J Mater Sci: Mater Med (2011) 22:2773–2780
DOI 10.1007/s10856-011-4445-x

Mechanical effects, antimicrobial efficacy and cytotoxicity of usnic acid as a biofilm prophylaxis in PMMA

Sunghwan Kim · Robert Greenleaf · Mark Carl Miller · Latha Satish · Sandeep Kathju · Garth Ehrlich · J. Christopher Post · Nicholas G. Sotereanos · Paul Stoodley

Received: 26 November 2010 / Accepted: 5 September 2011 / Published online: 22 September 2011

Abstract Experiments were performed to test the null hypothesis that the addition of a natural occurring antibiotic would not alter mechanical properties of polymethylmethacrylate (PMMA). Compression and four-point bending tests were used to assess mechanical properties of zirconium dioxide bearing bone cement (Type Zr) and barium sulfate bearing bone cement (Type Ba), mixed with the antibiotic usnic acid ("usnic"), used to create a surface resistant to biofilm formation. Addition of usnic had a statistically significant effect on the material properties. Compressive and bending strengths decreased as usnic was added and Type Zr was stronger than Type Ba although material properties remained above recommended minima. With implications of liver toxicity with large doses of usnic taken as a dietary supplement, cytotoxicity tests using bone cement coupons were performed and showed very little or no toxicity in primary cultures of rabbit skin derived fibroblasts. A simple test of usnic's efficacy as a biofilm prophylaxis in PMMA was also conducted. Bone cement coupons with usnic were tested for their effectiveness against methicillin resistant *Staphylococcus aureus*. Diminished biofilm formation on usnic-containing coupons indicated that usnic can be an effective anti-microbial agent.

S. Kim · M. C. Miller
Allegheny General Hospital, Pittsburgh, PA, USA

S. Kim · R. Greenleaf · M. C. Miller (✉) · N. G. Sotereanos
Orthopaedic Biomechanics Laboratory,
University of Pittsburgh, Pittsburgh, PA, USA
e-mail: mcmiller@wpahs.org

L. Satish · S. Kathju
Department of Surgery, Division of Plastic Surgery,
University of Pittsburgh, Pittsburgh, PA, USA

G. Ehrlich · J. Christopher Post
Drexel University, Allegheny General Hospital,
Pittsburgh, PA, USA

P. Stoodley
Engineering Sciences, University of Southampton,
Southampton, UK

1 Introduction

Polymethylmethacrylate (PMMA) based bone cement is used in total joint replacement surgery to stabilize the implant in bone tissue. The mechanical properties of bone cement are of crucial importance for the success of surgery since mechanical failure of the mantle can lead to aseptic loosening of the prosthesis [1]. Bacterial infection is also a major problem after total joint replacement surgery, and infections are reported in up to 24% of procedures [2–4]. Bacteria have been found to grow largely as a confluent biofilm on the surface of the implant, rendering them resistant to standard antibiotic therapy, and attempts to eradicate the infection with systemic antibiotics alone are usually ineffective. Therefore, removal and replacement of the infected implant is required to eliminate infection. Antibiotic-loaded bone cement has been studied and shown to be effective against infections following joint replacement surgery [5–11]. Local delivery of antibiotics by means of antibiotic-loaded acrylic cement, used to fix the implant or in revision as a temporary spacer has become a recommended practice in the management of infected arthroplasties [12, 13]. Antibiotics, however, may alter the mechanical behavior of the bone cement [14–17]. Furthermore, the increasing resistance of infecting organisms to commonly used antibiotics, such as gentamicin and tobramycin, is an emerging problem [18, 19]. This leads to a need for new antibiotics for which the mechanical

characteristics should be evaluated [20]. To the authors' knowledge, the effect of usnic on the material properties of bone cement has not yet been investigated.

Usnic acid ("usnic") is a natural antimicrobial agent effective against some pathogenic fungi and gram positive bacteria, including *Mycobacterium tuberculosis*, *Staphylococcus*, *Streptococcus*, and *Pneumococcus*. While the usnic dosage needed for stand-alone antibiotic action and the quantities taken as dietary supplements have shown some side effects [21], usnic is able to inhibit formation of bacterial biofilm, which can be found in permanent indwelling devices such as joint prostheses and heart valves [22, 23] and is much more resistant to antimicrobial agents [24, 25]. Small concentrations can be well-tolerated and elution of small amounts may prohibit biofilm formation while providing better mechanical properties to PMMA. Three parallel comparisons were performed. To test mechanical properties of PMMA after addition of usnic, we conducted compression and four point bending tests, preceding from the null hypothesis that addition of the antibiotic would not alter these properties. Cytotoxicity testing was also performed, using the hypothesis that there would be no difference in the viability of fibroblasts due to exposure with PMMA with up to 5% usnic. Finally, efficacy against methicillin resistant *Staphylococcus aureus* (MRSA) was examined with the hypothesis that usnic would show no difference compared to controls and gentamicin.

2 Materials and methods

Two commercial bone cements with different kinds of radiopacifying material were tested with and without antibiotics. The first type (Type Zr) was a zirconium dioxide bearing bone cement (Palacos LV Zimmer, Inc., Warsaw, IN). It contained of 40 g of powder and 20 ml of liquid monomer, where each packet of bone cement powder contained 33.6 g of polymethyl methacrylate, 6 g of zirconium dioxide and 0.4 g of benzoyl peroxide. One ampule of liquid contained 18.4 g of methyl methacrylate and 0.4 g of N, N-dimethly-p-toluidine. The second type (Type Ba) was a barium sulfate bearing bone cement (Simplex, Stryker, Kalamazoo, MI). It also used 40 g of powder and 20 ml of liquid. Packets of Type Ba powder contained 35.32 g of PMMA, 4 g of barium sulfate and 0.68 g of benzoyl peroxide. One ampule of Type Ba bone cement liquid contained 18.33 g of methyl methacrylate and 0.47 g of N,N-dimethly-p-toluidine. The antibiotic-loaded bone cement was prepared by mixing usnic powder with the PMMA powder in a vacuum mixing bowl (Mix-Evac, Stryker) before adding liquid monomer to the PMMA according to ISO 5833. After thorough blending, liquid monomer was added and mixed for 30 s in the manually driven blending chamber with an applied vacuum to reduce gas content which deteriorates the material properties of the bone cement [26]. The mixed bone cement was injected into molds with a syringe, following ISO 5833 and ASTM D638 standards meticulously throughout. Rough edges of specimens were removed by sanding. The specimens were stored for 24 h at ambient temperature (23°C) before testing according to the ISO 5833 recommendations. After measuring the size and mass of all the specimens, compression and four point bending tests were performed with an MTS Bionix 858 materials testing machine (MTS Systems, Eden Prairie, MN, USA). The following formulations were investigated: (1) bone cement without antibiotics, (2) bone cement with 1 g of usnic, (3) bone cement with 2 g of usnic and (4) bone cement with 4 g of usnic. All the results were analyzed using a two-way ANOVA and Tukey's honest significant difference applied as a post hoc test. For cytotoxicity tests for usnic acid, bone cement (Palacos R) coupons with two different concentrations of usnic (2.5 and 5%) were created in an identical manner and subjected to MTT assay.

2.1 Compression tests

For each usnic concentration, seven 6 mm diameter by 12 mm long cylinders were molded and tested in compression under displacement control at a cross-head rate of 20.0 mm/min according to ISO 5833 using the Bionix 858 machine. Specimens with visible bubbles were excluded from the analysis. The maximum compressive strength (force divided by original area) and the compressive modulus (slope of the stress–strain plot) were calculated for each specimen.

2.2 Four point bending tests

Seven $75 \times 10 \times 3.3$ mm^3 rectangular beams of each PMMA-usnic combination were molded and tested in four point bending according to ISO 5833 (cross-head rate of 5.0 mm/min). A linear variable differential transducer (LVDT) was integrated into the four point bending fixture to measure the deflection of the center of the specimen. The deflection was recorded at applied loads of 15 and 50 N in order to calculate the bending modulus according to ISO 5833. Ultimate bending strength was also obtained.

2.3 Cytotoxicity tests

Primary cultures of adult rabbit-derived fibroblasts were grown in standard tissue culture flasks in RPMI-1640 (Invitrogen Corp. Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts, West

Sacramento, CA) and 1% antibiotic/antimycotic solution (Sigma Chemical Corporation, St Louis, MO) at 37°C in a humidified chamber containing 5% CO_2. Once the cells reached 80% confluence, cells were sub-cultured by treating them with 0.25% trypsin–EDTA (Invitrogen Corp). The MTT Cell Proliferation Assay Kit (ATCC, Rockville, MD) was used to determine changes in the proliferation rates of cells treated with coupons prepared using 2.5 and 5% usnic acid mixed with bone cement (Palacos LV Zimmer, Inc., Warsaw, IN). The coupons were made 1 day and 1 week before they were employed in the cytotoxicity assay and then subjected to ethylene oxide treatment at 54°C for 1 h before exposing them to the assay. The assay, as previously described [27], is based on the conversion of yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells. The amount of formazan produced is proportional to the number of viable cells. In actively proliferating cells, an increase in MTT conversion is spectrophotometrically quantified at wavelength 570 nM. Comparison of this value to an untreated control provides a relative increase in cellular proliferation. Conversely, in cells undergoing apoptosis, MTT production decreases, reflecting the loss of cell viability. Cells were plated in 24-well flat bottom tissue culture plates at a density of approximately 1–1.2 $\times$ 10^4 cells/well and allowed to attach overnight at 37°C. The cells were then incubated with the coupons for 24 h. After the exposure periods, the cells were photographed and replaced with fresh medium. Next, 10 µl of the MTT reagent was added to each well, and the plate was incubated for 4 h at 37°C. The MTT crystals were then solubilized by adding 100 µl of the MTT detergent reagent to each well. Absorbance measurements were performed at 570 nm using a spectrophotometer. All experiments were performed in triplicate and repeated four times.

2.4 Efficacy against MRSA: biofilm formation on bone cement mixed with (+)-usnic acid assessed by confocal microscopy

To qualitatively assess the potential of usnic acid to inhibit biofilm formation by Staphylococci we exposed coupons made from bone cement to *S. aureus* CGS01. *S. aureus* CGS01 is a clinical MRSA strain isolated from a patient with a failed total elbow arthroplasty from which we had demonstrated the presence of biofilm growing on gentamicin impregnated bone cement [24]. Coupons were made from 0 or 8 g of usnic acid mixed with a 40 g pouch of powder (Type Zr) and then stirred in the liquid monomer. Small amounts of PMMA were separated, rolled into balls and pressed flat to form a coupon, approximately 1.5 cm diameter and 5 mm thick. All manipulations were performed under sterile conditions and with sterile materials. After curing, the coupons were secured to the bottoms of 35 mm polystyrene Petri plates using cyanoacrylate adhesive. 2 ml of sterile brain heart infusion (BHI) broth (Oxoid Ltd) was added so that the coupons were fully submerged. Each plate was inoculated with 20 µl from an overnight BHI broth culture of *S. aureus* CGS01. The cultures were incubated for 24 h at 37°C, 5% CO_2. The spent media was removed and the coupons rinsed by adding 2 ml phosphate buffered solution (PBS), giving a gentle swirl by hand and then aspirating off, to remove planktonic and loosely adhered cells. 2 ml of fresh sterile BHI broth was then added and the plate was re-inoculated with 20 ml of a fresh overnight culture. The process was repeated for 3 more days resulting in a 4 day culture with repeated inocula challenges. At the start of day 5, the coupons were rinsed twice with PBS then stained by pipetting 50 µl of BacLight viability kit (Molecular Probes, Invitrogen), made according to manufacturers specifications, onto the surface of the coupon so that the surface was completely wetted with the stain. After a 15-minute incubation, the plates were flooded with PBS and the coupons were imaged with a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system (Leica Microsystems, Exton, PA) using a 63$\times$ water immersion lens (NA 1.2). Reflected confocal microscopy using the 488 nm laser was used to image the surface of the bone cement coupon and was designated as blue. The BacLight viability kit stains live bacteria (or more strictly cells with intact cell walls) green and dead cells (with permeable cell walls) red. Duplicate coupons were prepared and multiple images were taken from each coupon.

3 Results

3.1 Mechanical properties

Figure 1 shows the material properties (compressive strength, Young's modulus, bending strength and bending modulus) obtained for both Type Zr and Type Ba. For Type Zr, all compressive strengths were approximately 90 MPa; bending strength decreased from 68 to 47 MPa with the addition of usnic. Young's moduli and bending moduli were between 2 and 3 GPa and relatively insensitive to the addition of usnic. For Type Ba, compressive strengths and bending strengths were approximately 80 and 50 MPa respectively. Young's moduli and bending moduli were very similar to those of Type Zr. For bending strength, a gradual decrease was observed as in Type Zr, but bone cement without antibiotic was lowest in compressive strength. Both the compressive and bending moduli were unaffected by the addition of usnic. Most material

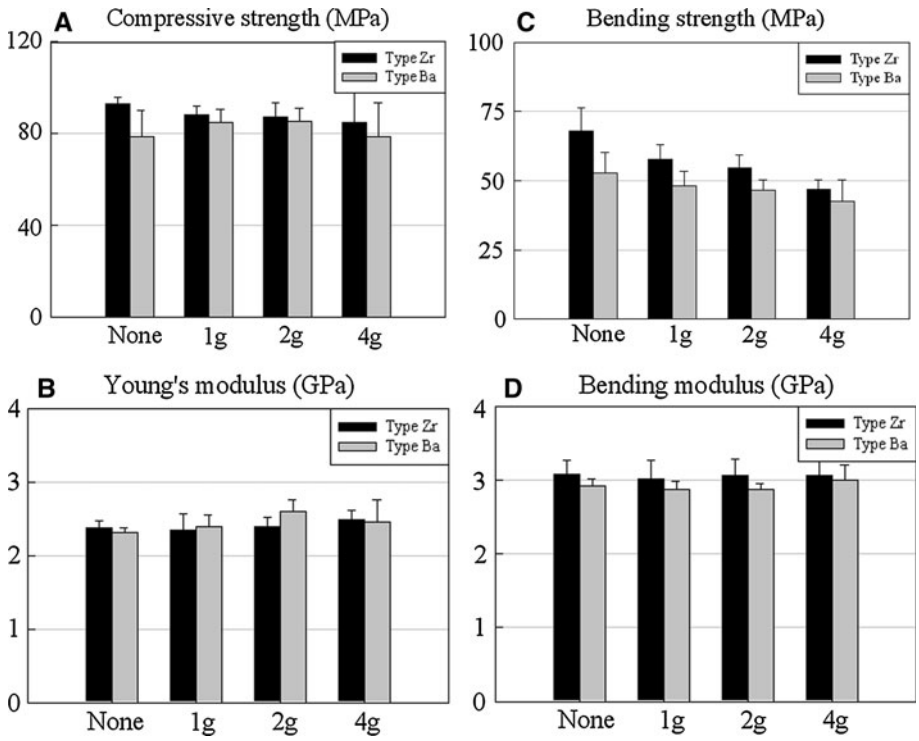

Fig. 1 Four material properties of PMMA with opacifying agent: Type Zr: Zirconium dioxide bearing PMMA. Type Ba: Barium sulfate bearing PMMA. **a** Compressive strength, **b** Young's modulus, **c** bending strength, **d** bending modulus

properties of Type Zr were higher than those of Type Ba except Young's modulus. Analysis with a two-way ANOVA found statistically significant differences due to bone cement type ($P < 0.001$) and concentration of usnic ($P < 0.002$). Post hoc testing found statistical differences in bending strength ($P < 0.001$) and bending modulus ($P = 0.017$) between the two bone cement types and bending strengths due to different concentrations of usnic ($P < 0.001$).

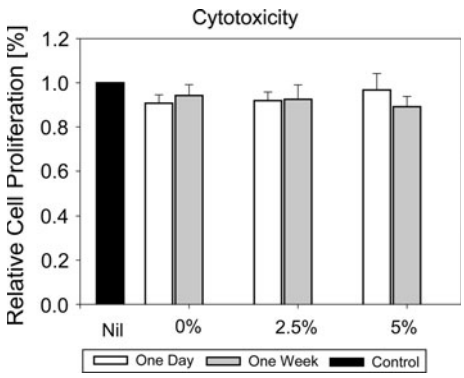

Fig. 2 Cytotoxicity tests. MTT cell viability tests on rabbit skin-derived fibroblasts revealed that coupons prepared using 2.5 and 5% usnic acid along with the bone cement did not elicit any significant cell death. Cells treated and non-treated with bone cement alone served as controls. The values are mean ± SEM of four independent studies performed in triplicate. Statistical analysis was performed by Student's *t*-test

3.2 Cytotoxicity

Coupons prepared using two different concentrations of usnic (2.5 and 5%) in the bone cement and left undisturbed for a day and for a week showed very little or no cytotoxic effects on primary cultures of fibroblasts derived from adult rabbit skin. (Fig. 2) No statistical difference in cytotoxicity compared to controls was found due to usnic or to extra curing time. As shown in Fig. 2, we observed a slight decrease in the viability of the cells in the coupons prepared using 5% usnic acid, but this decrease was not statistically significant. The morphology of the cells was unaltered by the presence of the coupons, whether or not usnic was present and whether or not the PMMA coupons were uncured or cured for either 1 day or 1 week. Micrographs with adequate resolution to show the cell morphology are shown in Fig. 3a–g.

3.3 Biofilm formation on bone cement mixed with usnic acid assessed by confocal microscopy

Figure 4a and b shows confocal micrographs of biofilm formation on bone cement with and without usnic acid respectively, where the first column in each figure displays the surface topography before introduction of bacteria, the second column shows the same surface with any live bacteria on that surface and the third column overlays the first two images. The reflected confocal images of the surface in the first column in the figures illustrate that both

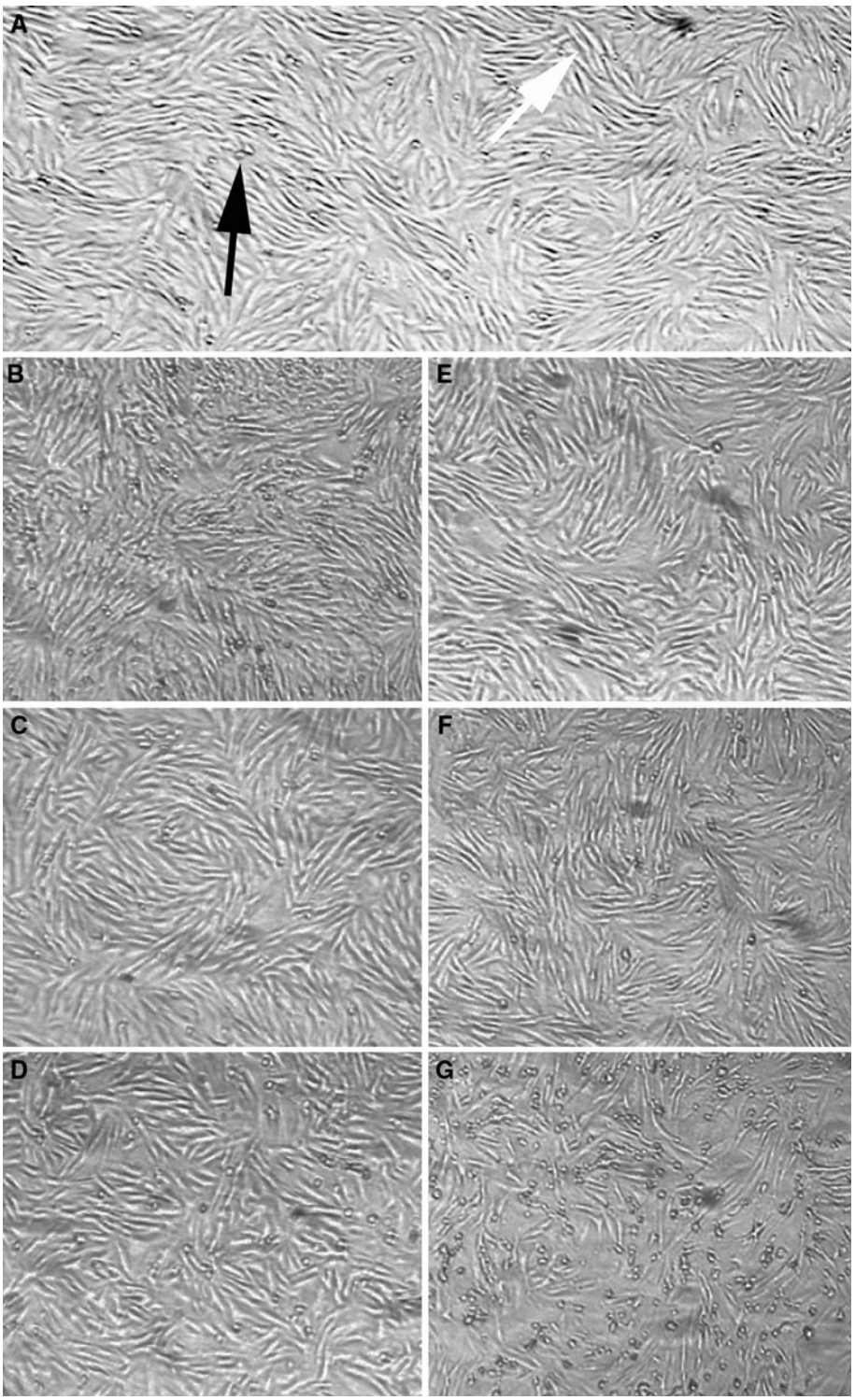

Fig. 3 Panels **a**–**g** Fibroblast morphology in wells with PMMA coupons: **a** control well (no coupon) showed attached elongated cells in confluency (see *white arrow*). There were occasional non-attached round cells (*black arrow*). **b**, **c**, **d** Fibroblast morphology after 24 h incubation in the presence of a 1 day cured PMMA. All of the cultures showed confluency. **b** 0% (+)-usnic acid. **c** 2.5% (+)-usnic acid. **d** 5% (+)-usnic acid. **e**, **f**, **g** Fibroblast morphology after 24 h incubation in the presence of a 1 week cured PMMA. The 5% coupon (panel **g**) showed a slight loss of confluency. **e** 0% (+)-usnic acid. **f** 2.5% (+)-usnic acid. **g** 5% (+)-usnic acid

surfaces were highly porous. The usnic acid, however, changed the structure of the surface from amorphous regions with bubble-like inclusions to unconnected rectangular striations distributed over the surface. These striations were probably crystals of usnic acid. Single cocci and dense patches of biofilm made up of clusters of live bacteria were clearly evident on the untreated bone cement (Fig. 4a, center column). This contrasted with the bone cement made with 8 g of (+)-usnic acid, which had few single cells and a sparse scattering of small (<10 μm) biofilm clusters (Fig. 4b, center column). Very few cells on both types of cement were dead (data not shown).

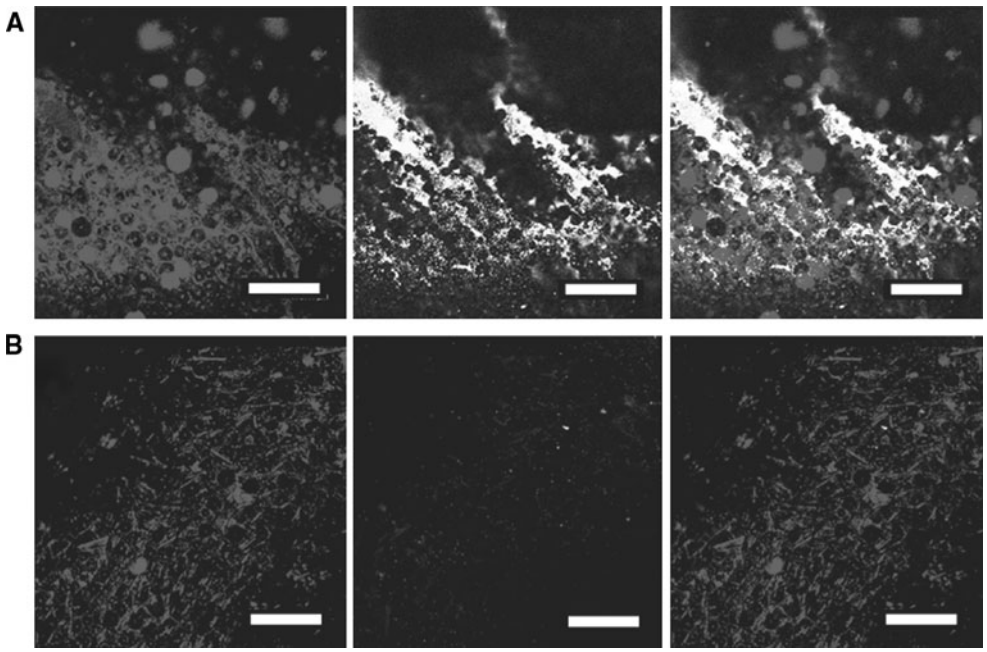

Fig. 4 Confocal micrographs showing bacteria growth on surfaces of PMMA with and without usnic. First column of the figure: the reflected confocal image before the addition of bacteria. Second column of the figure: the location of live bacteria on the same surface as shown in the first column, shown without the reflected confocal image. Third column of the figure: overlays of the first two columns. Row **a** control bone cement, row **b** bone cement with 8 g (+)-usnic acid. *Scale bar* = 200 μm

4 Discussion

In this study, we prepared two types of specimens from two formulations of bone cement to investigate the effect of usnic acid on the material properties of bone cements and found that compressive strength and bending strength were lessened with the addition of usnic whereas Young's modulus and compressive modulus were relatively unaffected. Although we used a vacuum mixing bowl and syringe injection to increase the quality of specimens by reducing voids, small voids were observed in the fracture plane of most bending specimens. Voids create stress concentration around them and subsequently contribute to failure. Overall, the results were similar to values in previous literature, which examined the effects of different antibiotics such as gentamicin and vancomycin on bone cement.[8, 9, 28–32] Our testing indicated that compressive strengths ranged from 75 to 100 MPa, bending strengths from 50 to 80 MPa, Young's moduli and bending moduli between 2 and 3 GPa. Although gradual decreases with increases in usnic content were observed in both compressive strength and bending strength for Type Zr and in bending strength only for Type Ba, all compressive strengths for all groups satisfied the ISO minimum requirement of 70 MPa. Unlike compressive and bending strength, Young's modulus and bending modulus were unaffected by the addition of usnic, which is very similar to the findings for other antibiotics previously reported by other groups [8, 9].

Between different bone cement types, bending strength and bending modulus were statistically different. For different antibiotic concentrations, only bending strength was statistically different. This means that bending properties were more affected by both the addition of usnic and the choice of bone cement than compressive properties. Young's modulus as measured from either a compression test or a four point bending test should theoretically be the same for an isotropic material, but differed in the present study. The compressive elastic modulus was higher than bending modulus, with the compressive modulus approximately 2.5 GPa and the bending modulus approximately 3 GPa for both Type Zr and Type Ba bone cement. This outcome is similar to the results of previous literature [5, 9, 28, 29]. One reason for this difference is that the bending modulus is calculated using beam theory which includes tension and compression on alternate sides of the beam while Young's modulus is only the slope of a compression test. In other words, the compressive and tensile elastic moduli differ for bone cement.

Except for Young's modulus, all the material properties for Type Zr were higher than for Type Ba. The reason for this difference in mechanical strength could be the difference in radiopacifying components, i.e., ZrO_2 and $BaSO_4$. Ginebra et al. [33] compared four cements containing either ZrO_2 or $BaSO_4$, and found that the addition of $BaSO_4$ decreased the mechanical properties and the effects were larger in tensile and flexural tests than compressive

tests [34]. They assessed the size of ZrO_2 and $BaSO_4$ by scanning electron microscopy and reported that ZrO_2 particles were approximately 10 μm and $BaSO_4$ particles were in the submicronic range. The difference in mechanical strength between bone cements with small particles and bone cements containing large particles can be described by two mechanisms. First, small particles are more likely to agglomerate than bigger particles, as shown by Liu et al. [35]. They reported that $BaSO_4$ is prone to agglomerate within the bone cement matrix and act as a stress concentrator, thereby impairing the material properties of bone cement. Secondly, small molecules surround the polymer beads and affect polymerization. These particles can remain as a layer on cement beads during mixing and might not become fully incorporated during polymerization [34].

Toxicity with usnic must be addressed because of some adverse reports related to its use as a slimming agent and dietary supplement [36]. While direct in vitro testing cannot allay all concerns, the results of the fibroblast testing did not indicate any significant cellular toxicity, nor any change in cellular morphology. Further testing would, of course, be necessary.

The results of confocal microscopy indicate usnic may prevent biofilm accretion on the PMMA surface because almost no cells were found on the surface of coupons which incorporated the usnic antibiotic. These results suggest that the usnic acid bone cement had prevented the bacteria from attaching, had lysed cells or had caused them to detach after initial attachment. Although the initial test dose of 8 g is more than would normally be added, the result shows that usnic acid mixed into bone cement has the potential of reducing biofilm formation by clinically relevant MRSA strains.

In conclusion, we confirmed that up to 5% of usnic acid could be added without reducing the compressive strength below the ISO recommended of value of 70 MPa. Statistical differences were found for bending strength and bending modulus between zirconium dioxide and barium sulfate bone cement. The addition of usnic statistically affected only bending strength, although gradual decreases of compressive strength and bending strength for antibiotic loaded bone cement were observed. Future mechanically-directed work should consider the effect of usnic on fatigue strength because fatigue strength of bone cement is also known to be affected by the addition of antibiotics [37].

References

1. Smeds S, Goertzen D, Ivarsson I. Influence of temperature and vacuum mixing on bone cement properties. Clin Orthop Relat Res. 1997;334:326–34.
2. Tunney MM, et al. Improved detection of infection in hip replacements. A currently underestimated problem. J Bone Joint Surg Br. 1998;80(4):568–72.
3. Trampuz A, et al. Sonication of removed hip and knee prostheses for diagnosis of infection. N Engl J Med. 2007;357(7):654–63.
4. Villa T, Carnelli D. Experimental evaluation of the biomechanical performances of a PMMA-based knee spacer. Knee. 2007; 14(2):145–53.
5. Askew MJ, et al. Effect of vacuum mixing on the mechanical properties of antibiotic-impregnated polymethylmethacrylate bone cement. J Biomed Mater Res. 1990;24(5):573–80.
6. Buchholz HW, Elson RA, Heinert K. Antibiotic-loaded acrylic cement: current concepts. Clin Orthop Relat Res. 1984;190: 96–108.
7. Cui Q, et al. Antibiotic-impregnated cement spacers for the treatment of infection associated with total hip or knee arthroplasty. J Bone Joint Surg Am. 2007;89(4):871–82.
8. He Y, et al. Effect of antibiotics on the properties of poly(methylmethacrylate)-based bone cement. J Biomed Mater Res. 2002;63(6):800–6.
9. Lautenschlager EP, et al. Mechanical properties of bone cements containing large doses of antibiotic powders. J Biomed Mater Res. 1976;10(6):929–38.
10. Levin PD. The effectiveness of various antibiotics in methyl methacrylate. J Bone Joint Surg Br. 1975;57(2):234–7.
11. Marks KE, Nelson CL, Lautenschlager EP. Antibiotic-impregnated acrylic bone cement. J Bone Joint Surg Am. 1976;58(3): 358–64.
12. Durbhakula SM, et al. Spacer endoprosthesis for the treatment of infected total hip arthroplasty. J Arthroplast. 2004;19(6):760–7.
13. Pitto RP, Spika IA. Antibiotic-loaded bone cement spacers in two-stage management of infected total knee arthroplasty. Int Orthop. 2004;28(3):129–33.
14. Armstrong MS, et al. Mechanical characteristics of antibiotic-laden bone cement. Acta Orthop Scand. 2002;73(6):688–90.
15. Klekamp J, et al. The use of vancomycin and tobramycin in acrylic bone cement: biomechanical effects and elution kinetics for use in joint arthroplasty. J Arthroplast. 1999;14(3):339–46.
16. Leone J, et al. Biaxial flexural modulus of antibiotic-impregnated orthopedic bone cement. J Biomed Mater Res B Appl Biomater. 2007;83(1):97–104.
17. Mohd Fuad D, et al. Biomechanical properties of bone cement with addition of cefuroxime antibiotic. Med J Malaysia. 2006; 61(Suppl A):27–9.
18. Neu HC. The crisis in antibiotic resistance. Science. 1992; 257(5073):1064–73.
19. Tunney MM, et al. Antimicrobial susceptibility of bacteria isolated from orthopedic implants following revision hip surgery. Antimicrob Agents Chemother. 1998;42(11):3002–5.
20. Baleani M, et al. Biological and biomechanical effects of vancomycin and meropenem in acrylic bone cement. J Arthroplast. 2008;23:1232–8.
21. Guo L, et al. Review of usnic acid and Usnea barbata toxicity. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2008;26(4):317–38.
22. Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol. 2003;57(2):677–701.
23. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother. 2001;45(4):999–1007.
24. Stoodley P, et al. Direct demonstration of viable *Staphylococcus aureus* biofilms in an infected total joint arthroplasty. A case report. J Bone Joint Surg Am. 2008;90(8):1751–8.
25. Francolini I, et al. Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces. Antimicrob Agents Chemother. 2004;48(11):4360–5.

26. Dunne NJ, Orr JF. Influence of mixing techniques on the physical properties of acrylic bone cement. Biomaterials. 2001;22(13):1819–26.
27. Pieters R, et al. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. Cancer Lett. 1988;41(3):323–32.
28. Linden U. Mechanical properties of bone cement. Importance of the mixing technique. Clin Orthop Relat Res. 1991;272:274–8.
29. Persson C, et al. Mechanical effects of the use of vancomycin and meropenem in acrylic bone cement. Acta Orthop. 2006;77(4):617–21.
30. Dunne N, et al. In vitro study of the efficacy of acrylic bone cement loaded with supplementary amounts of gentamicin: effect on mechanical properties, antibiotic release, and biofilm formation. Acta Orthop. 2007;78(6):774–85.
31. Holm NJ. The modulus of elasticity and flexural strength of some acrylic bone cements. Acta Orthop Scand. 1977;48(5):436–42.
32. Nelson RC, Hoffman RO, Burton TA. The effect of antibiotic additions on the mechanical properties of acrylic cement. J Biomed Mater Res. 1978;12(4):473–90.
33. Ginebra MP, et al. Mechanical performance of acrylic bone cements containing different radiopacifying agents. Biomaterials. 2002;23(8):1873–82.
34. Kjellson F, et al. Effect of iodixanol particle size on the mechanical properties of a PMMA based bone cement. J Mater Sci Mater Med. 2007;18(6):1043–51.
35. Liu C, et al. Some failure modes of four clinical bone cements. Proc Inst Mech Eng [H]. 2001;215(4):359–66.
36. Chitturi S, Farrell GC. Hepatotoxic slimming aids and other herbal hepatotoxins. J Gastroenterol Hepatol. 2008;23(3):366–73.
37. Davies JP, et al. Influence of antibiotic impregnation on the fatigue life of Simplex P and Palacos R acrylic bone cements, with and without centrifugation. J Biomed Mater Res. 1989;23(4):379–97.