

Full Length Research Paper

***In vitro* elution and dissolution of tobramycin and gentamicin from calcium phosphate**

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This study was conducted to investigate the *in vitro* drug release characteristics of tobramycin and gentamicin from calcium phosphate. Calcium phosphate beads were loaded with tobramycin and gentamicin separately to form 2 types of antibiotic beads to generate antibiotic drug delivery system for the treatment of osteomyelitis. Tobramycin and gentamicin concentrations were determined spectrophotometrically by measuring the absorbance at 400 nm. The standard graphs for tobramycin and gentamicin concentration versus absorbance reading were prepared as references to identify the concentration of the drugs release after incorporating calcium phosphate over 8 weeks. This study showed that incorporating tobramycin and gentamicin with calcium phosphate provided slow residual release of antibiotic from 30 min to 1344 h (8 weeks) and dissolution of calcium phosphate. In this respect, the drug delivery systems of tobramycin and gentamicin-incorporated calcium phosphate have potential of controlling drug release.

Key words: Tobramycin, gentamicin, calcium phosphate, drug delivery system, osteomyelitis.

INTRODUCTION

Osteomyelitis is difficult to treat because it is often associated with necrosis of bone and poor vascular perfusion accompanied by infection of the surrounding tissues. The treatment of osteomyelitis mainly involves operative debridement, surgical removal of necrosis tissue and prolonged antibiotic therapy (Nandi et al., 2009).

Conventional systemic delivery of antibiotics requires high doses of antibiotic and entails poor penetration into ischemic and necrotic tissue. This can cause systemic toxicity which is associated with renal and liver complications. Alternatively, antibiotic-incorporated bone cement beads specifically designed and directly implant to the infected bone that may enable to maintain a high antibiotic concentration for an extended duration without exceeding systemic toxicity (Zilberman and Elsner, 2008).

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Polymethacrylate (PMMA) bone cement and beads have been clinically used as carrier for local antibiotic delivery since 1970s (Kanellakopoulou and Giamarellos-Bourboulis, 2000). However, PMMA beads are preferable to microorganism adherence and growth on the biomaterial surface, despite, the release of antibiotics and it has potential to develop antibiotic resistance (Neut et al., 2001). Furthermore, a secondary surgery may be required to remove the cement because it is not biodegradable and may become a nidus for future infections (Liu et al., 2007). Thus, in this study, biodegradable carrier material by using calcium phosphate incorporated with tobramycin and gentamicin was formulated to treat bone infection.

Calcium phosphate such as β -tricalcium phosphate (β -TCP) and dicalcium phosphate dehydrate (DCPD) have been shown to be suitable carrier materials for local drug delivery in orthopaedic application (Ravelingien et al., 2010). Calcium phosphates have generated a great deal of interest in relation to hard tissue applications due to their bioactivity, biocompatible, biodegradable, osteoconductive, osteophilic nature, non-toxic, capable to fill bone cavities, non-mutagenic as well as non-inflammatory and not recognized as foreign material in the body. Most importantly, this biomaterial exhibit bioactive behaviour, being integrated into the tissue by the same processes active in remodeling healthy bone. As a result, this leads to osseointegration which is intimate physicochemical bond between the calcium phosphate implants and bone (Komath et al., 2000; Schmitz et al., 1999).

Tobramycin and gentamicin are used most frequently by surgeons for incorporation into bone cement (Kanellakopoulou and Giamarellos-Bourboulis, 2000; Walenkamp, 1997). Gentamicin remains the most effective antibiotic to incorporate with bone cement due to its high solubility, heat stability and bactericidal activity at low concentration (Campoccia et al., 2010; Faber et al., 2005). This drug was selected in this study because it is widely used for the treatment of osteomyelitis. Meanwhile, tobramycin is closely related to gentamicin with a similar spectrum of activity which is active against both Gram-positive and Gram-negative bacteria (Randelli et al., 2010), but less ototoxic and nephrotoxic than gentamicin (Scott et al., 1999) and its elution characteristics has been shown to elute at higher concentrations than gentamicin in bone cements (Sterling et al., 2003). Furthermore, tobramycin is usually substituted for gentamicin, because it is available as a pharmaceutical-grade powder, whereas gentamicin is not (Wininger and Fass, 1996).

The main purpose of this study is to investigate the release characterization of tobramycin and gentamicin from calcium phosphate bead. In addition, we also determined the relationship between concentration of antibiotics and absorbance as well as surface morphology before and after elution of antibiotic.

MATERIALS AND METHODS

Preparation of calcium phosphate beads

Commercially available calcium phosphate beads (Karios, France) were made with modification from manufacturer's instruction. Calcium phosphate bead was prepared by mixing 5 ml of liquid and 3 ml of starch soluble (BDH, United Kingdom) into a bowl. Then, 10 g of calcium phosphate powder was added and mixed vigorously until the mixture becomes a smooth homogenous liquid. The syringe was filled with liquid paste and slowly extruded on the Petri dish to form ~ 0.2 g bead. The beads were left to dry for overnight. Subsequently, the beads were sent to Malaysian Nuclear Agency (MNA) for gamma sterilization. Loading of the beads with tobramycin sulfate (Sigma-Aldrich, USA) and gentamicin sulfate (Calbiochem, USA) were carried out by dipping in 10 μ l tobramycin and gentamicin solution (0.12 and 0.05 mg/ml, respectively) at room temperature for 24 h.

Ninhydrin assay

Nine concentration levels of tobramycin and gentamicin solution were used to prepare the standard graph of these antibiotics versus OD reading in the range between 10 and 100 μ l. Five millimetre of antibiotic aliquot were mixed with freshly prepared 1.5 ml of ninhydrin reagent (2 mg/ml) by vortexing (30 s) and heated in a water bath at 95°C for 15 min. Then, the tubes were cooled in ice-water bath. Appropriate amount of aliquots were subjected to UV spectrophotometer reading (UV-1601, Shimadzu, Japan) at 400 nm against phosphate buffer as background reading (Frutos et al., 2000).

In vitro release tests of antibiotic

The releases of tobramycin and gentamicin from calcium phosphate were assayed in triplicate under sink condition. Each sample was immersed in 10 ml of PBS (pH 7.4) in a test tube and shaken horizontally in a shaking water bath (Sartorius, Germany) at 37°C and 50 rpm. After 30 min, 1, 2, 4, 8, 24, 48 and 72 h and then every week up to a total of 8 weeks, 5 ml of aliquot were withdrawn and subjected to ninhydrin assay as described previously. Five millimetre of fresh phosphate-buffered saline was added into the sample tube to replenish the release medium. Phosphate-buffered saline was used as a background UV reading.

Scanning electron microscopy (SEM)

Antibiotic beads were fixed in 4% glutaraldehyde and kept at 4°C for 24 h. Then, washed with 0.1 M sodium cacodylate buffers for 3 to 4 times and 10 min each and post-fixation in 1 % of osmium tetroxide about 2 h in 4°C. Dehydration was performed by sequential immersion in serial diluted ethanol solutions of 35, 50, 60, 70, 80, 90 and 100%. The samples were kept in absolute alcohol and critical point dried using CO₂ (Baltec CPD 300). The samples were then sputtered with palladium gold for scanning electron microscopy (SEM) (JOEL JSM 6400 and JEOL JSM 6701F) analysis.

Statistical analysis

Statistical evaluation for correlation of tobramycin and gentamicin versus absorbance was performed using linear regression model. The repeated measures analysis was used to compare mean concentration of tobramycin and gentamicin release from calcium

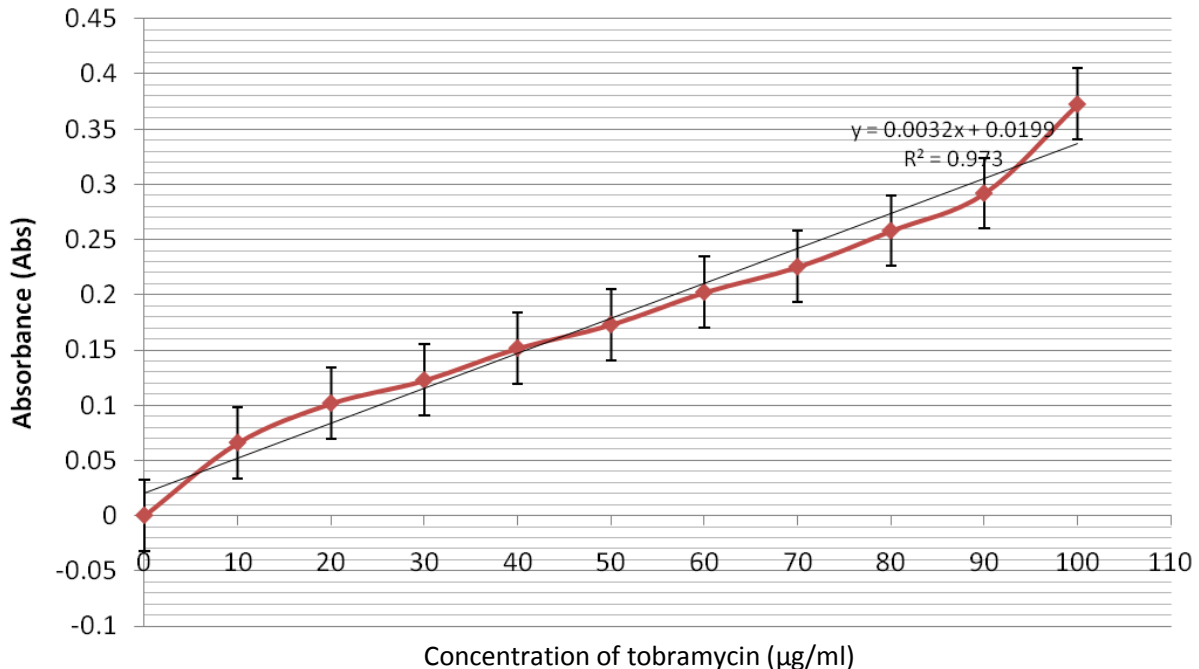


Figure 1. Calibration curve for absorbance versus concentration of tobramycin sulfate.

phosphate at different time. All results were expressed with mean (standard error of mean). $P < 0.001$ was considered to be a significant value. Meanwhile, the comparison of cumulative release of tobramycin and gentamicin release from calcium phosphate at 1344 h was evaluated using independent sample t-test. All results were expressed with mean (standard error of mean). $P < 0.001$ was considered to be a significant value.

RESULTS

Quantification analysis of tobramycin and gentamicin

The standard graph for tobramycin and gentamicin versus absorbance was identified as a reference to determine the concentration of tobramycin and gentamicin release from calcium phosphate bead according to the time interval.

The output in Figure 1 showed the results of fitting a linear model to describe the relationship between concentration of tobramycin and absorbance. According to the graph, r was 0.986, indicating a relatively strong relationship between the OD reading value and concentration of tobramycin. One unit increase in concentration of tobramycin resulted in an increased of 0.032 Absorbance for OD reading. The r^2 statistic indicated that the model as fitted explains 97.3% of the variability in absorbance. Since the P -value is less than 0.05, there was a statistically significant between absorbance and concentration of tobramycin.

Figure 2 was the linearity plot relating to absorbance and concentration of gentamicin. The graph showed that

the absorbance reading increased with increase of concentration of gentamicin. The r was 0.991, indicating a relatively strong relationship between the variables. One unit increase in concentration of gentamicin resulted in an increased of 0.021 Abs for OD reading. The r^2 statistic indicated that the model as fitted explains 98.2% of the variability in absorbance. The P -value is less than 0.05, therefore the absorbance is statistically significant with concentration of gentamicin.

In vitro tobramycin and gentamicin release from calcium phosphate bead

The characteristic for tobramycin and gentamicin release from calcium phosphate under *in vitro* conditions at various times during an 8 weeks interval clearly indicated that the rate of drug release was exponentially related to the release time. Statistical analysis showed that there was a significant different between drug release at different time release ($P < 0.001$). Drug release between 168 h (1 week), 336 h (2 weeks), 672 h (4 weeks) and 1344 h (8 weeks) was statistically significant compared with 0.5, 1, 2, 4, 6, 24, 48 and 72 h ($P < 0.001$). Figure 3 showed that the lag phase exhibited low initial burst effect from 0.5 h until 72 h. It appears that the antibiotic bead remain largely impermeable to PBS and thus, there was slower release of tobramycin and gentamicin from calcium phosphate. The lag phase was followed by a log phase, which can be attributed to a stage where the bulk degradation of the antibiotic bead reaches the maximum

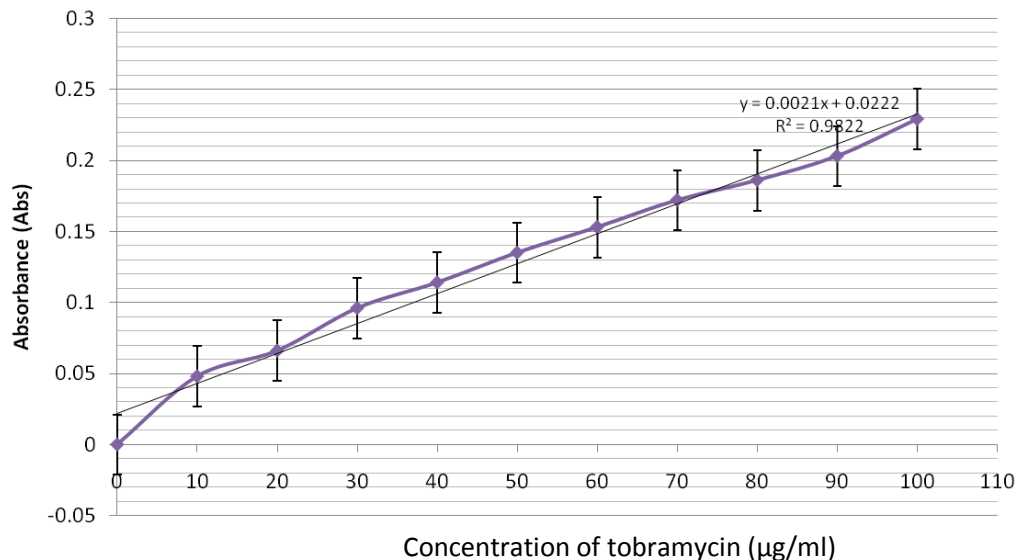


Figure 2. Calibration curve for absorbance versus concentration of gentamicin sulfate.

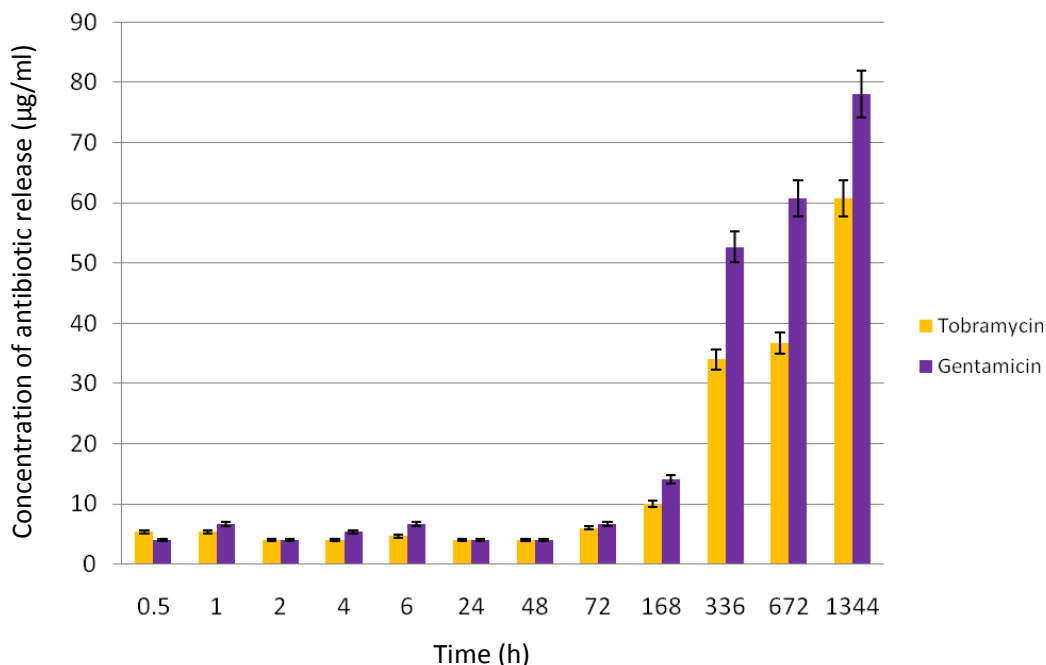


Figure 3. Concentration of tobramycin and gentamicin release from calcium phosphate bead over each sampling interval for the 8 weeks study period.

level allowing the tobramycin and gentamicin to diffuse out of the calcium phosphate. At this phase, the mean concentration release for tobramycin was increased from 168 h [10.0 (1.9) µg/ml], 336 h [34.0 (1.9) µg/ml], 672 h [36.7 (1.9) µg/ml] and 1344 h [60.7 (1.9) µg/ml] gradually. Meanwhile, the mean release rate for gentamicin also was increased from 168 h [14.0 (1.9) µg/ml], 336 h [52.7

(1.9) µg/ml], 672 h [60.7 (1.9) µg/ml] and 1344 h [78.0 (1.9) µg/ml].

In vitro release tests of tobramycin and gentamicin from calcium phosphate beads were assayed in triplicate. Statistical analysis showed that there was no significant difference between 3 samples of tobramycin-incorporated calcium phosphate and gentamicin-incorporated calcium

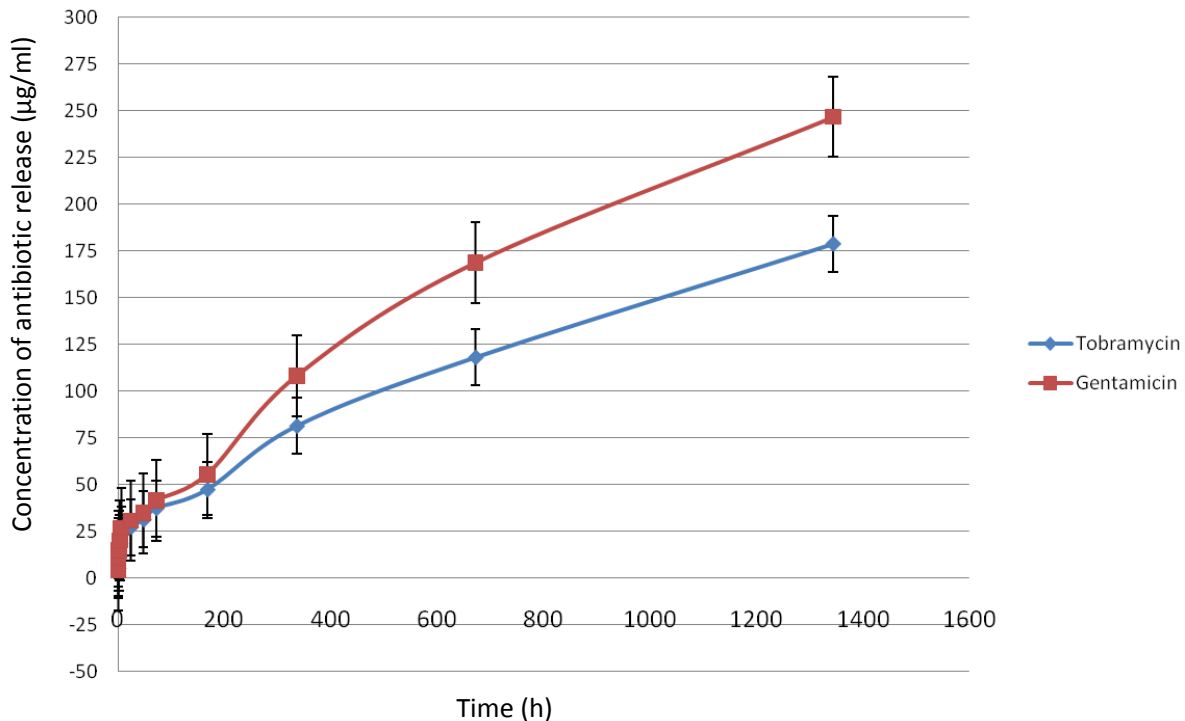


Figure 4. Cumulative *in vitro* release of tobramycin and gentamicin from calcium phosphate bead over each sampling interval for the 8 weeks study period.

phosphate on the release of antibiotics ($F_{\text{plate}} = 0.562 < F_{0.05, 2, 2} = 19$). This result indicated that antibiotics contained in each calcium phosphate bead was homogeneous.

The drug release for tobramycin-incorporated calcium phosphate was significantly different with gentamicin-incorporated calcium phosphate ($F_{\text{group}} = 175.54 > F_{0.05, 1, 2} = 18.51$). The concentration of gentamicin release from calcium phosphate [20.8 (0.5) µg/ml] was more than tobramycin release from calcium phosphate [14.9 (0.5) µg/ml] within the time release. Therefore, this result suggested that gentamicin released from calcium phosphate is higher than tobramycin.

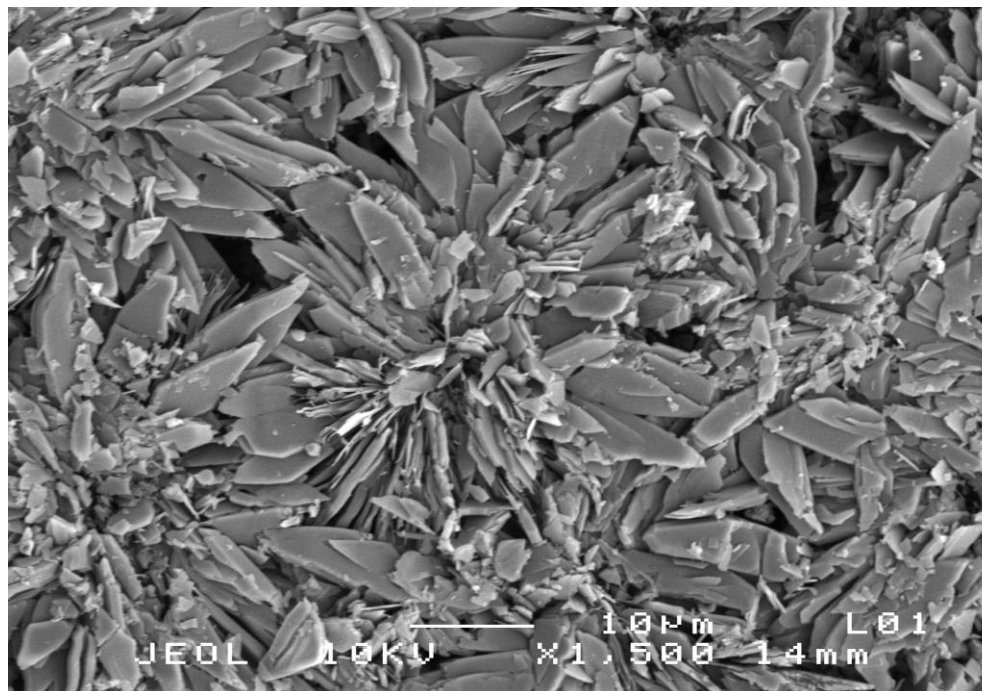
Cumulative release of tobramycin and gentamicin from calcium phosphate bead

The cumulative release of tobramycin and gentamicin from calcium phosphate under *in vitro* conditions at various times during an 8 weeks period clearly indicated that the rate of drug release was exponentially related to the release time (Figure 4). The initial burst release of tobramycin and gentamicin from calcium phosphate was slow release from 0.5 to 48 h. It appears that the antibiotic bead remain largely impermeable to PBS and thus, there was lower release of tobramycin and gentamicin from calcium phosphate. After the initial burst,

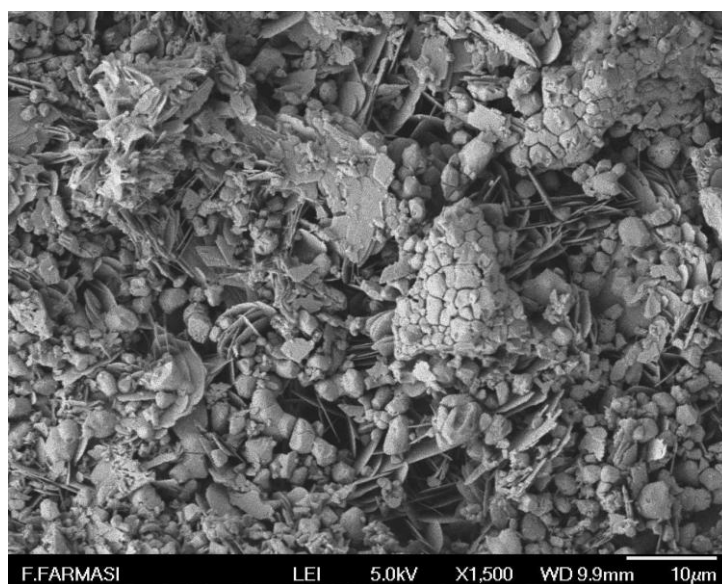
the release rate was increased rapidly from 72 to 1344 h (8 weeks) for tobramycin and gentamicin-incorporated calcium phosphate due to the bulk degradation of the antibiotic bead reaches the maximum level allowing for the tobramycin and gentamicin to diffuse out of the calcium phosphate. Statistical analysis showed that there was significant different mean concentration of drug release at 1344 h (8 weeks) between tobramycin-incorporated calcium phosphate and gentamicin-incorporated calcium phosphate ($P < 0.001$). The mean cumulative release of gentamicin from calcium phosphate [249.3 (2.4) µg/ml] was higher than tobramycin [178.7 (4.1)] at 1344 h.

Surface characteristic of tobramycin and gentamicin incorporated calcium phosphate before and after release assay

There was surface morphological changes of tobramycin-incorporated calcium phosphate and gentamicin-incorporated calcium phosphate during ninhydrin assay. The porosity of tobramycin and gentamicin-incorporated calcium phosphate was 40% and the pore size was less than 5 microns. Before the antibiotic beads were immersed in PBS, the shape of tobramycin and gentamicin-incorporated calcium phosphate beads was hemisphere with the surface structure of long plate like-



(a)

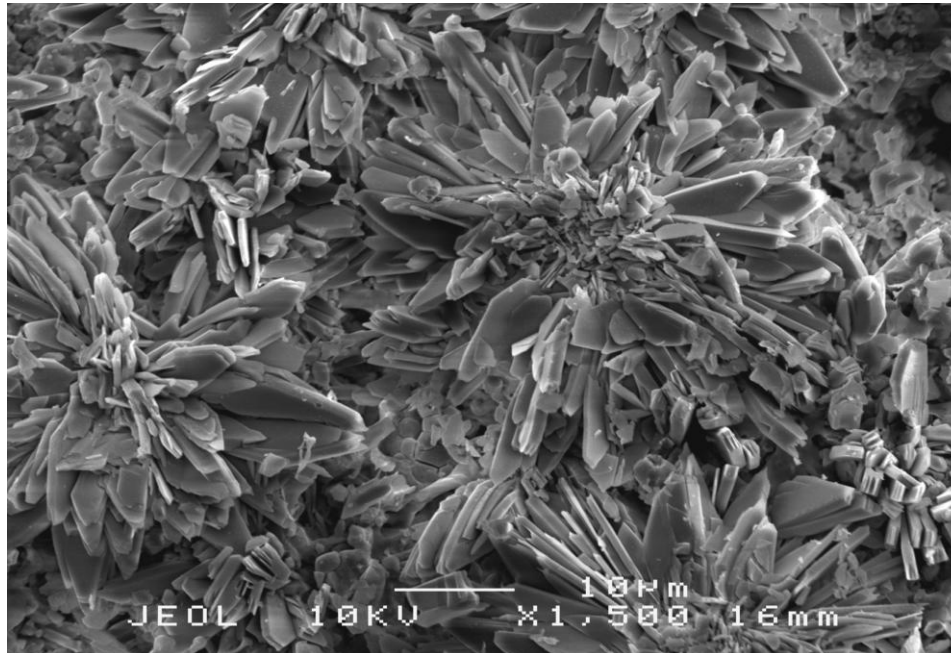


(b)

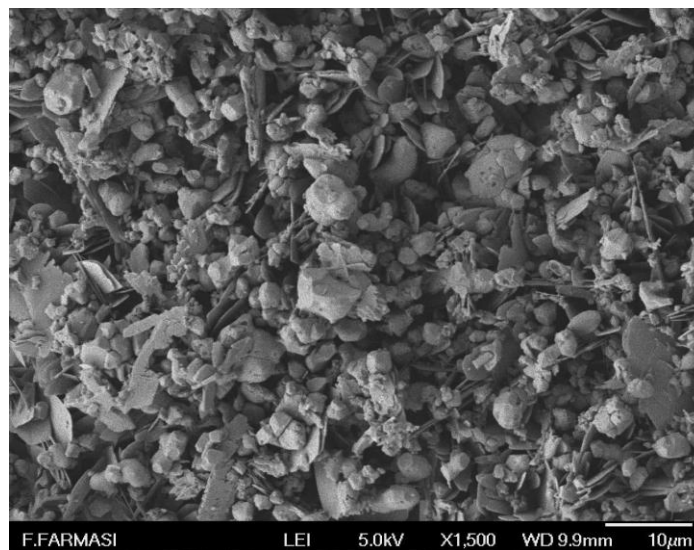
Figure 5. Scanning electron micrographs of the surface of tobramycin-incorporated calcium phosphate. (a) Tobramycin-incorporated calcium phosphate surface before the ninhydrin assay was performed (0 h) (1,500x). (b) Tobramycin-incorporated calcium phosphate surface after the ninhydrin assay was performed at 1344 h (8 weeks) (1,500x).

crystals radiating from a central nucleus. These plates were stacked together with narrow spacing (Figures 5a and 6b). Initially, the tobramycin and gentamicin-incorporated calcium phosphate were not damaged and remain intact. After the antibiotic beads immersed in PBS, the resorbing process involves dissolution of calcium phosphate crystal and then a precipitation of

calcium phosphate needle-like crystallites of micropores close to the dissolving crystal. In addition, voids, cracks and imperfections can be noticed on the surface of specimens through scanning electron micrographs (Figures 5b and 6b). Tobramycin and gentamicin-incorporated calcium phosphate degraded and decreased their size during 8 weeks incubation. The



(a)



(b)

Figure 6. Scanning electron micrographs of the surface of gentamicin-incorporated calcium phosphate. (a) Gentamicin-incorporated calcium phosphate surface before the ninhydrin assay was performed (0 h) (1,500x). (b) Tobramycin-incorporated calcium phosphate surface after the ninhydrin assay was performed at 1344 h (8 weeks) (1,500x).

degradation of these antibiotic beads would result in a gradual release of drugs through voids, cracks and imperfections of the surfaces.

DISCUSSION

The effectiveness of antibiotic delivery systems for the

local prevention of bacterial infections related to orthopaedic implants is strongly dependent on the drug release profile. If the drug is released too quickly, the entire drug could be released before the infection is suppressed. Conversely, if the release is delayed, the infection can develop further, thus making it difficult to manage the healing. The local antibiotic release profiles should exhibit a high initial release rate in order to

combat the bacteria which were introduced during the implantation then followed by a sustained release to combat bacteria introduced systemically afterwards (Zilberman and Elsner, 2008).

Low systemic toxicity and high local drug concentrations are the main advantages of local antibiotic delivery system (Stallman et al., 2003). The successful treatment of osteomyelitis involves local antibiotic delivery system which gives a sustained release of antibiotic for a period of about 6 to 8 weeks (Narahariseti et al., 2006). This study showed that incorporating tobramycin and gentamicin with calcium phosphate provided slow residual release of antibiotic from 0.5 to 1344 h (8 weeks). In this respect, the drug delivery system of tobramycin and gentamicin-incorporated calcium phosphate seems to be safer.

The release of antibiotics at level below the minimum inhibitory concentration (MIC) can evoke bacterial resistance at the release site and intensify infectious complications (Ravelingien et al., 2010). The MIC of tobramycin and gentamicin release should be reached at all time points during the *in vitro* release study (Schnieders et al., 2006). This study showed that the levels of concentration of drug release were sufficient to achieve the MIC for tobramycin and gentamicin (2.5 µg/ml) which is necessary to kill bacteria in surrounding tissue following surgery and not exceed a maximum limiting concentration that would be toxic to the cells in the implant.

Gentamicin and tobramycin are hydrophilic (Mingeot-Leclercq et al., 1995) which are drugs which tend to separate out into the water. The initial release may due to the possible uptake of water into the pores and drugs disperse into the water. After the initial release, the antibiotic beads undergo bulk degradation. In the case of composites, it can be observed that drug close to the surface has diffused out in the initial period. It may be taken that the remaining drug is entrapped deep within the pores and comes out only when the antibiotic beads actually starts to degrade or after water has penetrated sufficiently into the pores.

Biodegradation of tobramycin and gentamicin-incorporated calcium phosphate may be influenced by the combination of the physical factors and chemical factors. The physical factors tending to increase rate or extent of biodegradation include increases in porosity, reductions in crystal size, increase in number of crystal imperfection and decreases in grain size. The additives of the tobramycin and gentamicin into calcium phosphate may be the cause of the imperfections of the materials and the circular voids in may be due to tobramycin or gentamicin particle dissolved. The porosity of the polymer matrices depends on air entrapment during wetting and stirring of the calcium phosphate powder. Penetration of dissolution fluids into pores of the polymer matrices also depend on wet ability of the bone cement surface which makes antibiotic to be release from the surface (Van de

Belt et al., 2000). Meanwhile, the chemical factors depend on composition and ionic substitution in materials (Lu et al., 2002; Verron et al., 2010). In this study, starch was used as a binder to incorporate drug with calcium phosphate. Without a binder present, the drug is simply adsorbed onto the surface of the material. When placed in solution, the tobramycin and gentamicin can freely dissociate from the material surface and diffuse out of the highly porous calcium phosphate scaffold. However, the amount of drug released in an *in vitro* test also may be affected by the the quantity of drug added to the material, sample size and shape, the volume of buffer used and stirring of the test solution as opposed to static diffusion (Alexis, 2005; Silverman et al., 2007).

In vitro results may not correlate to the *in vivo* environment because the development of fibrous tissue around the beads and the lack of surrounding fluid reduce anticipated elution. On the other hand, the safety and positive effect of the device will be confirmed *in vivo* on established osteomyelitis induced in a rabbit model. Thus, the size of the antibiotic beads developed should be small enough to fit into the bone for the future study in animal models.

Conclusion

The advantage of tobramycin and gentamicin-incorporated calcium phosphate beads includes providing slow residual release of antibiotic for a definite time period and biodegradability of the carrier beads avoiding the need of second surgery for the their removal after therapy. Therefore, these preliminary *in vitro* drug elution study demonstrates that calcium phosphate incorporated with tobramycin and gentamicin appear to be a promising alternative antibiotic carrier for the treatment of osteomyelitis.

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