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Incorporation of chitosan in acrylic bone cement: Effect on antibiotic release, bacterial biofilm formation and mechanical properties

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Abstract Bacterial infection remains a significant problem following total joint replacement. Efforts to prevent recurrent implant infection, including the use of antibiotic-loaded bone cement for implant fixation at the time of revision surgery, are not always successful. In this in vitro study, we investigated whether the addition of chitosan to gentamicin-loaded Palacos® R bone cement increased antibiotic release and prevented bacterial adherence and biofilm formation by *Staphylococcus* spp. clinical isolates. Furthermore, mechanical tests were performed as a function of time post-polymerisation in pseudo-physiological conditions. The addition of chitosan to gentamicin-loaded Palacos® R bone cement significantly decreased gentamicin release and did not increase the efficacy of the bone cement at preventing bacterial colonisation and biofilm formation. Moreover, the mechanical performance of cement containing chitosan was significantly reduced after 28 days of saline degradation with the compressive and bending strengths not in compliance with the minimum requirements as stipulated by the ISO standard for PMMA bone cement. Therefore, incorporating chitosan into gentamicin-loaded Palacos® R bone cement for use in revision surgery has no clinical antimicrobial benefit and the detrimental effect on mechanical properties could adversely affect the longevity of the prosthetic joint.

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1 Introduction

Bacterial infection remains a major problem after total joint replacement (TJR) surgery, with implant infection, mainly with *Staphylococcus* spp. reported in up to 24% of procedures [1, 2]. As the infecting bacteria grow largely within a confluent biofilm on the surface of the implant, which render them resistant to current antibiotics, attempts to eradicate infection with systemic antibiotics are usually ineffective [3]. Therefore, removal and replacement of the infected implant is required to eliminate infection [3].

Gentamicin-loaded bone cement is normally used for prosthesis fixation at the time of revision TJR, in an attempt to eradicate bacterial infection and reduce the risk of infection of the newly implanted prosthesis [4, 5]. Increasingly, gentamicin-loaded bone cement is also being used for prosthesis fixation during primary TJR's in an attempt to prevent implant infection. However, the isolation of gentamicin resistant bacteria from infected prosthetic hip joints has raised concerns about the increased use of gentamicin-loaded bone cement [6–8]. Furthermore, we have shown that the bacteria which cause these infections can form bacterial biofilms on the surface of gentamicin-loaded bone cements despite the release of gentamicin [9].

Therefore, there is a clear need for the development of alternative antimicrobial strategies for the prevention and treatment of prosthetic joint infection. In recent years, functional biomaterials research has been directed towards the development of improved/new drug delivery systems with considerable attention given to chitosan based materials [10]. Chitosan is a co-polymer of glucosamine and *N*-acetylglucosamine deacetylated from the natural polymer chitin and in its crystalline form it is non-toxic, biodegradable and biocompatible. These properties have

resulted in chitosan been used for tissue engineering scaffolds, cell microcapsules and as a drug delivery carrier for antibiotics [10, 11]. Furthermore, chitosan has been shown to have intrinsic antibacterial activity when added to acrylic bone cement. Therefore, there is the real potential that chitosan added to bone cement could act as a drug delivery carrier for gentamicin, thereby increasing gentamicin release and preventing bacterial colonisation and biofilm formation. However, one concern is that the incorporation of chitosan into the bone cement may result in a reduction of the mechanical performance of the cement due to its degradation. Therefore, in this in vitro study, we added chitosan to unloaded and gentamicin-loaded bone cement and determined the effect this addition had on bacterial biofilm formation, gentamicin release and mechanical properties of the bone cement.

2 Materials and methods

2.1 Materials

The bone cements used were unloaded Palacos® R containing no gentamicin and Palacos® R + G which contains 0.5 g gentamicin per 40 g polymethylmethacrylate (PMMA) powder cement sachet (Heraeus-Kulzer, Hanau, Germany). Chitosan was purchased (Primex Ingredients ASA, Norway) and refined twice by dissolving it in dilute acetic acid solution. The solution was filtered and the chitosan was precipitated with aqueous sodium hydroxide and then dried in a vacuum oven for 24 ± 0.5 h at 40 ± 0.5°C. The extent of deacetylation of the chitosan powder was approximately 85%. The average molecular weight was 2,000–3,000 g mol^{-1}, as determined by gel permeation chromatography.

2.2 Preparation of bone cements

Different levels (1, 3 and 5% w/w) of chitosan were added to the polymer powder (40 g) of unloaded and gentamicin-loaded Palacos® R bone cement. The chitosan was uniformly dispersed within the cement powder using a small-scale turbo blender (Speed Mixer™ Model PAC 150FVZ; Rondol Technology Ltd., UK) at a speed of 1500 ± 50 rpm for 30 ± 1 s. After the chitosan was blended into the cement powder, both the polymer powder and liquid monomer were refrigerated for 24 ± 0.5 h at a temperature of 4 ± 0.5°C. Following refrigeration, the bone cements were vacuum mixed according to the manufacturer's instructions. Bone cement specimens for determination of gentamicin release and biofilm formation were then prepared by injecting the cement dough between two glass plates and, following curing, cutting 1.0 ± 0.1 cm^2 sections which were then stored under dark, sterile conditions at 22 ± 0.5°C. Bone cement samples used for mechanical testing were prepared by injecting the mixed bone cement into PTFE moulds which were allowed to cure for a minimum of 24 h. The samples were then removed from the mould. Rough specimen edges were removed by sanding with 1,200 μm grit silicon carbide abrasive. The cement samples used for measuring the compressive properties were cylinders of 12 ± 0.1-mm length and diameter 6 ± 0.1 mm. The samples for determining the bending properties were 75 ± 0.5 mm in length, 10 ± 0.1 mm in width, 3.3 ± 0.1 mm in thickness. Unloaded Palacos® R and Palacos® R + G, which contained no chitosan were used as controls in all experiments.

2.3 Determination of gentamicin release

Cement sections were placed in 5 ml of sterile water and stored in the dark at 37 ± 0.5°C. At designated time intervals (6, 24, 48 and 72 h), each section was removed, a 1 ml sample of the water taken and stored at −20°C until analysed and the section placed in 5 ml of fresh sterile water until the next time point. All samples were analysed for gentamicin by high performance liquid chromatography–mass spectrometry (Waters 2795 Alliance HT LC system; UK), with gentamicin release expressed relative to the surface area of the cement section [12]. For each bone cement, 12 sections were used (three sections at each of the four designated time intervals).

2.4 Bacterial biofilm formation

The three strains used were clinical isolates cultured as described previously from implants retrieved at revision TJR surgery [1]. The isolates chosen were *Staphylococcus epidermidis*, *S. capitis* and *S. aureus*, which are typical of the bacterial species that have been implicated most commonly in TJR infection. Biofilms were formed for each strain in triplicate on unloaded, gentamicin-loaded, chitosan-loaded and gentamicin/chitosan-loaded bone cement sections over designated time intervals (6, 24, 48 and 72 h) as described previously [9]. At each time point, the bone cement sections were removed, washed and biofilm formation quantified by total viable count. The unloaded Palacos® R cement containing 1–5% w/w chitosan specimens were included in the experiment to determine the intrinsic antibacterial activity of the chitosan.

2.5 Determination of mechanical properties

To simulate the degradation process, the bone cement specimens were placed in an incubator at 37 ± 0.5°C for

1, 7, 14, 21 and 28 days, with physiological saline (0.9% NaCl, pH = 7.4), which was not replaced. The compressive and bending properties for each bone cement mix were determined in accordance with ISO 5833 using a universal material test system (EZL 6000R, Lloyds Instrument Ltd., UK). All compression tests were conducted at a crosshead speed of 20 mm min^{-1} and the bending tests at 5.0 mm min^{-1}, respectively [13]. The compressive strength was determined based on the ultimate load recorded at specimen failure. The bending strength and bending modulus were calculated based on the ultimate load and the difference between the deflections under the loads of 15 and 50 N [13]. A total of 18 specimens of each bone cement mix were used for the determination of each property.

2.6 Statistical analysis

Data collated for all experimental tests were evaluated for statistical significance using a one-way analysis of variance with P-value < 0.05 denoting significance. Post-hoc tests were conducted using the Student-Newman-Keuls and Duncan methods. All the tests were conducted using commercially available software (InStat 3.06; GraphPad Software, USA).

3 Results

3.1 Cumulative gentamicin release

Figure 1 summarises gentamicin release as a function of time. For all the bone cements tested, gentamicin release was most rapid during the first 6 h and continued at a much lower rate thereafter. The addition of 1–5% w/w chitosan to the bone cement resulted in a significant decrease ($P < 0.0001$) in the total amount of gentamicin released over 72 h. After 72 h, the cumulative gentamicin release for Palacos® R + G and Palacos® R + G loaded with 1, 3 and 5% chitosan was 18.02 ± 0.08, 10.65 ± 0.76, 10.27 ± 4.45 and 12.51 ± 1.47 μg cm^{-2}, respectively.

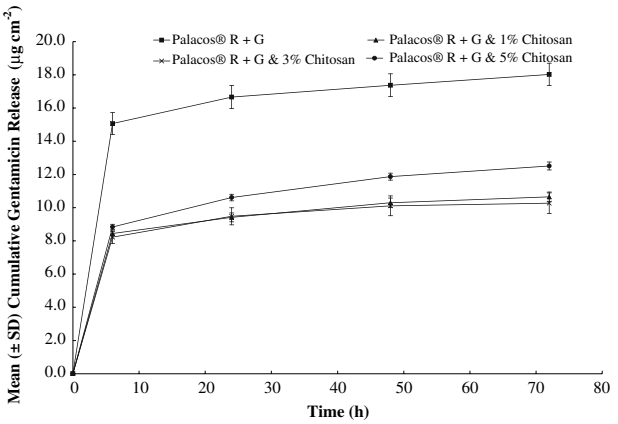

Fig. 1 Cumulative gentamicin release from gentamicin loaded Palacos® (Palacos® R + G) bone cement containing different levels of chitosan. Results are the mean ± SD of three replicates at each time point

3.2 Bacterial biofilm formation

Incorporation of 1–5% w/w chitosan to unloaded Palacos® R bone cement had no effect on bacterial colonisation and biofilm formation, with biofilms formed in similar numbers on chitosan-loaded and unloaded cement by the three strains tested at each time point (Fig. 2a–c). Furthermore, the addition of 1–5% w/w chitosan to gentamicin-loaded Palacos® R (Palacos® R + G) cement had an adverse effect on the efficacy of the bone cement in preventing bacterial colonisation and biofilm formation. This adverse effect was only apparent at 6 h for *S. epidermidis* and *S. capitis* isolates and was demonstrated by the fact that these strains adhered to the gentamicin-loaded cement containing chitosan but did not adhere to the gentamicin-loaded cement without chitosan (Fig. 2a, b). For *S. aureus*, gentamicin loading of the bone cement did not prevent bacterial colonisation and biofilm formation at any of the time points tested (Fig. 2c). Therefore, the adverse effect of incorporating chitosan, which was again only apparent at 6 h, was demonstrated by the fact that this isolate adhered in greater numbers to the gentamicin-loaded cement containing chitosan in comparison to the cement containing gentamicin only.

3.3 Mechanical properties

The mechanical properties of each bone cement type during the degradation period are shown in Fig. 3. Prior to degradation, no significant difference was observed for compressive strength ($P = 0.38$) and bending strength ($P = 0.07$) when comparing the control cements and the gentamicin-loaded bone cements containing chitosan (Fig. 3a, b). After the degradation period of 28 days, the compressive and bending strengths of the unloaded Palacos® R cement ($P = 0.07$) and gentamicin-loaded Palacos® R (Palacos® R + G) cement ($P = 0.08$) had not change significantly. In contrast, after the 28-day degradation period, the compressive and bending strengths of the gentamicin-loaded bone cements containing chitosan had decreased significantly ($P < 0.0003$).

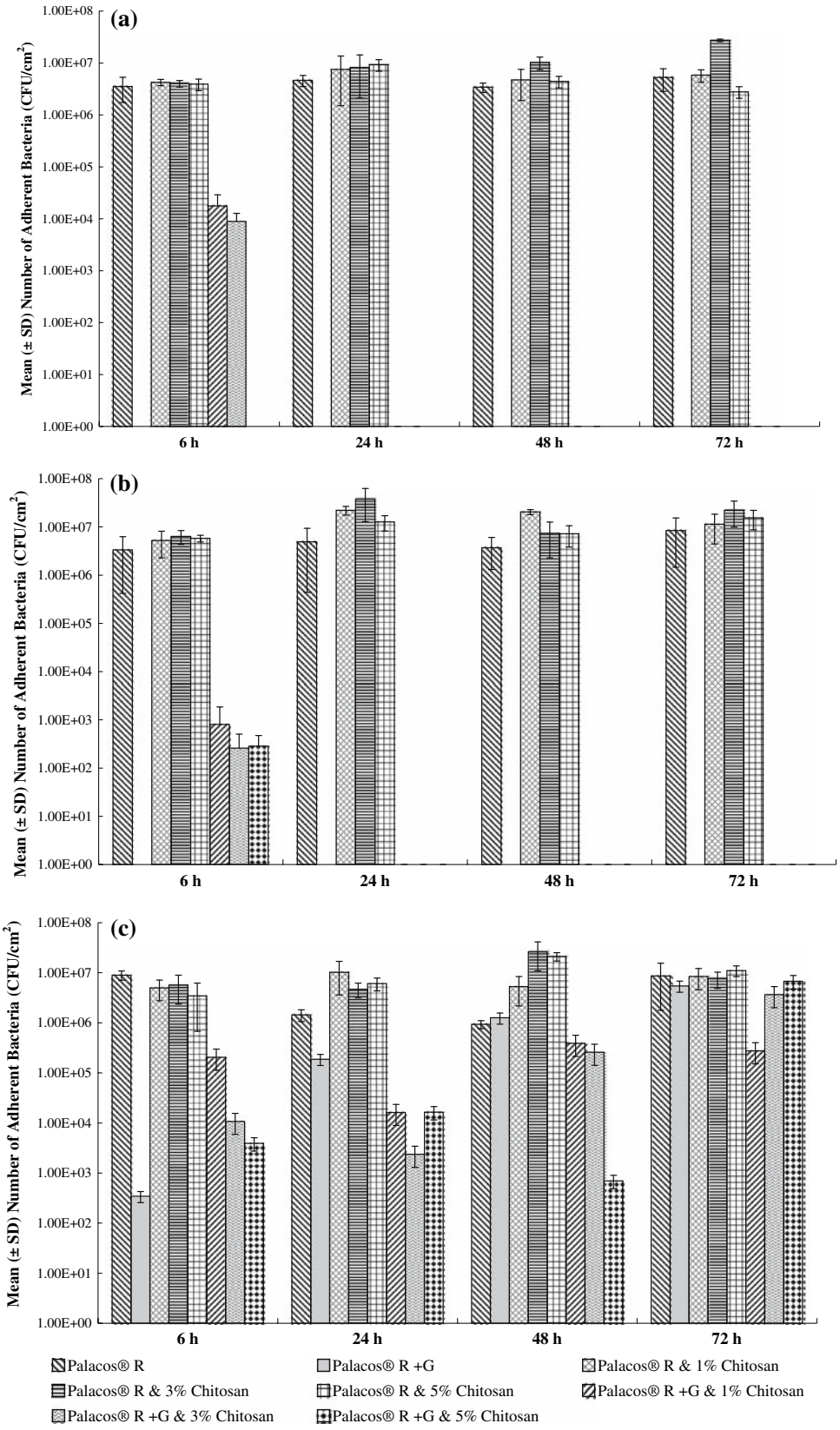

Fig. 2 Colonisation of unloaded (Palacos® R) and gentamicin-loaded (Palacos® R + G) bone cements containing different levels of chitosan by clinical prosthetic hip isolates; (**a**) *S. epidermidis* (**b**) *S. capitis* and (**c**) *S. aureus.* Results are the mean ± SD of three replicates at each time point

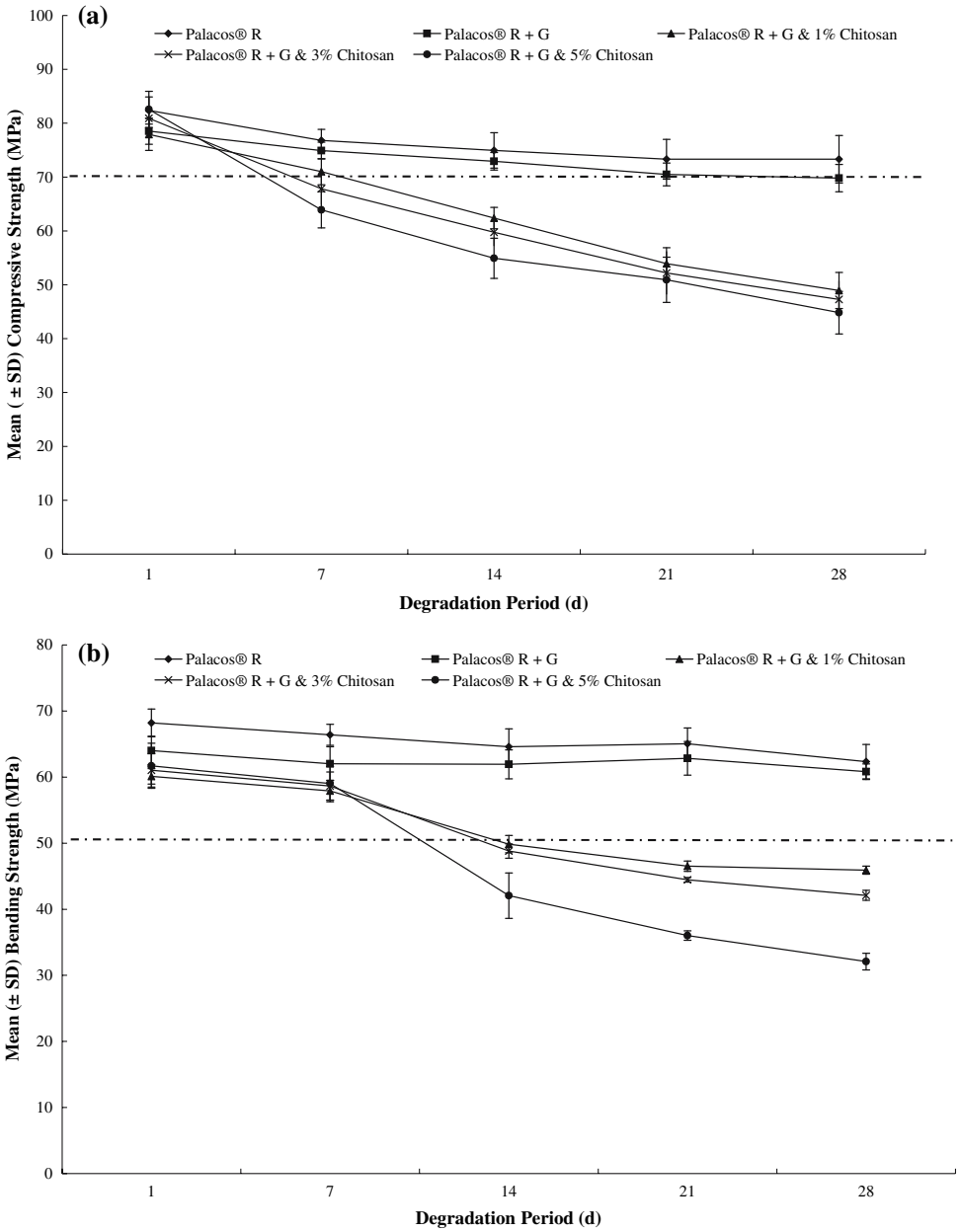

Fig. 3 Mechanical properties of unloaded (Palacos® R) and gentamicin-loaded (Palacos® R + G) bone cements containing different levels of chitosan; (**a**) compressive strength and (**b**) bending strength. Dashed line represents minimum ISO requirements. Results are the mean ± SD of three replicates at each time point

4 Discussion

Chitosan has previously been reported to possess intrinsic antibacterial activity. For example, it has been shown that chitosan can reduce the infection rate of experimentally induced *S. aureus* osteomyelitis in rabbits [14]. Furthermore, when added to hydroxyapatite and plaster of Paris to obtain a composite for sustained vancomycin or fosfomycin release, the composite material was able to inhibit *S. aureus* in vitro for as long as three months [15]. As chitosan is also used as a carrier for drug delivery, we hypothesised that chitosan added to bone cement could act as a drug delivery carrier for gentamicin. Moreover, although the exact mechanism by which antibiotics are released from PMMA cement is unknown, it has been suggested that surface elution and diffusion are the primary mechanisms of antibiotic release from the loaded material [16, 17]. Therefore, if cement porosity could be increased as a result of chitosan degradation facilitating a more efficacious route for antibiotic release, gentamicin release should be increased thereby preventing bacterial colonisation and biofilm formation on the bone cement surface.

However, the results of our study clearly show that the addition of chitosan to bone cement had a detrimental effect on gentamicin release which was significantly decreased as a result of the incorporation of chitosan. The reduction in gentamicin release from chitosan-loaded cement could potentially be as a result of the fact the

particle size of chitosan is significantly greater than that of gentamicin. Therefore, chitosan retained on the surface of the cement could impede the release of the smaller gentamicin particles from the cement. Furthermore, it is possible that the chitosan does not degrade sufficiently within the first 72 h to facilitate the release of the high local gentamicin concentrations required to eliminate existing infection and prevent infection of the newly implanted device. Significantly, when overt infection was simulated using *Staphylococcus* spp. clinical isolates, this decrease in gentamicin release had an adverse effect on the ability of the bone cement to prevent bacterial adherence and biofilm formation.

In addition, the incorporation of 1–5% w/w chitosan in unloaded Palacos® bone cement did not prevent or reduce bacterial adherence and biofilm formation on the surface of the bone cement suggesting that when chitosan is added to bone cement it does not possess intrinsic antibacterial activity. One potential explanation for this lack of effect is that the porous microstructure that develops through the degradation of a bioactive material such as chitosan could provide additional niches for the attachment of bacteria which subsequently form a bacterial biofilm. Interestingly, our results contrast with the results of a previous paper which reported that the incorporation of chitosan and chitosan nanoparticles resulted in a 1–2 $\log_{10}$ reduction in *S. aureus* and *S. epidermidis* adherence to bone cement not loaded with gentamicin [18]. However, there a number of differences between the two studies which may account for the contrasting results. For example, Shi et al. incorporated chitosan and chitosan nanoparticles at a bone cement powder ratio of 15% which is much higher than the 1–5% ratio used in the present study. Furthermore, Shi et al. used an attachment time of only 3 h which would be sufficient to allow adherence but not bacterial biofilm formation [18]. In contrast, we used a minimum attachment time of 6 h and examined biofilm formation at a number of additional time points, thereby more closely simulating in vivo conditions. Significantly, all three *Staphylococcal* spp. isolates used in the present study are clinical isolates cultured from retrieved prosthetic hip implants and are known biofilm formers. This is in contrast to Shi et al. who used *S. epidermidis* isolate ATCC 12228 which has been categorically shown to be a non-biofilm former in numerous previous studies [19–21] and would, therefore, not be suitable for use in bacterial adherence assays.

The mechanical properties of the PMMA cement used in orthopaedic surgery play an important role in determining the successful long-term stability of the prosthesis. In the compression and bending tests conducted prior to degradation, there was no significant difference in the compressive and bending strength of the control cement and the gentamicin-loaded bone cements containing chitosan. Furthermore, for all cements tested, the mechanical properties measured were in excess of the minimum values stipulated by the ISO standard for acrylic bone cement [13]. The unloaded Palacos® R and gentamicin-loaded Palacos® R did not demonstrate any significant changes in compressive or bending strength post degradation. In contrast, the compressive and bending properties of the gentamicin-loaded Palacos® R containing chitosan powder decreased and did not maintain acceptable ISO levels of compressive strength (70 MPa) or bending strength (50 MPa) on a repeatable basis after a degradation period of 7 days. These results clearly indicate that the addition of chitosan to bone cement has an adverse effect on the mechanical properties which could be detrimental to the longevity of the prosthetic joint.

5 Conclusions

The findings of our in vitro study suggest that the incorporation of chitosan in unloaded Palacos® R bone cement at concentrations of 1–5% does not prevent bacterial colonisation and biofilm formation by clinical prosthetic hip isolates. Similarly, the addition of chitosan powder to Palacos® R + G bone cement significantly decreased gentamicin release and did not increase the efficacy of the bone cement at preventing bacterial colonisation and biofilm formation. Furthermore, the mechanical performance of the bone cement containing chitosan was significantly reduced after 28 days of saline degradation, with the compressive and bending strengths of the cement not in compliance with the minimum requirements as stipulated by the ISO standard for PMMA bone cement. Therefore, incorporating chitosan into Palacos® R + G bone cement for use in revision surgery has no clinical antimicrobial benefit and the detrimental effect on mechanical properties could adversely affect the longevity of the prosthetic joint.

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