ORIGINAL ARTICLE

Antibacterial Efficacy and Drug-release Behavior Study of β-tricalcium Phosphate Micro-granules Against *Staphylococcus aureus* and *Escherichia coli*

Nor Hazliana Harun[1], Rabiatul Basria S.M.N. Mydin[1,2], Khairul Anuar Shariff[3], Nur Adila Rosdi[1,4], Davamunisvari Rames[1,4]

[1] Oncological and Radiological Sciences Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200 Bertam, Kepala Batas, Pulau Pinang, Malaysia.
[2] Department of Biological Sciences, 14 Science Drive 4; NUS Environmental Research Institute, National University of Singapore 117543, Singapore.
[3] School of Materials and Mineral Resources Engineering, Engineering Campus, Universiti Sains Malaysia, 14300, Nibong Tebal, Penang, Malaysia
[4] Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia

ABSTRACT

Introduction: This study aims to investigate different residue sizes of *β*-tricalcium phosphate (*β*-TCP) micro-granules as carriers to assess antibacterial activity and drug-control release behavior of ampicillin (AMP-) and antimycotic (AMC-). Incorporation of antibiotic into the *β*-TCP micro-granules and it sustain release behavior could be used as alternative solution to reduce the risk of osteomyelitis and bone infections risks. **Methods:** Three different residue sizes (less than 300 µm, 300 µm and 600 µm) were prepared and coated with antibiotics solution (20 µg/µl of ampicillin and 100X antimycotic solution) by using two methods; dip and stream coating. After 72 h, 1.5 mL of distilled water was added to the treated (*β*-TCP) micro-granules at two different pH value (5.0 and 7.4). The extracted solution was further analyzed by Kirby Bauer disc diffusion test and spectrophotometer assay. **Results:** The solution containing AMC-(*β*-TCP) micro-granules with the size of 300 µm residue produced the largest inhibition zones against *Escherichia coli* (*E. coli*). All residue sizes coated with AMP- showed no antibacterial activity against both strains; *Staphylococcus aureus* (*S. aureus*) and *E.coli*. Additionally, the release behavior of AMC-(*β*-TCP) micro-granules was found not depending on the pH, but on the size of residue. Complete drug release was rapidly observed within 48 h. **Conclusion:** Based on this findings, it showed AMC-(*β*-TCP) micro-granules had an antibacterial activity against Gram-negative strain. Specifically, it can reduced the growth rate of *E. coli* and the rapid release behavior of AMC-(*β*-TCP) micro-granules help in minimizing the risk-infections in early stage of implantation.

Keywords: Antibacterial activity, *β*-Tricalcium Phosphate micro-granules, *β*-TCP, *Escherichia coli*, *Staphylococcus aureus*

Corresponding Author:
Rabiatul Basria S.M.N. Mydin, PhD
Email: rabiatulbasria@usm.my
Tel: +604-5622351

INTRODUCTION

In recent year, osteomyelitis and bone infections incidence rate has be around 2 to 2.4 percent of cases (1,2). Osteomyelitis is well known disease cause inflammation of the bone marrow which specifically affected to a single bone or other parts of regions; marrow, cortex, periosteum and soft tissue. It can be caused by a variety of pathogens and one of the most frequent cases commonly caused by *Staphylococcus aureus* (*S.aureus*). *S.aureus*-induced osteomyelitis has posses great challenges in health industries, which contribute about 75% of cases (3,4). *S.aureus* are one of commensal inhabitants of the skin microflora and mucosal surfaces, hovewer, once it have accesed the bone under certain conditions, *S.aureus* colonization and biofilm development occurred. Direct interaction of *S.aureus* with the bone cells could trigger protein A (SpA) to bind to osteoblasts, thus disrupts normal bone formation (5). On the other hand, *E.coli* (Gram-negative strain) also been reported in few cases cause osteomyelitis among immunocompetent patients (6-8). It also frequently observed in children in which the entry points is from urinary and gastrointestinal tract (9).

Despite modern advances in antibiotics in treating pathogen infections, antibiotics have been provoked with development of bacteria's own defences against immune cells and had much higher toxicity effects due

to the increment in concentrations being used to have an effective treatment. According to the Ciampolini 2000, it was found that bacteria able to embed into osteocytes and produce a fibronogen layer in normal tissue thus avoid been attacked by immune cells and antibiotics (10). The above problems have urge researchers to have a new perspective on strategies control by focusing on bioceramic biodegradable material such as β-tricalcium phosphate (β-TCP) as one of the alternative solution.

β-TCP, calcium sulfate dihydrate and hydroxyapatite (HAp) have been extensively used in the last past century in orthopaedic surgery as bone grafts. However, only β-TCP showed a great balanced in absortion and effective calcium ions and phosphate ion release in great quantity for new bone formation developments (11). In addition, β-TCP usually been systhesized in powder forms and added together with antibiotics in different range of concentrations to act as antibacterial agents (12-14). In this study, we have modified the β-TCP with addition of ampicillin (AMP-) and antimycotic (AMC-) solution to improve antibacterial activity and drug release mechanisms. Antibacterial effectiveness of three different granular size of β-TCP were determined using Kirby-Bauer disk diffusion assay against standard representative strains; *S.aureus* and *E.coli*. Our findings provided valuable information into the real potential of β-TCP micro-granules as drug carrier.

MATERIALS AND METHODS

The B-TCP micro granular powders used in this study were weighted and mixed accordingly with the selected antibiotics; Ampicillin (AMP-) and antimycotic (AMC-) solution with concentration of 20 g/L and 100X were prepared in deionized water. The antibiotic/ β-TCP micro-granules were prepared through two methods; dip and stream coating. It was dried for 72 h at 37°C and be carried out in triplicate.

Preparation B-TCP micro granular powders

Calcium carbonate (CaCO3) and dicalcium phosphate dihydrate (DCPD) were used to fabricate β-tricalcium phosphate granules. CaCO3 and DCPD were mixed with ethanol using planetary ball milling machine at speed of 200 rpm for 6 h with Ca/P ratio of 1.5. After milling for 6 h, the mixture was dried in oven at temperature of 60°C for 24 h. The dried mixture was then crushed and compressed using uniaxially hand press machine with 50 MPa pressure. Pellet specimen was obtained and then sintered in furnace at 1100°C with heating rate 5°C/ minute and soak for 6 h. After sintering process, the pellet specimens were crushed using agate mortar and sieve to get below 300 µm, 300 µm and 600 µm micro granules powder.

Dip coating

It was done by weighing 15 mg of β-TCP powder and gently mixed with 1.5 mL of each antibiotics, respectively. Distilled water was used as control. The β-TCP micro-granules were incubated in the solutions for 24 h at room temperature (RT). Finally, the micro-granules were dried for 72 h at 37°C. This method was done by following the previous report (1).

Stream coating

Second coating method was used to coat β–TCP micro-granules with antimycotic known as stream coating. Similar weight of sample (15 mg) been transfer into 5 mL of syringe and the punch was pulled out and be placed directly onto the syringe outlet. Then, the particular syringe with 0.45µm syringe filter was filled with 1.5 mL of antibiotics solution. One mL of the solution was be removed through the filter and kept 0.5 mL beyond the micro-granules. The remained micro-granules were then transferred to centrifuge tube and dried at 37°C for 72h in a fumehood. Finally, all samples were gently swilled in distilled water to remove any excess of antibiotics. The double replicates are prepared.

Drug release

In order to assess the amount of antibiotic loaded, the samples were weighted agains and placed in the centrifuge tube filled with 1.5 mL of distilled water at two different pH value (5.0 and 7.4). The tube was sealed with parafilm and incubated at 37°C for 2 days. After treatment periods, the solutions are removed and placed into new centrifuge tube. The centrifuge tube with micro-granules are then filled with 1.5 mL of different pH distilled water. The solutions obtained are further tested using Kirby Bauer disc diffusion test and spectrophotometer method.

Kirby-Bauer disk diffusion assay

The antibacterial activity of the samples were tested using the Gram-positive *S. aureus* and Gram-negative *E. coli*. Both microorganisms were grown in Luria-Bertani (LB) broth at 37°C for 18-21 h. Firstly, l00 µL of bacteria *S.aureus* and *E.Coli* with optical density (OD) = 1.5 x 10^6 and OD = 8.0 x 10^8 respectively is loaded into the center of agar plate. The plate is gently swabbed and rotated the plate approximately 60 degrees each time to ensure an even distribution of the inoculum. The swab is discarded into an appropriate container. The plate is allowed to sit at room temperature. The β–TCP coated with antibiotic disc are placed on the surface of the agar by using forceps. The steps are repeated quadruplicate for each concentrations. The lid is replaced and the plates are inverted once all disks are in place. The agar plate are incubated at 37°C in the incubator for 24 h.

Spectrophotometric method of antibiotic with sulfuric acid

The calibration curve was built at concentrations of 0, 0.05, 0.09, 0.19, 0.38, 0.75 and 1.50 mL. The β–TCP samples analyzed were drawn up with 0.226 mL of deionized water, 0.524 mL of dissolved sulfuric acid 98% purity. After that, the mixture solutions are

sonicated for 5 minutes at 37°C. A spectrophotometer with the wavelength of 226 nm is used.

Statistical analysis

Each data was analyzed using the GraphPad Prism and one-way ANOVA was performed to calculate the statistically significant of the experiment data and the difference between mean values was compared by Tukey's test ($p < 0.05$).

RESULTS

Disk diffusion method

In our study, we evaluated the antibacterial effect of the released amounts of antibiotics combined with residue, 300 µm and 600 µm of *β*–TCP granules. There was no inhibition zone been measured for *β*–TCP mixed with both types of antibiotics against *S. aureus*. The combination of antimycotic with 300 µm *β*–TCP showed highest inhibition zone ranged between 10.0 ± 0.0 to 12.50 ± 1.0 mm at both pH (Table I and II). However, the association 600 µm *β*–TCP and antimycotic against *E. coli* have shown smaller inhibition zone around the disk, which is less than 7.50 ± 0.57 mm at pH 5.0 and 8.5 ± 0.57 mm at pH 7.4.

Drug release behavior in different pH measured by spectrophotometry

Figure 1 and Table III did show release behavior of AMP- and AMC- within 48 h at two different pH (5.0 and 7.4), corresponding to the altered pH during inflammation and biphasic processes situation. According to our results tabulated in Figure 1, we can conclude that AMP showed unstable released in acidic condition even at the highest concentration. Whereas, the AMC did show great release with the increasing of volumes (mL).

Thus, we further mixed AMC with the *β*-TCP micro-granules and it showed no significant difference between dip and stream coating methods. It showed a rapid release of AMC- from *β*-TCP micro-granules within 24 h and completely degrade after two days (Table III). All samples taken at later time points had an AMC- concentration below the detection limit. Both pH (acid and slightly alkaline) did also been proved to have no effects on the AMC/ *β*-TCP micro-granules release behavior study.

DISCUSSION

In this study, the addition of three different size (residue, 300 and 600 µm) of *β*–TCP to AMC- and AMP- showed a different drug release behavior. A Kirby-Bauer disk diffusion assay is used to screen the antibacterial activity of different sizes of *β*–TCP against *S. aureus* and *E. coli*. It revealed the appropriate size of *β*–TCP mixed with AMC- to enhanced the antibacterial applications with complete release of antibiotics from *β*–TCP as carrier. The presence of 300 µm of *β*–TCP combined with AMC- did showed a greater zone of inhibitions against *E. coli*

Table I: Comparison of coating assays with different amount and types of antibiotics against *E.coli* at pH 5.0.

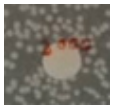
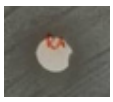
pH. 5.0		Residue of -TCP		300 m of -TCP		600 m of -TCP	
		Ampicillin	Antimycotic	Ampicillin	Antimycotic	Ampicillin	Antimycotic
Dip coating	15 L	No zone	8.5 ± 1.0 	No zone	10.25 ± 1.26 	No zone	7.0 ± 0.0 
	25L	No zone	9.5 ± 0.57 	No zone	10.5 ± 0.57 	No zone	7.5 ± 0.57 
Stream coating	15 µL	No zone	No zone 	No zone	11.0 ± 0.82 	No zone	6.5 ± 0.57 
	25 µL	No zone	9.75 ± 0.96 	No zone	12.25 ± 2.22 	No zone	7.0 ± 0.0 

Table II: Comparison of coating assays with different amount and types of antibiotics against *E.coli* at pH 7.4

pH 7.4		Residue of -TCP		300 m of -TCP		600 m of -TCP	
		Ampicillin	Antimycotic	Ampicillin	Antimycotic	Ampicillin	Antimycotic
Dip coating	15 L	No zone	11.0 ± 1.15 	No zone	12.5 ± 1.0 	No zone	8.0 ± 0.82 
	25L	No zone	7.5 ± 0.57 	No zone	11.0 ± 0.82 	No zone	8.5 ± 0.57 
Stream coating	15 µL	No zone	No zone 	No zone	10.0 ± 0.0 	No zone	8.0 ± 0.82 
	25 µL	No zone	No zone 	No zone	11.0 ± 0.0 	No zone	8.5 ± 0.57 

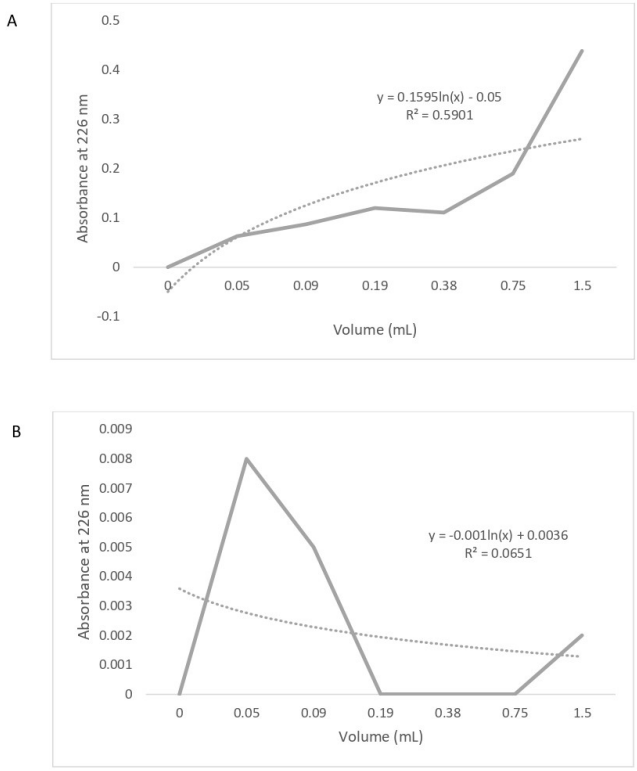


Figure 1: The release behavior of A) Antimycotic and B) Ampicillin

Table III: The absorbance of AMC- at the wavelength of 226 nm for Day 1 and 2 at two different pH (5.0 and 7.4)

Antimycotic		Residue of -TCP		300 m of -TCP		600 m of -TCP	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Dip coating	pH 5.0	0.003	-0.003	0.003	-0.003	0.021	-0.001
	pH 7.4	0.009	-0.001	0.005	-0.005	0.018	-0.003
Stream coating	pH 5.0	0.009	0.001	0.013	-0.009	0.020	0.002
	pH 7.4	0.000	0.001	0.012	0.001	0.001	-0.004

growth as compared to other sample sizes.

In addition, the porosity, granule and wide size of distribution of β–TCP did play role to enhance a better carrier for antibiotics. It also leads to better diffusion of antibiotics into the voids of β–TCP (15). Furthermore, this study proved a smaller size had a better antibacterial activity due to their higher surface-to-volume ratio as compared with the bigger ones (600 µm). However, there was no antibacterial activity against *S. aureus* strain for both combination of antibiotics with β–TCP. This study proved the coated β–TCP with AMC- as a carrier. Notably, this report showed a greater antibacterial activity of this combination on *E. coli* than that on *S. aureus*.

This difference was possible attributable from peptidoglycan layer which much thinner than Gram-

positive bacteria. *E. coli* have a double lipid membrane structure sandwiching the peptidoglycan layer with an additional lipopolysaccharide resulting a low permeability for foreign molecules. The mechanism underlying the antibacterial activity of this combination is based on the attraction between positively charged calcium and phosphate ions during dissolution of *β*–TCP and negatively charged of the bacterial membrane did also involve in antibacterial activity through ion-mediated killing. Cationic molecules such as calcium also had strong affinity and more easily bind to the cell wall of *E. coli* due to the negatively-charged teichoic acid residues and lipopolysaccharide molecules (16-18).

CONCLUSION

This study shown that an antibiotic (AMC-) can be incorporated with *β*–TCP micro-granules with three different sizes of residue and all sizes were found to have complete drug release within 48 h. The size of 300 µm did showed a better antibacterial activity against Gram-negative strains.

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