25] for each of the MIN derivatives without PEG (Table 1) showed that 9-Gly-NH-MIN and 9-hydrazino-Ac-NH-MIN retained significant biological activity, compared with unmodified MIN. However, the MIC of the two 9-PEG-MIN derivatives was dramatically elevated. The bulky PEG modification might have inhibited bacterial uptake of the MIN derivatives, and/or the binding of MIN to the 30S ribosomal subunits, resulting in the loss of bioactivity by the PEG-MIN derivatives. This result argues against permanent PEG tethering of MIN derivatives to medical implants.

Because of the loss of bioactivity of MIN after conjugation with PEG, we selected the acid-liable hydrazone bond to link 9-hydrazino-Ac-NH-MIN to solid surfaces, so that the drug could be released on demand upon acidification of the microenvironment by proliferating bacteria.[26]

Hydrazone bonds have been widely investigated for targeting drug delivery.[27] The bond is relatively stable in physiological pH. When the delivered drug was absorbed through endocytosis pathway, the hydrazone could be broken in response to the low pH in cytoplasmic endosomes.[28] To enable release on demand, the hydrazone bonds should remain largely intact for an effective number of days after implant insertion. For the attachment of antibiotics to the surface of implants by acid-labile linkers, we expect that the antibiotics should remain stably attached on the surface for weeks

Table 1. MIC of MIN derivatives with *S. aureus*

Compound	MIC (found) (μg/mL)	MIC (published) (μg/mL)
MIN	0.125	0.125
9-H$_2$N-MIN	2	1
9-Gly-NH-MIN	2	0.5–1
9-hydrazino-Ac-NH-MIN	2	NA
9-PEG$_{12}$-Gly-NH-MIN	500	NA
9-PEG$_{26}$-Gly-NH-MIN	1000	NA

Staphylococcus aureus subspecies *aureus* Rosenbach (ATCC 25923) were incubated in Mueller–Hinton broth at 37 °C for 14 h, noting the dilution midway between fully inhibited and uninhibited bacterial proliferation.[25]

to months. Due to the reversible property of hydrazone bonds in aqueous solution, the antibiotics attached to implant surfaces could be released gradually at neutral pH, but rapidly at acid pH induced by infection.
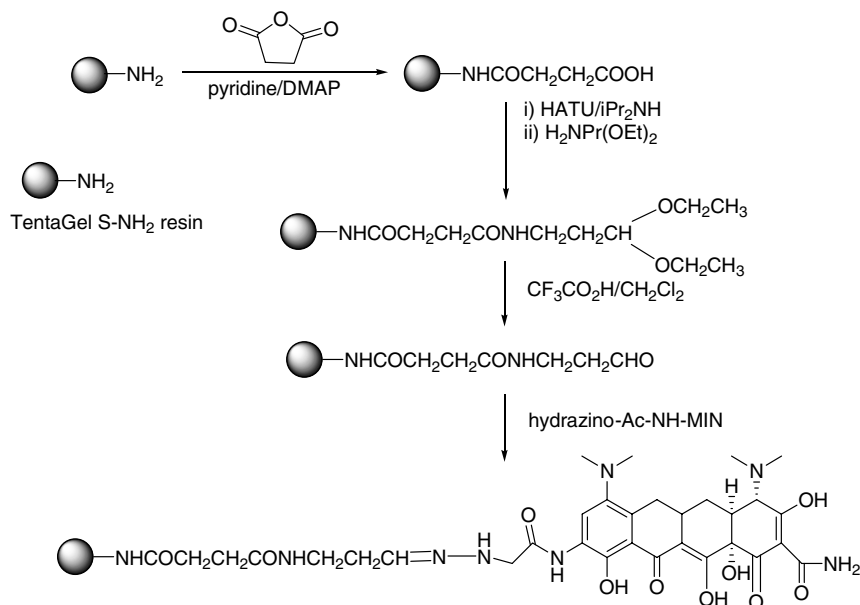
We selected TentaGel S-NH$_2$ polystyrene (PS) beads as a model to investigate the release of 9-hydrazino-Ac-NH-MIN from a polymeric surface. The amino groups on 500 mg of TentaGel S-NH$_2$ (Fluka 86364, 0.26 mmol/g) resin were transformed to carboxylates by reaction with 1.5 equiv of succinic anhydride in the presence of 0.1 equiv of *N,N*-dimethylaminopyridine (DMAP) catalyst dissolved in 4 mL of pyridine (Scheme 2). The reaction mixture was kept at 50 °C with shaking for 3 h. A ninhydrin test showed no free amino groups available on the surface of the resin beads. The carboxylates on the resin were activated by HATU and *i*Pr$_2$EtN at room temperature for 30 min, then coupled with H$_2$NPr(OEt)$_2$ at room temperature for 3 h. The resin was washed with Me$_2$NCHO and CH$_2$Cl$_2$, then dried under vacuum. Next, the resin was treated with 50% CF$_3$CO$_2$H in CH$_2$Cl$_2$ for 30 min to yield aldehyde-modified resin, which was washed with Me$_2$NCHO and CH$_2$Cl$_2$, then dried under vacuum overnight. 9-Hydrazino-Ac-NH-MIN at 0.1 M in CHCl$_3$ was then reacted with the aldehyde termini to yield the acid-labile hydrazone-linked 9-Ac-NH-MIN polystyrene beads. The resin was washed with physiological buffer (0.15 M NaCl, 0.01 M Na$_2$HPO$_4$, pH 7.4, PBS), Me$_2$NCHO and CH$_2$Cl$_2$, then dried under vacuum overnight. PS-Succ-NHPr-hydrazone-9-Ac-NH-MIN resin beads were stripped with 10% HOAc in CH$_2$Cl$_2$ to determine the total loading of 9-hydrazino-Ac-NH-MIN, 18.2 mg/g resin, by absorbance at 350 nm.[29]

The stability of the hydrazone bond was investigated by incubating 5.6 mg aliquots of PS-Succ-NHPr-hydrazone-9-Ac-NH-MIN resin beads at 37 °C under physiological conditions in 1.5 mL of 0.1 M Na$_2$HPO$_4$, pH 7.4, and under conditions of anaerobic bacterial metabolism in a confined microenvironment in 0.1 M NaOAc, pH 5.0. The concentration of the released 9-hydrazino-Ac-NH-MIN in the buffers was determined by absorbance at 350 nm[29] (Fig. 1).

Scheme 2. Reaction scheme for synthesis of PS-Succ-NHPr-hydrazone-9-Ac-NH-MIN.
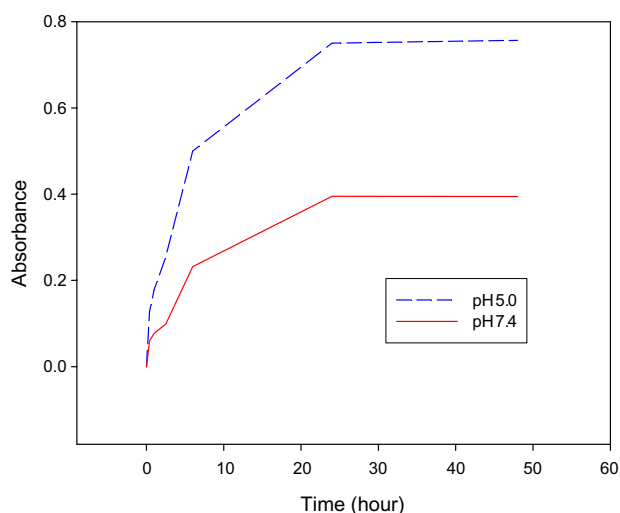

Figure 1. Release of 9-hydrazino-Ac-NH-MIN from PS-Succ-NHPr-hydrazone-9-Ac-NH-MIN resin beads as a function of pH. Solid line: 0.1 M Na_2HPO_4, pH 7.4. Dashed line: 0.1 M NaOAc, pH 5.0.

The time course of 9-hydrazino-Ac-NH-MIN release from the beads showed much faster release at pH 5.0 than at pH 7.4. After 24 h at pH 5.0, released MIN reached 36.7 μg/mL, or 54% of the MIN loading measured after coupling (Fig. 1). That value is 18 times greater than the MIC of 2 μg/mL. After 24 h at pH 7.4, released MIN reached 18.8 μg/mL, or 27.6% of the MIN loading measured after coupling. The leveling of the A_{350} trace after 24 h is probably the result of the instability of hydrazino-MIN. Hence, the hydrazone bond is not stable enough to keep 9-hydrazino-Ac-NH-MIN on the surface for an extended period of time.

We also attached 9-hydrazino-Ac-NH-MIN to the surface of Ti6Al4V foil. We prepared H_2NPrSi-O-Ti6Al4V alloy foil as described previously for Ti6Al4V pins.[17]

The amine was then coupled with 4 equiv of glyoxylyl-8-amino-3,5-dioxaoctanoic acid in the presence of HATU/iPr$_2$EtN in Me$_2$NCHO. 9-Hydrazino-Ac-NH-MIN at 0.1 M in CHCl$_3$ was coupled to the surface of the aldehyde-PEG-NHPrSi-O-Ti6Al4V foil for 4 h to yield Ti-O-SiPrNH-PEG-hydrazone-9-Ac-NH-MIN. The foil was washed with CH$_2$Cl$_2$, Me$_2$NCHO and double deionized H$_2$O, then dried under vacuum overnight. On the surface of a 1 cm^2 Ti6Al4V foil with a maximum monolayer loading of 200 pmol,[30] as we found for VAN,[18] the amount of strippable MIN would be too low to measure by UV absorbance.

To determine bacterial viability on Ti6Al4V foil substrates, control Ti6Al4V alloy foils and Ti-O-SiPrNH-PEG-hydrazone-9-Ac-NH-MIN alloy foils were sterilized by incubation with 70% EtOH, and washed five times with autoclaved PBS. *S. aureus* subspecies *aureus* Rosenbach were cultured in Mueller–Hinton broth at 250 rpm, 37 °C for 14 h. Using a 0.5 McFarland standard, the bacteria were diluted to 1×10^4 cfu/mL in Mueller–Hinton broth. The sterilized Ti6Al4V foils were cultured with the *S. aureus* at 37 °C for 24 h, after which the pH was still 7.3–7.4. For Ti6Al4V foil, we immersed the MIN-bonded foil in the minimum volume of 200 μL of broth. The foils were washed three times with PBS to remove loosely adherent bacteria, and assessed for bacterial adhesion and viability by staining with the Live/Dead® BacLight™ viability kit, which fluorescently labels viable bacteria green and dead bacteria red. After labelling, the Ti6Al4V alloy foils were washed three times with PBS to remove nonspecific stain and visualized by confocal laser fluorescence microscope on a Fluoview 300 (Olympus, Melville, NY). Challenge by *S. aureus*, however, showed live bacteria on all foil samples. At physiological pH, the concentration of 9-hydrazino-Ac-NH-MIN that could be released into the medium was insufficient to inhibit the growth of *S. aureus*. Even if all the attached monolayer of MIN were

released into the broth, the resulting MIN concentration of 1.2 µg/mL would have been below the MIC of 2 µg/mL. The flat foil has much less surface area than the porous resin beads.

For the construction of medical implants with covalently bound broad-spectrum antibiotics for self-protection against Gram-negative colonization, we designed and synthesized three MIN derivatives. Conjugation of long hydrophilic PEG spacers with MIN at the C9 position resulted in dramatic loss of bioactivity, which argued against the utility of permanent linkage of MIN to the surface of medical implants by PEG spacers.

Knowing that 9-hydrazino-Ac-NH-MIN showed reasonable antibiotic activity, we attached it to the surfaces of polystyrene beads and Ti6Al₄V foils by an acid-labile hydrazone bond that would permit release of free 9-hydrazino-Ac-NH-MIN to enter bacteria, triggered by bacterial acidification of the implant microenvironment. The release kinetics showed a clear difference between the rates of 9-hydrazino-Ac-NH-MIN released at pH 7.4 and pH 5.0. However, the hydrazone bond may not be stable enough for long term medical implant application. We are also considering methylmaleimide[31] linkers to enable this strategy.

Acknowledgments

References and notes

1. Toubes, E.; Segreti, J. *Curr. Infect. Dis. Rep.* **2002**, *4*, 433.
2. Garvin, K. L.; Hanssen, A. D. *J. Bone Joint Surg.* **1995**, *77A*, 1576.
3. Duggan, J. M.; Georgiadis, G. M.; Kleshinski, J. F. *Infect. Med.* **2001**, *18*, 534.
4. Zimmerli, W.; Trampuz, A.; Ochsner, P. E. *N. Engl. J. Med.* **2004**, *351*, 1645.
5. Haddad, F. S.; Masri, B. A.; Campbell, D.; McGraw, R. W.; Beauchamp, C. P.; Duncan, C. P. *J. Bone Joint Surg. (Brit.)* **2000**, *82*, 807.
6. Loll, P. J.; Axelsen, P. H. *Ann. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 265.
7. van de Belt, H.; Neut, D.; Schenk, W.; van Horn, J. R.; van der Mei, H. C.; Busscher, H. J. *Acta Orthopaed. Scand.* **2001**, *72*, 557.
8. Frazier, D. D.; Lathi, V. K.; Gerhart, T. N.; Hayes, W. C. *J. Biomed. Mater. Res.* **1997**, *35*, 383.
9. Montali, A. *Injury* **2006**, *37*, S81.
10. Liu, X.; Chu, P. K.; Ding, C. *Mater. Sci. Eng. R Rep.* **2004**, *47*, 49.
11. Jose, B.; Antoci, J. V.; Zeiger, A. R.; Wickstrom, E.; Hickok, N. J. *Chem. Biol.* **2005**, *12*, 1041.
12. Puleo, D. A.; Kissling, R. A.; Sheu, M. S. *Biomaterials* **2002**, *23*, 2079.
13. Jennissen, H. P. *Ann. N.Y. Acad. Sci.* **2002**, *961*, 139.
14. Walsh, C. *Nature* **2000**, *406*, 775.
15. Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. *Chem. Rev.* **2005**, *105*, 425.
16. Zreiqat, H.; Akin, F. A.; Howlett, C. R.; Markovic, B.; Haynes, D.; Lateef, S.; Hanley, L. *J. Biomed. Mater. Res. A* **2003**, *64*, 105.
17. Edupuganti, O. P.; Antoci, V., Jr.; King, S. B.; Jose, B.; Adams, C. S.; Parvizi, J.; Shapiro, I. M.; Zeiger, A. R.; Hickok, N. J.; Wickstrom, E. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2692.
18. Antoci, V., Jr.; King, S. B.; Jose, B.; Parvizi, J.; Zeiger, A. R.; Wickstrom, E.; Freeman, T. A.; Composto, R. J.; Ducheyne, P.; Shapiro, I. M.; Hickok, N. J.; Adams, C. S. *J. Orthopaed. Res.* **2007**, *25*, 858.
19. Schierholz, J. M.; Beuth, J. *J. Hospital Infect.* **2001**, *49*, 87.
20. van Ogtrop, M. L.; Andes, D.; Stamstad, T. J.; Conklin, B.; Weiss, W. J.; Craig, W. A.; Vesga, O. *Antimicrob. Agents Chemother.* **2000**, *44*, 943.
21. Zhanel, G. G.; Homenuik, K.; Nichol, K.; Noreddin, A.; Vercaigne, L.; Embil, J.; Gin, A.; Karlowsky, J. A.; Hoban, D. J. *Drugs* **2004**, *64*, 63.
22. Sum, P. E.; Lee, V. J.; Testa, R. T.; Hlavka, J. J.; Ellestad, G. A.; Bloom, J. D.; Gluzman, Y.; Tally, F. P. *J. Med. Chem.* **1994**, *37*, 184.
23. Milatovic, D.; Schmitz, F. J.; Verhoef, J.; Fluit, A. C. *Antimicrob. Agents Chemother.* **2003**, *47*, 400.
24. Barden, T. C.; Buckwalter, B. L.; Testa, R. T.; Petersen, P. J.; Lee, V. J. *J. Med. Chem.* **1994**, *37*, 3205.
25. NCCLS; *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*; 5th ed.; National Committee of Clinical Laboratory Standards: Wayne PA, 2000; Vol. NCCLS document M7-A5.
26. DeLand, F.; Wagner, H. N., Jr. *J. Lab. Clin. Med.* **1970**, *75*, 529.
27. Ulbrich, K.; Subr, V. *Adv. Drug Deliv. Rev.* **2004**, *56*, 1023.
28. van Dyke, R. W. *Subcell Biochem.* **1996**, *27*, 331.
29. Hasan, T.; Khan, A. U. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4604.
30. Puleo, D. A. *J. Biomed. Mater. Res.* **1997**, *37*, 222.
31. Drummond, D. C.; Daleke, D. L. *Chem. Phys. Lipids* **1995**, *75*, 27.