J Mater Sci (2010) 45:5244–5251
DOI 10.1007/s10853-010-4566-5

Comparison of antibacterial properties of commercial bone cements and fillers with a zinc-based glass polyalkenoate cement

A. W. Wren · N. M. Cummins · M. R. Towler

Received: 17 November 2009 / Accepted: 27 April 2010 / Published online: 11 May 2010

Abstract Postoperative infection following invasive surgical procedures is a significant cause for concern, particularly in spinal reconstructive surgery. The objective of this study is to compare the antibacterial efficacy of a novel zinc-based glass polyalkenoate cement (Zn-GPC) based on $0.04SrO–0.12CaO–0.36ZnO–0.48SiO_2$ glass, to a number of commercially available bone cements and fillers including Simplex P + Tobramycin (S_{Tob}), Spineplex (S_{pine}) and Novabone Putty (N_{Put}). The agar diffusion test was performed on each material against *Escherichia coli*, *Staphlococcus epidermidis*, *Pseudomonas aeruginosa* and *Staphlococcus Aureus*. S_{Tob} was found to produce large inhibition zones in each of the bacteria tested and was statistically significantly higher than any other material. The experimental Zn-GPC (B_{TSC}) was found to exhibit antibacterial properties in both *E. coli* and *S. epidermidis*. Neither S_{pine} nor N_{Put} showed any inhibitory effect in any of the bacteria tested. A study was also performed to determine the effect of antibiotic release from S_{Tob} and Zn-GPC (B_{Tob}) containing the antibiotic tobramycin (Tob). Antibacterial efficacy was found to increase with respect to maturation with B_{Tob}, whereas S_{Tob} was found to decrease significantly over the time period of 0–14 days. The final objective is to investigate any change in agar composition during the agar-diffusion test. Little change was observed for S_{Tob} as antibiotic release cannot be determined using EDX. There was, however, an increase in Zn levels when analysing B_{TSC} which suggests that Zn is playing a role in the antimicrobial nature of the Zn-GPC. No significant changes were observed for S_{pine} or N_{Put}.

A. W. Wren (✉) · M. R. Towler
Inamori School of Engineering, Alfred University, Alfred, New York 14802, USA
e-mail: wren@alfred.edu

N. M. Cummins
Materials and Surface Science Institute, University of Limerick, Limerick, Ireland

Introduction

Biomaterial-associated infections are of major concern in surgery. Microorganisms are frequently introduced onto implant surfaces and subsequently into the body [1]. It has been reported that infection rates range from 0.8–1.2% in orthopaedic surgery (total hip arthroplasty) and 3.6–8.1% (closed fracture) to 17.5–21.2% (open fractures) in trauma surgery [2]. Sepsis can ultimately lead to failure of the arthroplasty, causing the need for revision surgery, prolonged hospitalization and even death [3]. The field of biomedical engineering relies heavily on the interaction between the biomaterials and the surrounding physiological environment [4] and recently additives have been included in biomaterial design to increase antibacterial properties in an effort to extend the longevity of the implant. Some examples include antibiotic coatings, antibiotic-loaded collagen sheets and cement spacers [5] and antibiotic-loaded bone cements (ALBC). Commercially, ALBCs consist of a PMMA-based cement with additions of antibiotics such as tobramycin, gentamicin [6, 7], vancomycin and erythromycin [7]. However, concerns related to the use of ALBCs include the long-term effectiveness of the released drug [6, 7] and promotion of bacterial resistance which is likely due to the long-term sub-therapeutic levels of antibiotic eluted from the cement [6]. Other additives such as chitosan and collagen have been added to materials to promote cell growth [7]. Chlorhexidine (CHX) has also been added to dental glass polyalkenoate cements (GPCs) to further improve antibacterial properties [8].

However, CHX was found to interfere in the setting reaction of commercially tested GPCs resulting in reduced mechanical properties [8].

GPCs, traditionally used in dental applications, generally contain ions that impart an antibacterial nature when used in an oral aqueous environment. The antimicrobial nature of these cements is predominant due to the release of the fluoride ion (F^-) [9–11] and also related to setting occurring at a low pH [12]. GPCs have the additional ability to uptake and re-release F^- ions in a F^--rich aqueous environment, which can impart a prolonged antibacterial response [10]. However, translating GPCs from dental to orthopaedic applications highlights concerns relating to the composition of the material, in particular the presence of aluminium (Al) in the glass phase which can cause neurological [13, 14] and bone metabolic defects [15]. The GPCs produced in this work have zinc ions (Zn^{2+}) substituting for Al ions (Al^{3+}) as numerous studies report on the antibacterial efficiency [12, 16–18] and positive bone metabolic effects of Zn [19, 20].

For this study, a range of commercially available materials were selected for testing. Simplex + Tobramycin (Stryker Howmedica, Limerick, Ireland) and Spineplex (Stryker Howmedica, Limerick, Ireland) both of which are PMMA-based materials used in load-bearing applications in the skeleton. Novabone putty (Gian Medical, Birmingham, UK), a bioglass-based bone filler was also included alongside an experimental Zn-GPC formulation. Each material was tested against a number of known bacteria that cause infection in surgical procedures; *Escherichia coli*, *Staphlococcus epidermidis*, *Pseudomonas aeruginosa* and *Staphlococcus aureus*. Of these, *S. epidermidis* and *S. aureus* are responsible, in most instances, for biomaterial related infections [21], due to the presence of adherent, multilayered biofilms on the surfaces of implanted biomedical devices which are less susceptible to antibiotics when compared to their planktonic counterparts [22]. *P. aeruginosa* has been found to infect surgical procedures such as Total Knee Arthroplasty (TKA) [23], and is reported to be the cause of osteomyelitis in pelvic bones [24]. It has also, along with *E. coli*, been found to constitute 42% of polymicrobial infections in the spine [21]. This infection rate is preceded by *S. aureus* and *S. epidermidis* which are the primary cause of infection in the majority of postoperative spinal infections [21]. These species are prevalent in human epithelia residing on most skin and mucous membranes and are known to cause nosocomial infections in newborns, severely ill and immuno-compromised patients [25]. Further studies on biopsies of the spine by Heyer et al. [26] reveal the presence of all these bacteria, highlighting the relevance of this study.

For this study, a novel GPC formulation alongside Simplex + Tobramycin, Spineplex and Novabone Putty, were tested against *E. coli*, *S. epidermidis*, *P. aeruginosa* and *S. aureus*.

Materials and methods

Materials

- Simplex P + Tobramycin (S_{Tob})—Stryker Howmedica, Limerick, Ireland (#0473S024).
- BT 101 + 10 wt% TSC (B_{TSC})—experimental GPC.
- Spineplex (S_{pine})—Stryker Howmedica, Limerick, Ireland (#V1104).
- Novabone Putty (N_{Put})—Gian Medical, Birmingham, UK (#0610D5).

Glass synthesis

A strontium–calcium–zinc–silicate glass formulation (0.04SrO/0.12CaO/0.36 ZnO/0.48 SiO_2, mol. fraction) was synthesized. The glass was prepared by weighing out appropriate amounts of analytical grade reagents (Sigma-Aldrich, Dublin, Ireland) and ball milling for 1 h. The mixture was then dried in an oven (100 °C, 1 h), fired (1480 °C, 1 h) in a platinum crucible and shock quenched in water. The resulting frit was dried, ground and sieved to retrieve a glass powder with a maximum particle size of 45 m.

Polyacrylic acid (PAA)

The PAA used in this study (E9, M_w 80,800) was supplied by Advanced Healthcare Limited (Kent, UK). The polyacrylic acid was ground and sieved to retrieve <90-μm particles.

Tri-sodium citrate (TSC)

The TSC used in this study was obtained from Reagacon (Shannon, Ireland) and was incorporated into the cement at 10 wt% addition.

Tobramycin sulphate (Tob)

The Tob used in this study was obtained from Stryker Howmedica (Limerick, Ireland) and was incorporated into the cement at 2.5 wt% addition; a concentration similar to that contained in S_{Tob}.

Cement preparation

Cements were prepared by thoroughly mixing the glass powder with the PAA and distilled water (BT 101) on a

Table 1 Formulation of cements used in this study

	Cement formulations				
	Glass	PAA	H_2O	TSC	Tob
BT 101	1	0.37	0.37	–	–
B_{TSC}	1	0.37	0.37	0.075	–
B_{Tob}	1	0.37	0.37	–	0.018

glass plate using a dental spatula at a powder to liquid ratio of 2:1.5 g/mL. Additions of 10-wt% TSC/2.5-wt% Tob were also added to the cement formulations as outlined in Table 1.

Sample preparation

Three specimens of S_{Tob}, BT_{TSC}, S_{pine} and N_{Put} were used for testing in each bacterium. Each sample was produced by filling moulds (2 mm × 8 mmØ) with the cement and leaving them to set for 1 h prior to testing. Samples were produced under standard sterile laboratory conditions. A short maturation study was undertaken to determine the antibacterial properties of the experimental GPC containing Tobramycin (B_{Tob}) against S_{Tob}. For this study, both the materials were immersed in similar concentrations of sterile water for 1, 7 and 14 days. The cement was then tested in *S. epidermidis* at 0, 1, 7 and 14 days. A similar quantity of antibiotic was added to the GPC formulation, 2.5 wt%, as is contained in S_{Tob}.

Agar disc-diffusion test

The antibacterial activity of the cements was evaluated against *E. coli* strain DH5α, *B*, *S. epidermidis* strain NCIMB 12721, *P. aeruginosa* strain PA01 and *S. aureus* strain A35-4 using the agar disc-diffusion method. Luria agar and broth were used for the culture of *E. coli*, BHI agar and broth were used for culturing *S. epidermidis, P. aeruginosa* and *S. aureus*. All organisms were grown at 37 °C. Preparation of the agar disc-diffusion plates involved seeding the agar plates with a sterile swab dipped in a 1/50 dilution of the appropriate 16-h culture of bacteria. Three discs of each material were placed on the inoculated plates and the plates were cultured for 24 h at 37 °C. The agar diffusion test was performed under standard laboratory sterile conditions using a fumigation hood with sterile swabs for inoculation of bacteria.

Electronic calipers were used to measure zones of inhibition at three different diameters for each disc and zone sizes were calculated as follows:

$$\text{Inhibition Zone (mm)} = \frac{\text{Halo}\,\phi - \text{Disc}\,\phi}{2} \quad (1)$$

All cements were analysed in triplicate and mean zone sizes ± standard deviations were calculated.

Preparation of agar specimens

Agar strips (3 × 10 × 5 mm) were prepared for investigation by EDX from the agar diffusion test. The specimens were cut from the assay, extending from the cement disc, through any inhibition zone, through to the bacterial colony. The agar specimens were then placed on a glass slide and incubated at 37 °C in an air-assisted oven for 24 h until dry.

Quantitative EDX

A Hitachi SU-70 field emission scanning electron microscope (FE-SEM) equipped with an Oxford Instruments Energy Dispersive X-ray spectroscopy (EDX) system was used to obtain secondary electron images and carry out chemical analysis of the agar samples. All EDX spectra were collected at 20 kV and quantitative EDX converted the collected spectra into concentration data using standard reference spectra obtained from pure elements under similar operating parameters.

Statistical analysis

One-way analysis of variance (ANOVA) was employed to compare the antibacterial efficacy of the experimental materials in relation to (1) specific bacterial strains, (2) maturation when implanted with an antibiotic. Comparison of relevant means was performed using the post hoc Bonferroni test. Differences between groups was deemed significant when $p \leq 0.05$. Statistical analysis was performed using SPSS software for windows version 16 (SPSS Inc. Chicago, IL).

Results and discussion

The primary objective of this study is to investigate the antibacterial response of a number of commercially available materials and one experimental material in an in vitro model. The first bacterium tested was a gram negative aerobic bacteria, *E. coli*, an opportunistic pathogen reported by Rafiq et al. [5] to account for 6% of all organisms infecting Total Hip Arthroplastys (THR) from the period 1974–2005. Research by van de Brand et al. [27] also found that *E. coli* was present in 60% of cultures where intermittent catheterization was required in joint

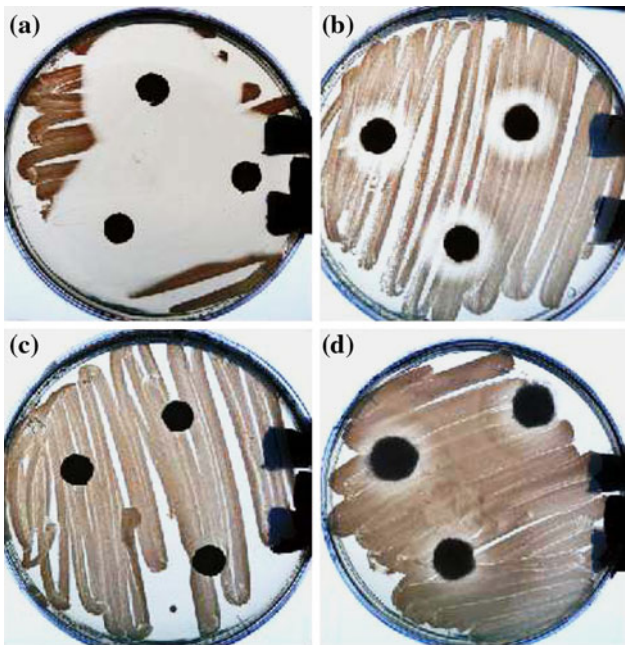

Fig. 1 Cements tested in *S. epidermidis* where **a** S_{Tob}, **b** B_{TSC}, **c** S_{pine} and **d** N_{Put}

arthroplasty. In relation to this study the biomaterials tested had more success in eliminating *E. coli* than any of the other bacteria examined. Figure 1 shows sample images of the agar diffusion test performed with *S. epidermidis*. Figure 2a shows the commercial materials and experimental GPC tested against *E. coli*.

From Fig. 2a, it can be seen that S_{Tob} proved to be the most effective in killing *E. coli*; achieving inhibition zones of 8.3 ± 1.69 mm. However, the experimental GPC, B_{TSC} produced inhibition zones of 5.9 ± 0.05 mm. Both S_{pine} and N_{Put} did not produce any zones of inhibition in *E. coli*. Statistical analysis was performed between each material for variance, and this was done for each bacterium. It was found that there are significant differences between the two cements that showed inhibition, S_{Tob} and B_{TSC} ($p = 0.001$). S_{Tob} and B_{TSC} showed significant differences when compared with all other materials tested (Fig. 2a) also there was found to be no significant difference between S_{pine} and N_{Put} ($p = 1.000$), as these materials showed no inhibition. This was similar for all bacterium tested where S_{pine} and N_{Put} showed no inhibition for *S. epidermidis*, *P. aeruginosa* and *S. aureus* ($p = 1.000$) as shown in Fig. 2b–d. S_{Tob}

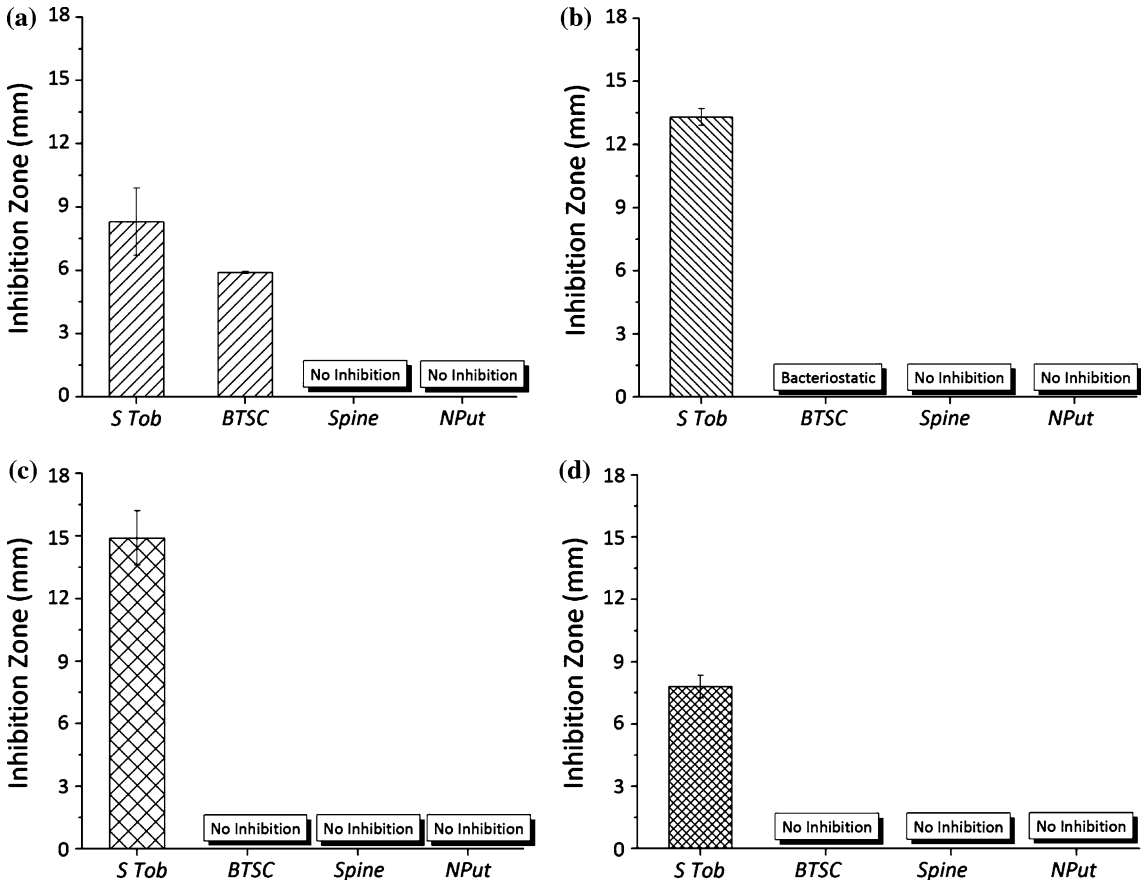

Fig. 2 Antibacterial properties of experimental materials in **a** *E. coli*, **b** *S. epidermidis*, **c** *P. aeruginosa* and **d** *S. aureus*

Table 2 Analysis of variance (Bonferroni post hoc) between biomaterials and bacteria spp

	E. coli	*P. aeruginosa*	*S. epidermidis*	*S. aureus*
S_{Tob} vs. B_{TSC}	0.001	0.000	0.000	0.000
S_{Tob} vs. S_{pine}	0.000	0.000	0.000	0.000
S_{Tob} vs. N_{put}	0.000	0.000	0.000	0.000
B_{TSC} vs. S_{pine}	0.000	1.000	1.000	1.000
B_{TSC} vs. N_{put}	0.000	1.000	1.000	1.000
S_{pine} vs. N_{Put}	1.000	1.000	1.000	1.000

The mean difference is significant at the 0.05 level

showed significant differences in each bacterium where it produced inhibition zones of 13.3 ± 0.4 mm for *S. epidermidis*, 14.9 ± 1.3 mm for *P. aeruginosa* and 7.8 ± 0.5 mm for *S. aureus*. These results were also significantly different to all other materials (Table 2) as no other material produced clear inhibition zones for *S. epidermidis*, *P. aeruginosa* and *S. aureus*. However, some antibacterial efficiency was observed with B_{TSC} when tested in *S. epidermidis* (Fig. 1). Inhibition zones were not recorded as isolated colonies of bacteria were present within the inhibition zone. This indicates that B_{TSC} is bacteriostatic in relation to *S. epidermidis* and not bacteriocidal. B_{TSC} is likely to be inhibiting the growth and proliferation of these bacteria as opposed to killing the cells outright.

With respect to each bacterium, S_{Tob} was found to be bacteriocidal and outperformed each of the competing materials. This is due to the presence of the antibiotic Tobramycin (Tob). Tob is generally used to treat gram negative bacterial infections such as skin or bone, lower respiratory tract and central nervous system (CNS) infections [28]. Studies by Le Goffic et al. describe Tob mechanism of action in relation to *E. coli*. Tob was found to occupy two binding sites on the ribosomes of the bacterial cell. The primary one is likely responsible for the inhibition of protein synthesis, whereas the secondary one is related to the misreading of the messenger RNA [29]. Tob has also previously been used to treat *P. aeruginosa* infections in patients with cystic fibrosis [30, 31], and has been described by D'Arrigo et al. to show excellent in vitro and clinical activities against susceptible strains of *E. coli* and *S. aureus* by inhibiting plasma membrane functions and by targeting ribosomal sites [32]. However, overuse of antibiotics in medicine promoted the evolution of some species of bacteria to infer resistance. These include the Methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) strains which are responsible for a considerable amount of hospital acquired infections [33–35]. The primary reason for increased usage of antibiotics in implant-related infections is that bacteria change their biological behaviour when adhering to implant surfaces [2]. They produce a biofilm which forms a protective barrier and they can also reduce their metabolic activity and increase their generation time. As antibiotics act on growing bacteria, an increased minimum inhibitory concentration (MIC) of antibiotic is required to affect bacteria with reduced metabolic activity. Studies have found that 800-fold higher MIC for Tob in adhesive *P. aeruginosa* than for non-adhesive [2, 7]. Concerns also arise regarding the quantity of antibiotic released and the longevity around the implant site. After the high initial release of antibiotics, the long-term low concentration around implants may also contribute to antibiotic-resistant strains [2, 7]. The effectiveness of such materials is dependent on the rate and manner of which the drug is released, and this is directly related to the host material [7]. Drug release from materials is achieved through mechanisms of water pore penetration, soluble matrix dissolution and diffusion of the solubilized drug via matrix imperfections (pores/cracks). However, PMMA-based materials display a biphasic release pattern characterized by a high initial release followed by a long tail of low, ineffective and largely incomplete release that continues for days or months. Studies reveal that less than 10% of the trapped drug is eventually released from the cement [7]. Another concern is that antibiotics such as Tob can have harmful side effects in the body. Tob belongs to a group called aminoglycosides which are known to be nephrotoxic and ototoxic, depending on the choice of aminoglycoside and duration of therapy [36]. Increasing the dosage of antibiotic to reach the MIC when combating adherent biofilms may subsequently result in more traumas to local tissues.

To determine the efficacy of antibiotic release from S_{Tob} and a GPC (B_{Tob}), testing was performed on *S. epidermidis* over a period of 0–14 days. Figure 3 shows the comparison

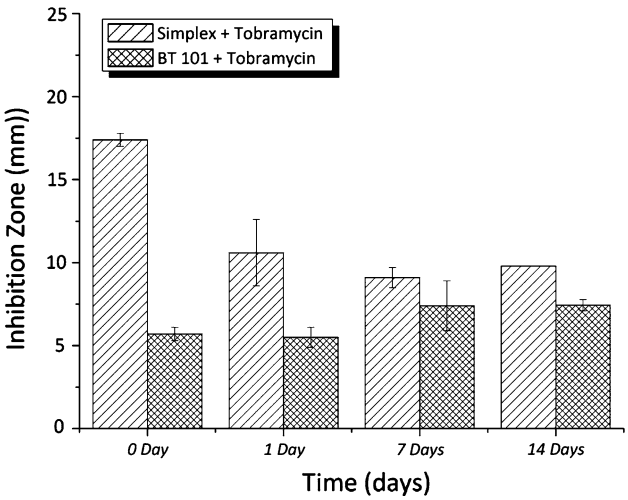

Fig. 3 Comparison of S_{Tob} and B_{Tob} in *S. epidermidis* with respect to maturation

Table 3 Analysis of variance (Bonferroni post-hoc) between S_{Tob} and B_{Tob} in *S. epidermidis* with respect to maturation

Time (days)	S_{Tob}	B_{Tob}
a		
0 vs. 1	0.000	1.000
0 vs. 7	0.000	0.043
0 vs. 14	0.000	0.041
1 vs. 7	0.589	0.021
1 vs. 14	1.000	0.020
7 vs. 14	1.000	1.000
b		
S_{Tob}. vs. B_{Tob}	0 day	0.000
	14 day	0.001

The mean difference is significant at the 0.05 level

of S_{Tob} and B_{Tob} in *S. epidermidis* with respect to maturation.

From Fig. 3 it can be seen that there is a significant decrease in antibacterial efficacy from 0 (17.4 ± 0.4 mm) to 14 days (9.8 ± 0.01 mm) regarding S_{Tob}. However, there is no significant change when comparing 1, 7 and 14 days ($p = 0.589$, 1.000, 1.000; Table 3a). This suggests that the set material is retaining some of the antibiotic in the bulk and that the surface antibiotic has solubilized and was expended after initial testing at 0 days. B_{Tob}, on the other hand, was found to show a significant ($p = 0.041$) increase in inhibition over the period of 0 (5.7 ± 0.4 mm) to 14 days (7.4 ± 0.3 mm). It was expected that B_{Tob} would perform better than the results obtained here as GPCs are ion leachable materials. However, this may be the reason for the lower results found as compared to S_{Tob}. It may be possible that ion processes within the cement are reducing the efficacy of the antibiotic. Considering this, however, antibacterial efficacy was found to increase with respect to maturation. Table 3b confirms that there remains a significant change in inhibition between S_{Tob} and B_{Tob} at initial testing, 0 days and after 14 days. Further testing to 30 or 90 days may provide more conclusive results regarding the longevity and efficacy of antibiotic implanted materials.

Further testing was performed to determine what was causing the antibacterial efficacy of the materials. This was achieved by performing EDX on agar specimens from the agar extracted from the area immediately surrounding the samples, whether inhibition was present or not. Figure 4 shows the EDX results obtained from (a) S_{Tob}, (b) B_{TSC}, (c) S_{pine} and (d) N_{Put}.

It is evident from Fig. 4a that there is little change in the composition of the agar regarding S_{Tob}. In relation to S_{Tob}, the antibacterial properties are as a result of antibiotic release from the cement. Antibiotics are complex organic structures and techniques such as EDX are ineffective as only compositional elemental analysis is possible. Alternative methods would have to be employed to quantitatively determine antibiotic content. Figure 4b reveals the

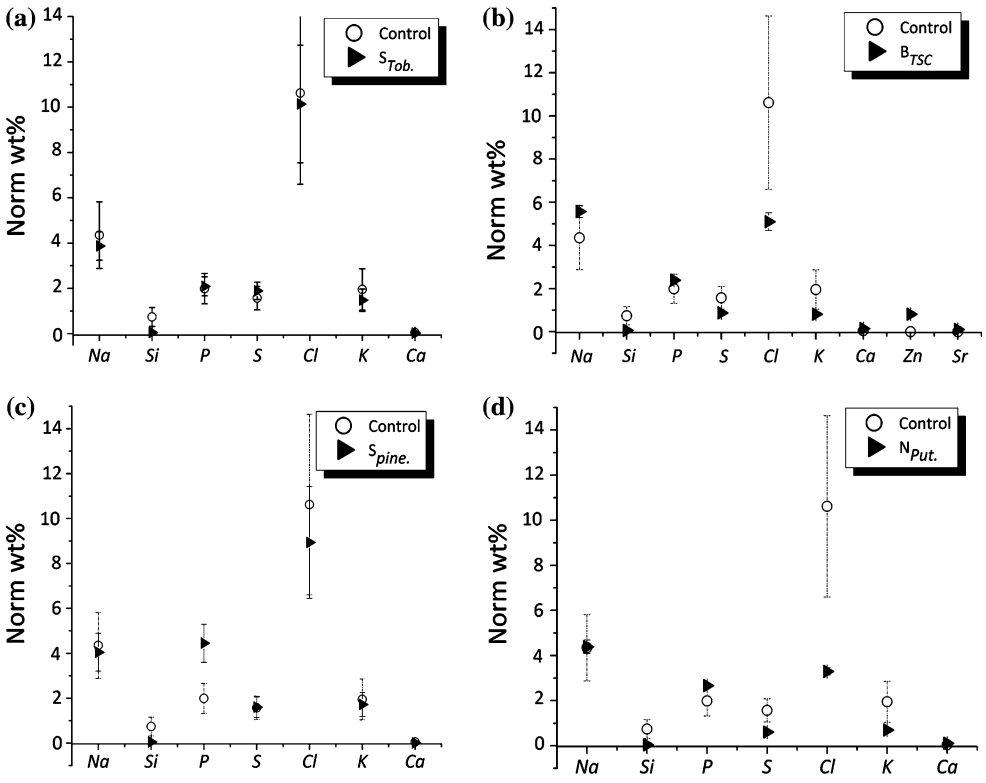

Fig. 4 EDX of agar samples comparing control agar with agar from plates containing materials. **a** Simplex + Tobramycin, **b** BT101 + 10 wt% TSC, **c** Spineplex, **d** Novabone Putty

presence of approximately 1-wt% Zn, whereas no Zn was found in the control agar. There was also a small quantity of Sr (0.1 wt%) detected, where again, no Sr was present in the control agar. It is difficult to determine the precise mechanism by which B_{TSC} is exhibiting antimicrobial properties as TSC present in the cement is known to be an antibacterial agent itself [37, 38]. Previous study by the authors determined that TSC prolongs the setting [39] of this cement facilitating greater ion release [40]. It has also been previously been reported that by increasing the concentration of TSC and Zn ion release, the antibacterial efficacy of the material increases [40]. Zn is an antibacterial ion which acts as an inhibitor of multiple activities in the bacterial cell, such as glycolysis, transmembrane proton translocation and acid tolerance [41]. It has also been cited by Shashibhushan et al. [18] that Zn can interfere with substrate transport and oxidation and can react with thiol groups on the bacterial cell wall. Zn can also change protein structure leading to inhibition of specific metabolic enzymes, thereby causing growth inhibition [18]. Another possible reason for inhibition by B_{TSC} could be explained by the mechanism of action of TSC, which is known to chelate Ca [42], which in this case slows the setting reaction in these cements [39]. However, regarding bacterial inhibition, TSC may be binding any Ca being used by the bacteria for metabolism. There was no antibacterial effect attributed to S_{pine} and N_{Put}, however, there were slight changes in the EDX patterns. Figure 4c shows that there was an increase in the phosphorus (P) concentrations as compared to the control agar. This may be due to bacteria metabolising as they proliferate on the surface of the agar. There was also a very slight increase in P content in the agar surrounding N_{Put} (Fig. 4d). These materials exhibited no antibacterial properties when tested against these bacteria as S_{pine}, in particular, contains no antimicrobial compounds or ions. This cement is implanted and remains as an inert material exhibiting no bioactivity. N_{Put} is based on a Bioglass formulation suspended in a gelatin binder, however, the composition failed to inhibit the proliferation of the bacteria tested.

To conclude, S_{Tob} was the only material to exhibit antibacterial properties when tested in each bacterium. However, the ongoing discussion as regards the overuse of antibiotics suggests that additional ways of combating infection would be welcome. B_{TSC} was the only other material to exhibit antibacterial properties and this was only evident against *E. coli* and *S. epidermidis*. The advantage of using a GPC over an acrylic cement is that beneficial ions can be absorbed and re-released when in an ion-rich environment. However, further research is required to evaluate at what extent this occurs. S_{pine} and N_{Put} are two commercially available materials that were found to exhibit no antibacterial properties, and as such this leaves room for development of more bioactive materials with properties suitable for use in orthopaedics.

References

1. Subbiahdoss G, Kuijer R, Grijpma DW, Van der Mei HC, Busscher HJ (2009) Acta Biomater 5:1399
2. Diefenbeck M, Muckley T, Hofmann GO (2006) Injury 37:S95
3. An YH, Friedman RJ (1997) J Microbiol Meth 30:141
4. Dillow AK, Tirrell M (1998) Curr Opin Solid State Mater Sci 3:252
5. Rafiq I, Gambhir AK, Wroblewski BM, Kay PR (2006) Int Orthop 30:532
6. Squire MW, Ludwig BJ, Thompson JR, Jagodzinski J, Hall D, Andes D (2008) J Arthroplasty 23(1):110
7. Zilberman M, Elsner JJ (2008) J Cont Rel 130:202
8. Takahashi Y, Imazato S, Kaneshiro AV, Ebisu S, Frencken JE, Tay FR (2006) Dent Mater 22:647
9. Marczuk-Kolada G, Jakoniuk P, Mystkowska J, Luczaj-Cepowicz E, Waszkiel D, Dabrowski JR, Leszczynska K (2006) Postepy Hig Med Dosw 60:416
10. Seppa L, Forss H, Ogaard B (1993) J Dent Res 72(9):1310
11. Hayacibara MF, Rosa OPS, Koo H, Torres SA, Costa B, Cury JA (2003) J Dent Res 82(4):267
12. Daugela P, Oziunas R, Zekonis G (2008) Stomatol, Baltic Dent Maxillofac J 10:16
13. Polizzi S, Pira E, Ferrara M, Bugiani M, Papaleo A, Albera R, Palmi S (2002) Neuro Toxicol 23:761
14. Reusche E, Pilz P, Oberascher G, Linder B, Egensperger R, Gloeckner K, Trinka E, Iglseder B (2001) Human Pathol 32(10):1136
15. Firling CE, Hill TA, Severson AR (1999) Arch Toxicol 73:359
16. Sawai J (2003) J Microbiol Meth 54:177
17. Sawai J, Shinobu S, Igarashi H, Hashimoto A, Kokugan T, Shimizu M, Kojima K (1998) J Ferm Bioeng 86(5):521
18. Shashibhushan KK, Basappa N, Subba Reddy VV (2008) J Indian Soc Pedod Prev Dent 26(2):S56
19. Yamaguchi M, Ma ZJ (2001) Cal Tissue Int 69:158
20. Yamaguchi M, Ma ZJ (2001) J Bone Miner Metab 19:38
21. Singh B, Lim MR (2007) Oper Tech Orthop 17:199
22. Mack D, Becker P, Chatterjee I, Dobinsky S, Knobloch JK-M, Peters G, Rohde H, Herrmann M (2004) Int J Med Microbiol 294:203
23. Papagelopoulos PJ, Mavrogenis AF, Giannitsioti E, Kikilas A, Kanellakpooulou K, Soucacos PN (2007) J Arthroplasty 22(3):457
24. Andonian S, Rabah DM, Aprikian AG (2002) Urology 60(4):698
25. Arciola CA, Campoccia D, Gamberini S, Donati ME, Pirini V, Visai L, Speziale P, Montanaro L (2005) Biomaterials 26:6530
26. Heyer CM, Al-Hardi A, Mueller K-M, Stachon A, Nicolas V (2008) Acad Radiol 15:901
27. van den Brand ICJB, Castelein RM (2001) J Arthroplasty 16(7): 850
28. Du J, Jasti B, Vasavada RC (1997) J Control Release 43:223
29. Le Goffic F, Tangy F, Moreau B, Capmau M-L (1979) J Antibiot 32(12):1288
30. Ratjen F, Doring G, Nikolaizik WH (2001) Lancet 358:983
31. Moriarty F, Elborn S, Tunney M (2005) J Microbiol Meth 61:171
32. D'Arrigo M, Ginestra G, Mandalari G, Furneri PM, Bisignano G (2009) Phytomed. doi:10.1016/j.phymed.2009.07.08 (in press)
33. Talan DA (2008) Ann Emerg Med 51(3):299
34. Harris LG, Richards RG (2006) Injury 37:S3

35. Rabaud C, Mauuary G (2001) Pathol Biol 49:812
36. Lode H (1998) Curr Ther Res 59(7):420
37. Weijmer MC, Debets-Ossenkopp YJ, van de Vondervoort FJ, Ter Wee PM (2002) Neph Dial Trans 17:2189
38. Weijmer MC, Van den Dorpel M, Van de Ven PJG, Ter Wee PM, Van Geelen JACA, Groeneveld JO, Van Jaarsveld BC, Koopmans MG, Le Poole CY, Schrander-van der Meer AM, Siegert CEH, Stas KJF (2004) J Am Soc Neph 16:2769
39. Boyd D, Clarkin OM, Wren AW, Towler MR (2008) Acta Biomater 4:425
40. Wren AW, Boyd D, Thornton R, Cooney JC, Towler MR (2009) J Biomed Mat Res B: App Biomat 90-B(2):700
41. Daugela P, Oziunas R, Zekonis G (2008) Stomatol, Baltic Dent Maxillofac J 10:16
42. Schneiders W, Reinstorf A, Pompe W, Grass R, Biewener A, Holch M, Zwipp H, Rammelt S (2007) Bone 40:1048