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Fabrication of ciprofloxacin loaded alginate/cockle shell powder nanobiocomposite bone scaffold

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ABSTRACT

Orthopedic implant infection is one of the most challenging issues in bone tissue engineering industry. Hence, local delivery of antibiotics incorporated into a fabricated bone scaffold possibly provides a more rapid bacteria inhibitory effect. In this study, pure ciprofloxacin loaded alginate/cockle shell powder nanobiocomposite bone scaffolds are fabricated with 5 wt% and 10 wt% ciprofloxacin respectively and tested for drug encapsulation, drug release and antibacterial properties towards common implant infecting bacterial strains (*Staphylococcus aureus* and *Pseudomonas aeruginosa*). Results from the studies showed a low drug encapsulation and drug release regardless of the concentration of drugs loaded with no significant differences noted ($p<0.05$). However, bacterial inhibition studies through direct contact and using eluted samples from drug release studies showed some inhibitory effects towards the growth of both bacterial strains tested. These findings were further justified with microscopy observations on biofilm and bacterial colony formation. Mineralization studies conducted additionally indicated that the scaffolds characteristics was not compromised due to drug loading. Although pure ciprofloxacin may not be the most suitable antibiotic to be incorporated into the nanobiocomposite bone scaffold, the study did provide some insight to the possible use of the scaffold for future drug delivery applications.

Keywords: *Cockle shell powder; alginate; ciprofloxacin; drug delivery and antibacterial*

INTRODUCTION

Bone transplantation becomes a necessary requirement when critical injuries that are unable to undergo auto-recovery occurs. Reconstructive and transplantation surgery are still dampened with the lack of donor tissues that are further complicated by immunosuppression or infection issues (Jovic et al., 2020). The drawbacks on conventional methods such as autographs and allographs call for the ever-developing field of bone tissue engineering to provide an alternative method in bone transplantation (Kanczler & Oreffo, 2008). Bone implants fabricated in forms of bone scaffolds or injectable materials made of biomaterials are gaining popularity in recent years to be used as a template or a temporary matrix for cell

proliferation and extracellular matrix settlement for bone regeneration (Hutmacher, 2000). Even with the presence of wide selection of bone substitute materials, the field of orthopedic implantation constantly battles implant rejections due to infection's which could lead to severe consequences or even death (Ribeiro et al., 2012). This scenario is further hindered with the lack of biomaterial-based grafts that could be used for effective repair of infected bones (Zou et al., 2020).

The adherence of bacteria on implant devices and its subsequent biofilm formation presents a great challenge for a successful antibiotic treatment (Zilberman & Elsner, 2008). Surgical removal of infected tissues and a heavy regime of antibiotic treatment are the current choice of treatment with a motif of reducing as much as possible the bacterial load from an infected tissue (Zimmerli & Sendi, 2017). Common bacteria's involved in orthopedic implant infections include *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Corvec et al., 2012) that are known to develop biofilms as a mode of defense. Biofilms formed by bacteria that colonize foreign implant materials are a common contributors for immune system and antibiotic treatment failure (Seebach & Kubatzky, 2019). The staphylococcus subspecies based infection on the other hand often requires a combination of intravenous and oral antibiotic therapy for an effective eradication of the biofilm-embedded bacteria. Even so, the high resistance of most biofilm-embedded bacteria against most antibiotic often leads to survival of dormant cells within biofilms or implant niches that could possibly contribute to frequent reinfections that may result in implant failure or non healing bone defects (Moriarty et al., 2016). Early antibiotic intervention embedded within an implant material therefore forms an attractive alternative to provide an in-built defence mechanism towards initial bacterial invasions. This, in recent years has formed an important part of biomaterial implant fabrication that is necessary to reduce the consequences of implant infections and thus reducing rejection rates and implant failures.

Commonly used antibiotics such as ciprofloxacin is well-known for its high penetrative ability and broad-spectrum characteristics which could potentially be used for prevention of bacterial infection on orthopedic implant devices (Krishnan et al., 2015). The ability to produce a localized drug delivery effect by incorporation of drugs within a bone implant would be able to provide a better therapeutic effect against implant infections compared to systemic delivery of drugs (Mouriño & Boccaccini, 2009). A designed implant material could either be passively modified through surface modification, or actively modified through incorporation of pharmacologically active substance such as antibiotics with an aim in preventing bacterial infection. In this study, we take into account of active modification by exploring the ability to produce a localized drug delivery effect by fabricating a bone scaffold loaded with pure ciprofloxacin.

In our earlier studies, we have successfully fabricated a biomaterial based nanobiocomposite bone scaffold using alginate and nano cockle shell powder that has shown promising properties of an ideal bone graft (Bharatham et al., 2014). The biocompatible aragonite calcium carbonate polymorph from the mollusc shells has been shown to possess excellent physiochemical characteristics and cellular response as an ideal bone grafting material when formulated with alginate polymers. The ease of conversion of the cockle shell powder into a nano phase material (Islam et al., 2012) further adds to the advantages in regards to increase in surface modification and binding factors that could influence the efficiency of the nanobiocomposite bone scaffold designed as potent drug carrier. The ability to integrate drugs during its fabrication phase and its subsequent release would allow further exploration of the developed material into potential bone implant material with a drug delivery system with subsequent antibacterial properties.

In this study ciprofloxacin an antibiotic drug commonly used in treating bone infections such as osteomyelitis (Lili et al., 2019) and known to be highly effective against *Staphylococcus aureus* (Tsai et al., 2008) is used to test for the efficacy of the nanobiocomposite scaffold fabrication for drug delivery. In addition, surface characteristic modification of the scaffold material in regards mineralization properties that contributes positively towards osteoconduction that might be altered due to drug incorporation is also evaluated in this study.

METHODOLOGY

Fabrication of nanobiocomposite bone scaffold

Bone scaffolds were fabricated based on previous studies (Su et al., 2021) using 30% alginate and 70% nano-cockle shell powder incorporated with either 5wt% or 10wt% pure ciprofloxacin. The fabrication steps of the bone scaffolds includes mixing of nano-cockle shell powder with hydrocolloid alginate solution that was homogenized at 400 rpm prior to adding of drugs. Citric acid solution was then added into the mixture until a moldable consistency was achieved. The mixture was then filled into a custom made cylindrical mold and allowed to set at 37˚C for 24 hours. Non-drug incorporated scaffolds were used as controls in the study.

Mineralization study of the nanobiocomposite bone scaffold

Mineralization study was performed on formulated scaffolds both control and drug loaded scaffolds using 10x simulated body fluid (SBF). The scaffolds were immersed in SBF for 24 hours, washed with distilled water and fixed in 2.5% glutaraldehyde solution for another 24 hours prior to scanning electron microscope (SEM) evaluation (Su et al., 2021).

Drug release evaluation

Drug encapsulation efficiency

Encapsulation efficiency studies were conducted according to Krishnan et al., (2015). Scaffolds fabricated with ciprofloxacin were powdered and soaked in 5 mL of phosphate buffer solution (PBS) prior to be centrifuged at 8000 rpm for 15 minutes. Presence of drugs in the supernatants were evaluated using spectrophotometer at 277 nm in triplicate and the concentration was determined based on a standard curve. The drug encapsulation efficiency was then calculated using the formula below:

$$Drug\ encapsulation\ efficiency = \frac{Amount\ of\ drug\ encapsulated}{Amount\ of\ drug\ added}\ x\ 100\%$$

Drug release study

Drug release study was conducted based on modified methods of Cao and Zhang [Cao et al., 2017; Zhang et al., 2012). Scaffolds fabricated with ciprofloxacin were immersed in 10 mL of simulated body fluid (SBF) and incubated at 37 °C. The elution sample were taken at 1, 5, 12 and 24 hours as well as on days 3, 6, 10, 14, 18 and 21. During each sampling period, 1 mL of the elution sample was stored in the Eppendorf tube and replaced with fresh simulated body fluid (SBF) in order to prevent super saturation of drug. Concentration of eluted samples were determined by spectrophotometer at 277 nm in triplicate and was further used for bacterial inhibition studies.

Antibacterial activity evaluation

Bacterial inhibition study

Bacterial inhibition studies were tested on elution samples collected from various time points of drug release study as well as through direct contact with bone scaffold samples on Staphylococcus aureus (*S. aureus*) and Pseudomonas aeruginosa (*P. aeruginosa*). Bacteria's were incubated at 37 °C in growth medium and the culture was adjusted by spectrophotometric measurement at 625 nm to provide a final density of 1.0 x 108 CFU / mL in broth. 100 µL of each bacterial strain were spread on Mueller Hinton agar in triplicates respectively prior to be placed for culturing with 6 mm diameter filter papers containing 10 µL of the elution samples obtained from the drug release study. The agar plates were incubated at 37 ºC overnight and the diameter of the inhibition zone on the agar plates were measured in millimetres (Isa et al., 2016).

Biofilm formation study

Biofilm formation study were conducted using *S. aureus* and *P. aeruginosa* sub-cultured in 50 ml Luria broth (LB) containing 1.0 x 106 CFU/mL bacteria. Drug containing scaffolds and control scaffolds were then immersed and incubate in the LB broth at 37 ºC shaking incubator with 80 shakes/minute for 24 hours. After 24 hours the scaffolds were removed and washed with phosphate buffer solution (PBS) thrice to remove unattached bacteria's (Punyani et al., 2007) prior to be preserved for microscopy examination.

Statistical analysis

All experimental data were expressed as mean ± standard error mean (SEM) and quantitative results were analysed using one-way ANOVA with significant value of $p<0.05$.

RESULTS

Fabrication of nanobiocomposite bone scaffolds

The texture of scaffolds obtained when formulated with different amount of citric acid is shown in Table 1. The formulation of the scaffolds fabricated was altered using varying amount of citric acid in which the best outcome for the nanobiocomposite scaffold were achieved when the materials were fabricated with 30 µl of 50% citric acid for 1g of scaffold. This formulation produced a scaffold structure that was easily moldable and gradually hardens providing sufficient work time to be made into its final shape.

Table 1

Formulation used in fabrication of the nanobiocomposite bone scaffolds and its resulting texture observed

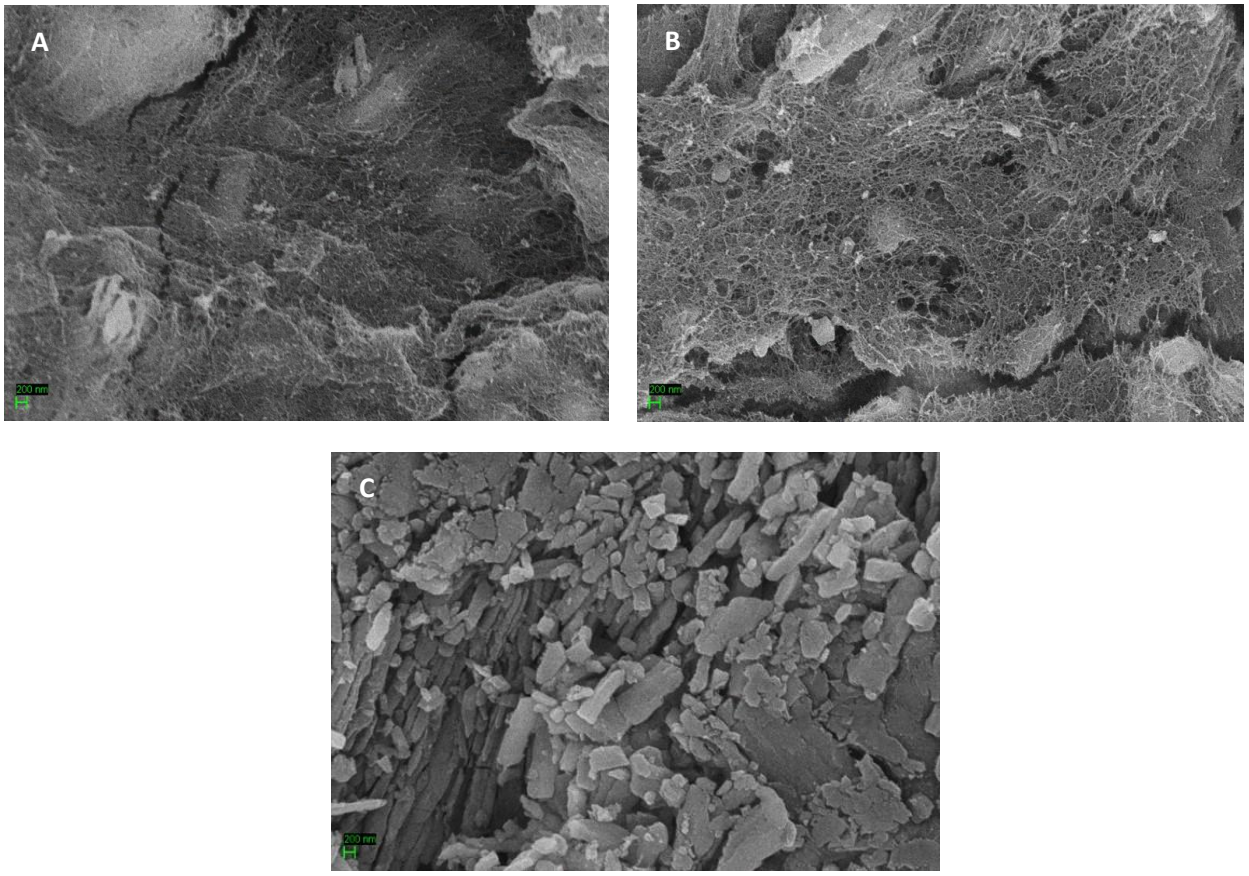
Nano Cockle shell Powder: Alginate (w/v)	Deionized water (mL)	50% Citric acid (µl)	Texture
70:30	6	200	Hard and unable to be molded
70:30	6	100	Unable to be molded
70:30	6	50	Unable to be molded
70:30	6	30	Moldable and gradually hardens
70:30	6	20	Paste-like texture, with runny consistency.

Mineralization study

Figure 1 shows the scanning electron microscopy observation of the scaffold surface on both control as well as drug loaded scaffold. The results showed similar mineralization pattern with formation of apatite layers visualized as network like patterns on all scaffold surfaces, indicating no alteration in mineralization ability of the scaffolds despite being loaded with drugs.

Figure 1

Scanning electron microscopy observation of surface mineralization



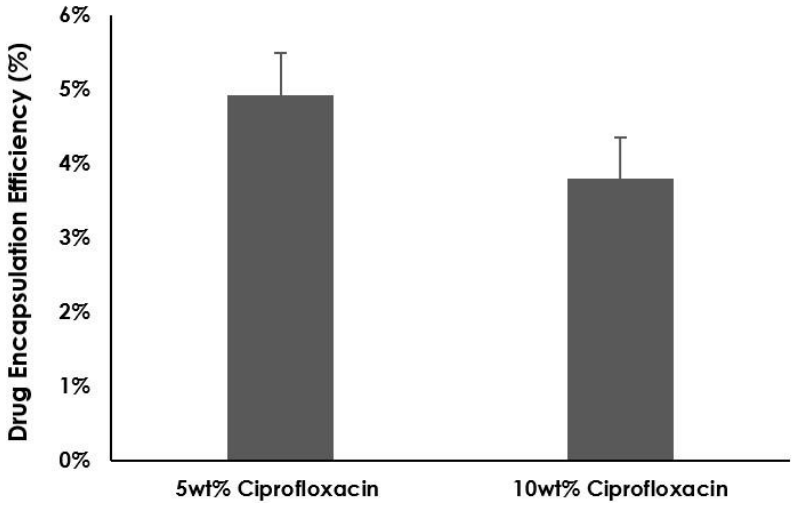
Note: SEM micrograph of surface mineralization on (A) control scaffold, (B) 5wt% ciprofloxacin scaffold, (C) unmineralized surface of control scaffold for comparison at (x10). Scale 200nm.

Drug release evaluation

Fabrication of the nanobiocomposite scaffold with 5wt% and 10wt% ciprofloxacin both resulted in a low drug encapsulation efficiency of 4.92 ± 0.569 % and 3.8 ± 0.551 % respectively and was found to be not significant as shown in Figure 2. In regards to drug release, we report similar low drug release profile (Figure 3) from both scaffolds formulated with 5wt% and 10wt% ciprofloxacin over the study period. An initial burst release pattern was observed at the first hour with a significantly higher release of drugs observed in 5wt% ciprofloxacin loaded scaffolds compared to 10wt% ciprofloxacin loaded scaffolds. This was then followed by a significant drop in drug released within the next 5 hours for both concentration. Then after, drug release for all subsequent time point from the scaffolds remained plateau regardless of the concentration of drugs used. Presences of drug concentration in samples were no longer detectable spectrophotometrically on Days 14, 18 and 21.

Figure 2

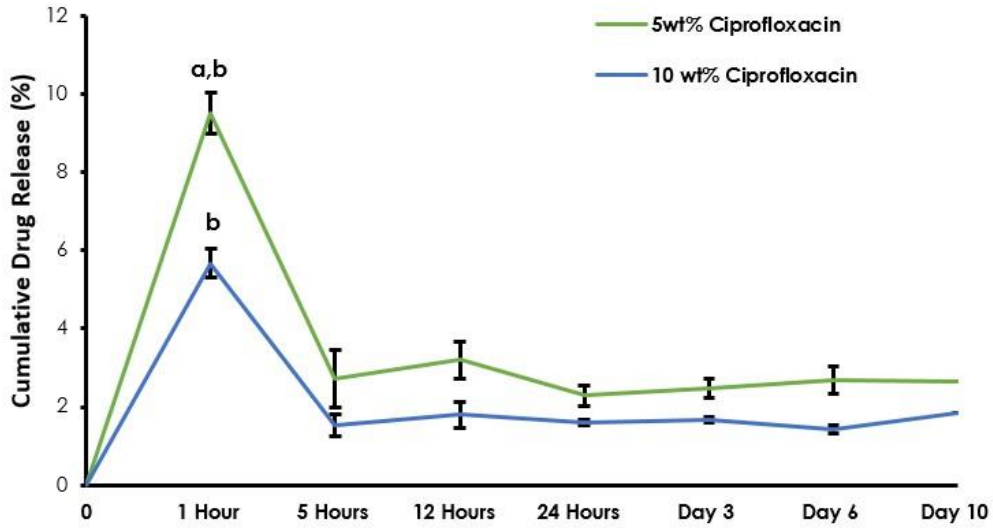
Drug encapsulation efficiency of ciprofloxacin loaded nanobiocomposite bone scaffold



Note: Drug encapsulation efficiency of ciprofloxacin loaded nanobiocomposite bone scaffold. Data are displayed in the form of mean ± SEM, n=3.

Figure 3

Cumulative drug release from ciprofloxacin loaded bone scaffolds



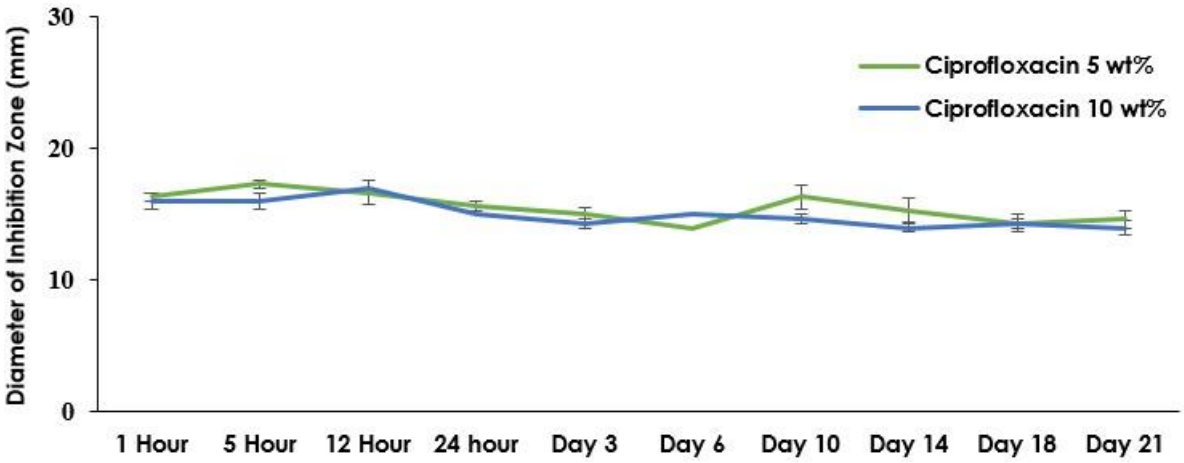
*Note: Cumulative drug release of ciprofloxacin loaded bone scaffolds. Data are displayed in the form of mean ± SEM, n=3. *a: significant difference when compared to 10 wt% ciprofloxacin scaffolds (p<0.05) at 1 hour. *b: significant difference when compared to other time points (p<0.05).*

Antibacterial activity evaluation

Figure 4 and 5 shows the results of the bacterial inhibition assay conducted using the eluted samples obtained from drug release study on *S. aureus* and *P. aeruginosa*. The results were found to be parallel to those of the drug release as no significant differences in the inhibitory effect was observed across the tested time point. Inhibitory zone diameters for *S. aureus* and *P. aeruginosa* were found to be in the range of 14 – 17 mm and 17 – 21 mm respectively. A stronger antibacterial activity in regards to inhibitory zone diameter (Figure 6) was obtained for scaffolds loaded with 5 wt% ciprofloxacin against *P. aeruginosa* (44.33 ± 0.67 mm) compared to *S. aureus* (40.33 ± 0.33 mm) when drug loaded scaffolds were directly placed on to the bacterial laden culture plates in the direct contact inhibition study.

Figure 4

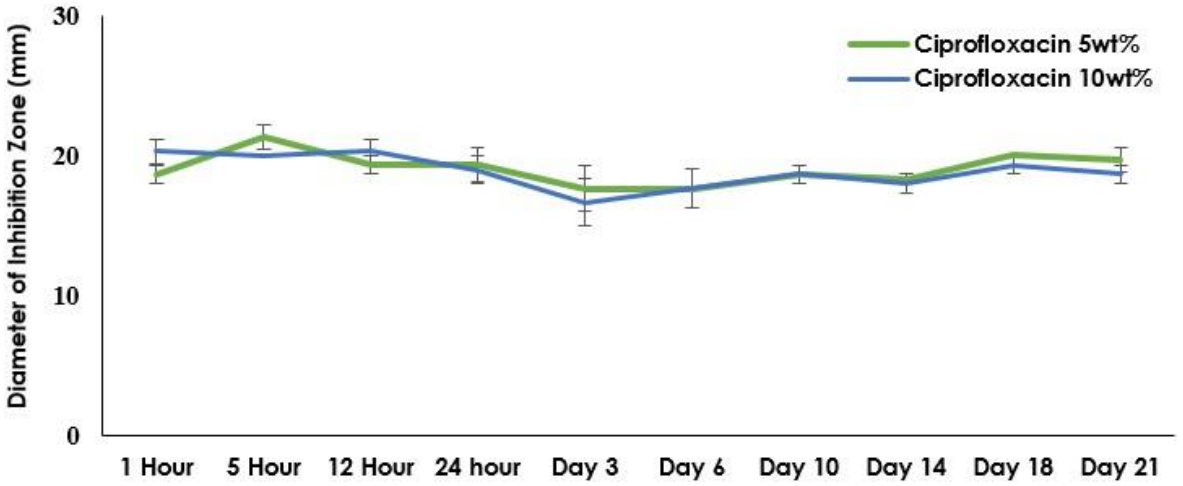
Antibacterial activity against S. aureus



Note: Antibacterial activity of eluded drug from ciprofloxacin loaded scaffolds against S. aureus.

Figure 5

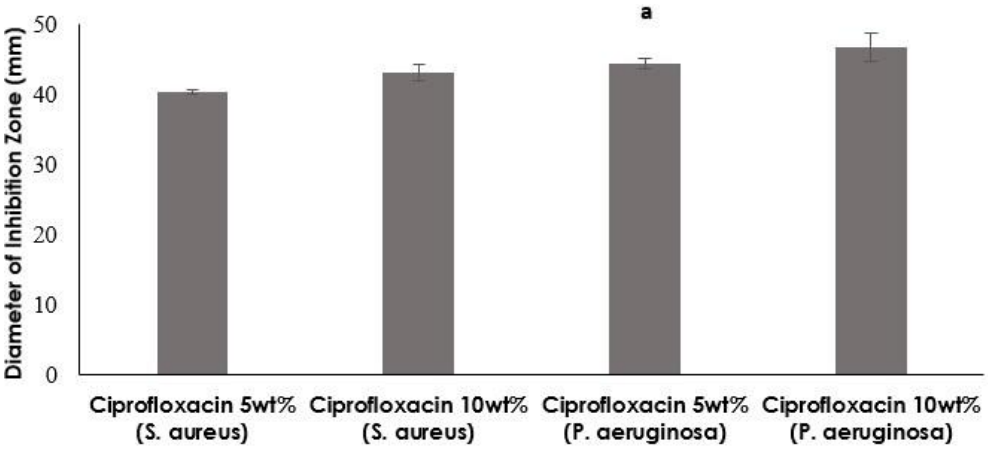
Antibacterial activity against P. aeruginosa



Note: Antibacterial activity of eluded drug from ciprofloxacin loaded scaffolds against P. aeruginosa..

Figure 6

Antibacterial activity via direct contact inhibition studies

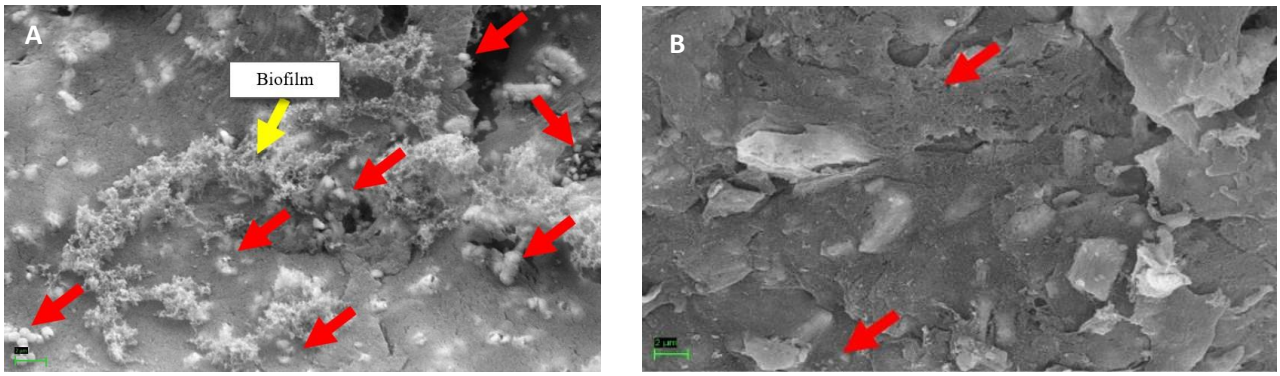


*Note: Antibacterial activity shown through direct contact inhibition studies on ciprofloxacin loaded scaffolds towards S. aureus and P. aeruginosa. Data are displayed in the form of mean ± SEM, n=3. *a: significant difference when compared to 5%wt ciprofloxacin scaffold that was tested against S. aureus ($p<0.05$).*

Figure 7 demonstrates the scanning electron microscopy (SEM) observations of bacterial growth and biofilm formation on control and 5wt% ciprofloxacin loaded scaffold surfaces respectively after being immersed in *S. aureus* bacteria solution for 24 hours. Biofilm formation and a higher number of bacterial colonies were evident in control scaffolds compared to drug loaded scaffolds. Figure 8 and 9 shows the bacterial growth on surface of scaffolds observed via H&E histology observations of drug loaded scaffolds immersed in *S. aureus* and *P. aeruginosa* bacteria solutions respectively as compared to the control scaffolds. Scaffolds loaded with ciprofloxacin regardless its concentration showed a lower number of bacterial colony growth on its surface as opposed to control scaffolds.

Figure 7

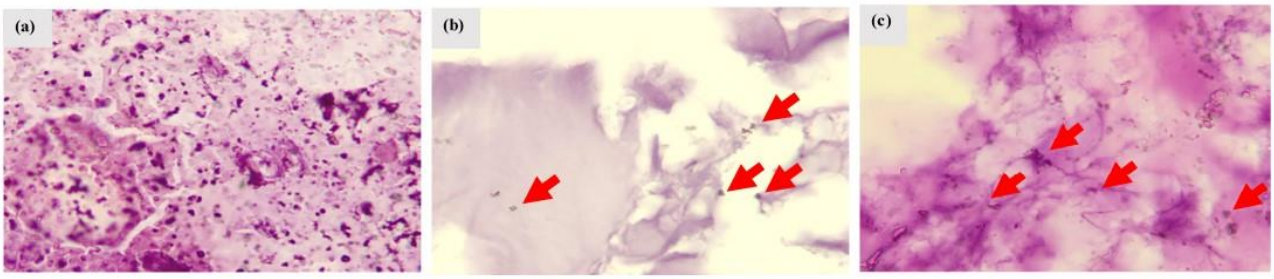
SEM of bacterial growth and biofilm formation



Note: SEM micrograph of (A) control scaffold and (B) 5wt% ciprofloxacin loaded scaffold tested against S. aureus at x3000 demonstrating the growth of cocci-shaped bacteria colonies (red arrow) and biofilm formation (yellow arrow). Scale 2µm.

Figure 8

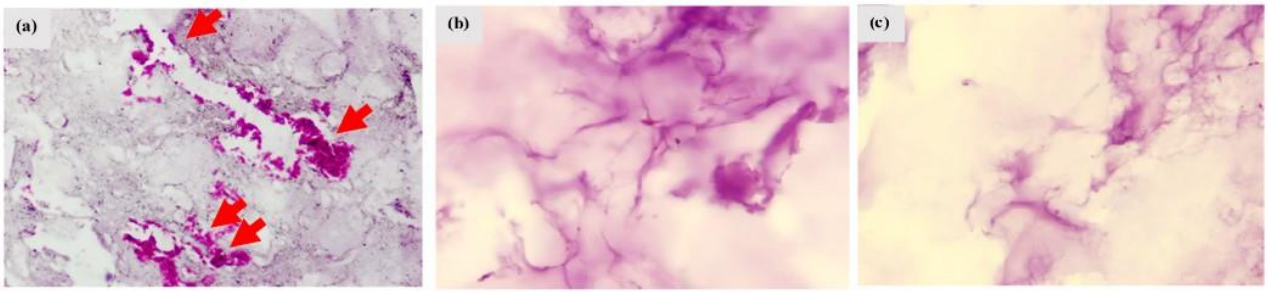
S. aureus bacterial growth on scaffold surfaces



Note: Histology observations of scaffold surfaces of (a) control scaffold, (b) 5wt% and (c) 10wt% ciprofloxacin loaded scaffold tested against S. aureus at (x100) using H&E staining. Multiple purple dots in (a) and red arrows in (b) and (c) indicates the growth of bacterial colonies.

Figure 9

P. aeruginosa bacterial growth on scaffold surfaces



Note: Histology observations of scaffold surfaces of (a) control scaffold, (b) 5wt% and (c) 10wt% ciprofloxacin loaded scaffold tested against P. aeruginosa at (x100) using H&E staining. Red arrow in (a) indicates growth of bacterial colonies.

DISCUSSION

Fabrication of scaffolds for bone tissue engineering could be undertaken via various approaches. The moldable version of scaffolds often provide better flexibility for design and shape that could enhance specificity. The most ideal scaffold texture should result in a moldable scaffold with sufficient level of hardness. Scaffolds that are moldable into different sizes and shapes provide an attractive option as it allows the tailoring of the scaffold structure to suit the size or the need of a defect area in accordance to the magnitude of the injury (Turnbull et al., 2018). The need for sufficient hardness is also important to ensure the success of the scaffold in supporting regeneration of new tissues in regards to the mechanical strength of the bone (Calori et al., 2011). In this study, the use of citric acid at a concentration of 30 µl provided the most suitable formulation to produce an ideal textured scaffold in regards to its biocomposite formulation. It is a known fact that presence of citrate molecules are known to bind strongly to apatite nanocrystals directly regulating crystallinity aspects of bone minerals that contributes to the overall strength of bone tissues (Sun et al., 2014). Use of citric acid in fabrication of the scaffold material is an ideal choice as it is able to shorten the setting time of the mixture samples while contributing to the structural hardness and possible increase in mechanical strength of the biomaterial (Sheikh et al., 2015). The advantages of using citric acid in the fabrications of bone scaffolds can be traced back to the citric acid-based polymer/hydroxyapaptite composites that formed a novel class of biomaterial based orthopaedic implants with added advantages for bone tissue engineering (Sun et al., 2014). It is also worthy to note that composite presence of citrate either on the biomaterial or exogenous supply can help accelerate osteoblast responses.

A key point in determining the successful fabrication of a bone scaffold relays in its ability to undergo sufficient mineralization in order to facilitate bone cell regeneration that may be altered with incorporation of chemicals and drugs during the fabrication process. In this study, any possible characteristic alteration in the nano cockle shell-alginate bone scaffold in undergoing mineralization due to the presences of ciprofloxacin was observed through the formation of surface apatite layers when immersed into a simulated body fluid (SBF) solution. SBF is a medium that contains human plasma-like ion concentration that is widely used to determine the possibility of a

bone scaffold to undergo the process of mineralization (Kretlow, J. D. & Mikos, A. G. 2007). The ability of a scaffold to undergo mineralization is a key component in determining the success of the scaffold material in increasing the bone regeneration process (Shin et al., 2017). It is worthy to note the enhanced responses of mineralized biomaterial compared to a non-mineralized material in which the former has shown better mechanical properties as well as better cellular responses (Xinchen et al., 2020). Therefore, the ability of the fabricated scaffold to undergo mineralization forms an important characteristic of a good quality scaffold and observation from this study indicated that incorporation of ciprofloxacin did not deter the mineralization process of the drug loaded scaffolds fabricated.

Drug encapsulation efficiency and drug release study are important aspects in predicting the in vivo behavior of drugs that are formulated into a scaffold (Wallace et al., 2012). Both this studies were carried out in a medium with neutral condition (pH 7.40) to mimic conditions of blood plasma in human body. We postulate that the direct loading of the drug into the scaffold mixture did not favour the solubility aspect of the drug in the mixture. Ciprofloxacin's solubility is very much pH dependent and the drug is known to show higher solubility in lower pH medium (Ross, D. L. & Riley, C. M. 1990 ; Breda et al., 2009). From our understanding, the nanobiocomposite scaffold formulated has an alkaline pH due to the presences of calcium carbonate originating from the cockle shell powders that could have contributed to the low solubility of the drug in the mixture. Reduced solubility of the drug resulted in lower drug encapsulation efficiency regardless of the drug concentration used. However, the interaction of ciprofloxacin to the alginate group in the formulation of the scaffold could have contributed to some amount of ciprofloxacin to be bounded into the scaffold mixture that were detected in the study. In regards to this, binding of ciprofloxacin is highly depended on concentration of alginate used, which was a constant factor in the formulation of the scaffolds. This could have contributed to a lack of carboxyl group (-COOH) from the alginates to form bonds with the amino group ($-NH_2$) of ciprofloxacin (Lecaroz et al., 2006) despite the increase in drug dosage. Earlier reports on the use of alginate as carries for ciprofloxacin has reported a significant increase in drug loading of ciprofloxacin when the concentration of alginate used is increased (Hariyadi, D.M. & Hendradi, E. 2020). The increase in concentration of drug used in this study were unable to produce any significant differences as available carboxyl group remained constant. Scaffold that was formulated with 5 wt% ciprofloxacin however showed slightly better drug encapsulation thus was able to have better drug release comparatively. The characteristic of ciprofloxacin that gradually crystallizes in medium of neutral pH (Salem et al., 2005) could have potentially contributed to the reduction in drugs detected after the initial release. The low solubility of the drug in the neutral pH could also be a factor that the drug concentration remained low throughout the study.

Bacterial inhibition assay allowed an initial evaluation of antibacterial properties of the nanobiocomposite scaffold when formulated with ciprofloxacin. The study was conducted using the eluted samples obtained from drug release study as well as through direct contact inhibition studies on *S. aureus* and *P. aeruginosa*. Despite the low percentage of drug encapsulated and released from the scaffold, some extend of inhibitory effects against both bacterial strain was evident from the eluted samples. These findings can be attributed to the fact that ciprofloxacin is an antibiotic from quinolone group that is known to act actively against *P. aeruginosa* with less vigorous activity against *S. aureus* (Oliphant, C. M. & Green, G. M. 2002; Stewart, P. S. & Costerton, J. W. 2001). On the other hand, biofilm formation study was conducted to observe the ability of the scaffolds to inhibit the bacterial colony growth on its surfaces. Formation of biofilm layers on surface of scaffolds are major contributing factor towards antibiotic resistance that eventually leads to failure in implants (Stewart, P. S. & Costerton, J. W. 2001). Less bacterial colonies were observed on the drug loaded scaffolds as opposed to control scaffold surfaces which indicated that ciprofloxacin formulated into the scaffold is able to give a localized antibacterial activity against the bacterial strains tested. The drug effects in both concentration tested was found to be more prominent against *P. aeruginosa* with no obvious bacterial colonies being observed compared to the control scaffold. This finding are in agreement with the antibacterial evaluation using direct contact inhibition studies that showed a higher antibacterial activity of the drug towards *P. aeruginosa*. The ability of the drug loaded scaffolds to reduce the formation of bacterial colonies on the scaffolds surface as compared to the control scaffolds indicates a direct contact antibacterial property with the scaffold surface despite its poor drug encapsulation and release. This form of contact killing active surface strategies in recent years have come into highlight to reduce probability of infections in implant devices taking into count the concept of pre-incorporating antibiotics for active release in order to reduce bacterial adhesion (Romanò et al., 2019). In this study the presence of ciprofloxacin incorporated into the scaffolds which is postulated to be in-situ due to its inability to be efficiently released from the scaffold did help prevent the formation of biofilm layer on the surface of the scaffold as observed through the histology studies.

CONCLUSION

Incorporation of drugs into an implant material is becoming an important aspect of drug delivery in the field of bone tissue engineering in recent years. The concept forms an attractive alternate to address fast availability of drugs at the target site due to lack of vasculature in the bone. In this study, an attempt to incorporate ciprofloxacin, an antibiotic drug into alginate/cockle shell powder nanobiocomposite bone scaffold was undertaken. The incorporation of drugs into the scaffold formulation did not alter the ability of the scaffold to undergo mineralization indicating that the characteristic of the scaffold in regards to osteoconductivity is unlikely to be

affected. Although the drug encapsulation efficiency and drug released from the drug loaded scaffolds was found to be low despite the concentration of drugs used, antibacterial activity and inhibitory effects against the bacteria *S. aureus* and *P. aeruginosa* were still noted. From the study, we have deduced that some optimization steps may be needed in order to increase the drug encapsulation efficiency with possibility of changing pure form of ciprofloxacin to an hydrochloride form that has better solubility. Varying the content of alginate used in the formulation could be another possible variable factor that could be manipulated to increase drug binding sites. Regardless the need of further studies, the possibility of drug loading into the formulated scaffold promises a more precise directed way for drug-delivery based implant materials in the ever changing paradigm of bone tissue engineering.

AUTHOR CONTRIBUTIONS

The following study was undertaken by Wong Huai Li as part of a research project under the supervision of B. Hemabarathy Bharatham for scaffold design and fabrication, Jacintha Santhanam for antibacterial related evaluation and Ng Shiow Fern for drug related studies. The manuscript was written by Wong Huai Li and B. Hemabarathy Bharatham who also supervised, edited and reviewed the content.

ETHICS APPROVAL

Not applicable.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in this work.

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