Fabrication and characterization of chitosan-titanium oxide nanotubes scaffolds reinforced with tiger milk mushroom

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Abstract. Chitosan-based scaffolds have been reported to promote cellular activities but lack mechanical strength which is much sought after for bone regeneration. The current research work aided to reinforce chitosan-based scaffolds with tiger milk mushroom (TMM) powder, a naturally occurring polysaccharide. Scaffolds of chitosan-titanium oxide nanotubes (TNTs) reinforced with tiger milk mushroom (TMM-CTNTs) were fabricated via direct-blending and freeze-drying methods. Prior to that, TNTs were hydrothermally synthesized and blended with chitosan solution and TMM powder at 1-5 weight percent (wt %). The pore size, microstructure, porosity, swelling, degradation, compressive modulus and functional groups of resultant scaffolds were characterized. These cylindrical scaffolds of TMM-CTNTs showed pore size of 48 – 68 μm. The addition of TMM from 3 wt% to 5 wt% in scaffolds reduced the porosity from 81.7 % to 79.9 %. The compressive modulus of 3 wt%-5 wt% TMM-CTNTs scaffolds increased from 0.013 MPa – 0.038 MPa. The incorporation of TMM influenced the swelling property of scaffolds. The swelling percentage of TMM-CTNTs reduced from 400% to 373% as TMM powder was introduced from 1 wt% to 5 wt%. The degradation ratio increased from 0.959% to 2.385 % as TMM powder was introduced from 1 wt% to 5 wt%. The Fourier-Transform Infrared (FTIR) spectra of TMM-CTNTs scaffolds revealed the presence of β-glucan which verified that the processing methods in this study preserved the medicinal property of TMM. A preliminary in vitro test, MTT assay, was used to study proliferation rate of MG63 (osteoblast-like cells) cultured on TMM-CTNTs scaffolds with different weight percent of TMM. Notably, the cells proliferation of MG63 showed high biocompatibility at 3 days of culture.

Keywords: titanium oxide nanotubes, tiger milk mushroom, chitosan

1. Introduction

The evolution in biomaterial research field has been tremendously become a major attention to researchers in the development of orthopedic tissue engineering. Manufacturing of artificial composite scaffolds using natural bioactive polymer is in high demand compared to synthetic polymer. The physical and chemical characteristic of natural bioactive polymer are similar to the structure of extra cellular matrix (ECM) in human tissues. The designed scaffolds should be capable to mimic ECM functions as a transporter of nutrient, oxygen and metabolic by product at the defect sites within a few
weeks of implantation. It is essential for scaffolds to be nontoxic and safe to be used in order to promote normal cellular activities [1].

Chitosan, a natural abundant resource, is a semi-synthetic polymer which is derived from chitin [2]. This linear polysaccharide composed of glucosamine and N-acetyl glucosamine units which can commonly be found in skeletal of fungi, crustaceans and insects. Chitosan has excellent biodegradability, anti-bacterial activity, biocompatibility and non-antigenicity that make it suitable for tissue engineering. Ideal scaffolds should be designed with porous three-dimensional (3D) network, high mechanical strength and controllable degradation rate for renewal and replacement of new bone tissue. However, the fabrication of chitosan-based scaffolds has been not much attractive to researchers due to its insufficient mechanical strength for load bearing usage [3], uncontrollable degradation rate [4] and high acidity and concentration solution [5]. Up to now, there are only a few studies reported on designing scaffolds using chitosan as a based material in orthopedic tissue engineering [6-7]. Chitosan as stand-alone material for scaffolds fabrication could not fulfill ideal requirement as temporary three-dimensional structure for cell growth. To overcome these challenges, reinforcement of chitosan-based scaffolds with organic and inorganic compound can produce nanocomposites scaffolds with improved properties.

Designation of scaffolds template in nano range can effectively enhance surface area and permeability of scaffolds. Therefore, introduction of titanium oxide nanotubes (TNTs), inorganic ceramic materials, is the best material selection in designing porous scaffolds with firm structure. Titanium oxide nanotubes possess great unique features of large surface area, good corrosion resistance, excellent biocompatibility and enhanced bioactivity especially in terms of osteoblast adhesion and proliferation [8-9]. Previous study by Lim et al. involved the preparation of chitosan-TNTs scaffolds which showed improved compressive modulus and excellent biocompatibility by promoting enhanced adhesion, proliferation and early differentiation of MG63 cells [2].

Lignosus rhinocerusis commonly known as tiger milk mushroom (TMM), is a medical mushroom which is under Polyporaceae family has been used by native communities in treating cough, fever, asthma, joint pain, cancer and food poisoning. The sclerotium part of TMM has been investigated in a few studies and showed tremendous medicinal benefits, biopharmacological properties and functional food potentials. There are a few research works revealed that TMM function as anti-cancer, anti-inflammatory, immune-modulatory and antioxidant activities [10-12]. These fabulous properties naturally exist in TMM due to β-glucan, a bioactive compound presence in tuber part of sclerotium. Current research in tissue engineering have not explored the potential of β-glucan material in scaffolds. Therefore, chitosan, TNTs and TMM are desired compounds since they are safe and have been reported with high in vitro efficacy. This spectacular invention may be useful in improving mechanical strength and enhanced scaffolds compatible performance with living tissues during cellular activities for bone regeneration.

In this paper, TMM-CTNTs scaffolds were fabricated using direct blending and freeze-drying method. The pore size, microstructure, porosity, swelling, degradation, compressive modulus and functional groups of resultant scaffolds were characterized. A preliminary in vitro test of cell proliferation on TMM-CTNTs scaffolds using MG63 (osteoblast-like cells) was conducted. The effects of TMM weight percent in chitosan scaffolds on the physical properties and proliferation of MG63 were studied.

2. Materials and methods

2.1. Materials
Titanium dioxide (TiO$_2$) powder, chitosan powder (middle viscous, 80 % deacetylated), sodium bicarbonate, sodium pyruvate, and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich (Germany). NaOH pellets were purchased from R&M Chemicals (UK). Acetic acid, hydrochloric acid and ethanol were purchased from Fisher Scientific (USA). Chemicals for the preparation of phosphate buffered saline (PBS) including sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic, monopotassium and phosphate were purchased from Sigma Aldrich (Germany). Tiger Milk Mushroom (TMM) powders were purchased from LiGNO (Malaysia). 24-well plates and 96-well plates were purchased from Orange Scientific (Belgium). Minimum essential media (MEM), fetal bovine serum (FBS), penicilline/streptomycin, 0.25 % trypsin and 3-[4,5-dimethylthiazole-2-yl]-2,5-
diphenyltetrazolium bromide (MTT) were purchased from Gibco (USA). Distilled water was used throughout the sample preparation.

2.2. Fabrication of tiger milk mushroom incorporated chitosan-titanium oxide nanotubes (TMM-CTNTS) scaffolds

Titanium oxide nanotubes (TNTs) were hydrothermally synthesized by mixing 2 g of bulk TiO₂ powder in 40 mL of 10 M NaOH in a Teflon container, put in a stainless steel reactor, and heated 150 °C for 72 h [13]. The hydrothermal product was filtered and washed with distilled water and 0.1 M of hydrochloric acid (HCl) until pH 7 was achieved. The white filter cake was dried overnight in the oven (Binder, United Kindom) at 60 °C and finally was sintered at 400 °C for 5 h. The fabrication of tiger milk mushroom incorporated into chitosan-titanium oxide nanotubes (TMM-CTNTs) scaffolds was referred to a method developed by Lim et al. [2] The titanium oxide nanotubes (TNTs) and tiger milk mushroom (TMM) powder (LiGNO, Malaysia) at different weight percent (wt%) were mixed with chitosan solution using a hotplate at 700 rpm for 5 h (see table 1). Chitosan solution was prepared by dissolving 1 g of chitosan flakes in 100 mL of 0.2 M acetic solution at room temperature. The solution was constantly stirred at 300 rpm for 3 h. Next, the colloidal suspension was transferred into a 24-well plate and kept frozen at -20 °C for 24 h. The frozen scaffolds were freeze-dried at -40 °C for 24 h. Finally, scaffolds were rehydrated with absolute alcohol for 1 h and then followed by 30 min rehydration of 70 % alcohol and 50 % alcohol respectively. Rehydrated scaffolds were finally dried prior to characterization.

| Sample identification | Weight percent of titanium oxide nanotubes (wt%) | Weight percent of Tiger milk mushroom (wt%) | Chitosan solution (mL) |
|-----------------------|-----------------------------------------------|---------------------------------------------|------------------------|
| TMM0                  | 16                                            | 0                                           | 24                     |
| TMM1                  | 15                                            | 1                                           | 24                     |
| TMM2                  | 14                                            | 2                                           | 24                     |
| TMM3                  | 13                                            | 3                                           | 24                     |
| TMM4                  | 12                                            | 4                                           | 24                     |
| TMM5                  | 11                                            | 5                                           | 24                     |

2.3. Microstructural, physical and chemical characterization of tiger milk mushroom incorporated chitosan-titanium oxide nanotubes (TMM-CTNTS) scaffolds

The pore size and surface morphology of TMM0 – TMM5 scaffolds at different weight percent of TMM powder ranging from 0 to 5 % were characterized using Field Emission Scanning Electron Microscope (FESEM) (FEI QUANTA 400F, United States) at 10 – 20 kV. The pore size of scaffolds at different area was measured and the mean values were taken. The surface morphology of scaffolds was viewed under 500x magnification. The porosity of scaffolds was measured in percentage between 0 to 100 % and determined using equation (1):

\[
\text{Porosity} = \left[ 1 - \frac{V_{\text{skeletal}}}{V_{\text{bulk}}} \right] \times 100 \%
\]

V_{\text{skeletal}} defined as the skeletal volume of scaffolds (cm³) measured using a pycnometer (Duran, Germany). On the other hand, V_{\text{bulk}} was the bulk volume of scaffolds (cm³) determined from formula of
TA.XT Plus Texture Analyzer (Stable Micro System, United Kingdom) was used to determine compressive modulus via the plot of stress against strain. The load cell of 500 N was used to compress scaffolds until 40% of its original thickness at a crosshead speed of 1 mm/min. Prior to that, the dimension and thickness of scaffolds was measured using the vernier caliper.

The water absorption capacity of the scaffolds was determined by performing a swelling ratio test. The dry weight ($W_d$) of all scaffolds at 6 different weight percent of TMM powder ranging from 0-5% was measured and placed in 24-well plate. Two mL of pure minimum essential media (MEM) was pipetted into each well. Scaffolds were fully immersed in MEM and incubated for 24 h. Next, MEM from each well was removed and the wet weight for all scaffolds was measured and recorded. The swelling ratio was determined using equation (2):

$$\text{Swelling ratio (\%) } = \frac{(W_w - W_d)}{W_d} \times 100$$

where $W_w$ and $W_d$ are the weight of wet and dry scaffolds respectively.

Degradation test was conducted by immersion of scaffolds in 2 mL of MEM in a 24-well plate. Prior to that, the initial weight ($W_o$) of scaffolds was measured and recorded. The scaffolds at different weight percent of TMM were incubated at 37 °C for 28 days and changed medium every 2 days. After 28 days of immersion, scaffolds were washed with PBS after MEM was removed. After removal of PBS, wet scaffolds were kept frozen at -20 °C for 5 h. The samples were freeze dried at temperature -40 °C for 24 h. The weight ($W_f$) of freeze dried scaffolds was measured and recorded. The percentage of degradation was determined using equation (3):

$$\text{Degradation rate (\%) } = \frac{(W_o - W_f)}{W_o} \times 100$$

Each testing was performed triplicate on all scaffolds at different weight percent of TMM powders. The data were taken from calculated means values. The chemical compound of TMM powders and TMM-CTNTs scaffolds samples were identified using Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer, United States) at 400 – 4000 cm$^{-1}$ wavelength.

2.4. In vitro cell culture

MG63 cells (ATCC, USA) were cultured in a cell culture flask and maintained using pure MEM added with 5% FBS and 1% sodium pyruvate. The cells in the culture flask were incubated at 37 °C with 5% CO$_2$ level. Every 2 days, media were replaced to ensure cells supplemented with sufficient nutrients. Subculture and seeding of cells were proceeded when 90 – 100% confluence of cells was achieved. During subculturing or passaging, 0.25% trypsin was used to detach cells. Later, some of detached cells were transferred from previous culture to a new culture flask. During seeding procedure, trypsinization method was applied where cells were detached from the culture flask. All the detaching cells were transferred to falcon tube for centrifugation. Cells pellet were produced via centrifugation of cells detached with complete medium in falcon tube at 1800 rpm for 5 min. Finally, cells pellet was resuspended for a few times in complete medium. Prior of that, scaffolds samples which were placed in 24-well plate need to be sterilized under ultra violet (UV) irradiation for 15 min before being seeded with cells. The total number of cells were calculated and diluted to 2.8 x 10$^5$ cells ml$^{-1}$. Thirty thousand cells were transferred to each scaffold and empty well of polystyrene plate that served as a control. Both scaffolds and controls were top up with 2 mL of complete medium before the plate of scaffolds was placed in an incubator at 37 °C for 3, 7 and 14 days. Culture medium was changed every 2 to 3 days to maintain sufficient supplement for cells.

2.5. Cell proliferation

3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the proliferation of cells seeded on scaffolds and well (as control) for incubation duration of 3, 7 and 14 days. MTT assay relies on the reduction of MTT-formazan by mitochondrial succinate dehydrogenase
of living cells. The formation of formazon as product was observed by colour change from yellow to dark purple crystal. In details, 1 mL of pure medium was pipetted in each well after discarded the cultured medium. Next, 100 μL of MTT solution with concentration of 5 mg mL\(^{-1}\) was transferred to each well before the plate containing cells seeded on scaffolds was incubated for 4 h. After 4 h of incubation, 1 mL of dimethyl sulphoxide (DMSO) was pipetted into each well and resuspended well for a few times using a micropipette. Triplicate of 300 μL purple crystal solution which was formazon product was pipetted to a 96 well-plate. The results of MTT test were evaluated in optical density (OD) values and determined by Versa Max Microplate Reader (Molecular Devices, USA) at wavelength of 570 nm. This MTT test at different incubation period was performed 3 times.

2.6. Statistical analysis
Analysis on quantitative results of \textit{in vitro} proliferation was presented as means ± S.D (N=3) and performed via one-way ANOVA (analysis of variance) analysis. This analysis was performed using LSD and Duncan post-hoc test with p < 0.05 was measured as statistically significant.

3. Results and discussion

3.1. Physical and chemical characterization
Pore size and microstructures of scaffold samples (TMM0 – TMM5) were examined at magnification of 500x using FESEM. Figure 1 (TMM0 –TMM5) shows the FESEM images of TMM-CTNTs scaffolds at weight percent (wt%) of 0 – 5 % TMM. All images show 3D porous scaffolds with good interconnectivity network. The incorporation of TMM affected the macroporous size and interconnectivity diameter. The pore size of C-TNTs scaffold without TMM (TMM0) was measured averagely around 110.9 μm (see table 2). Addition of TMM into TMM1, TMM2, TMM3, TMM4 and TMM5 scaffolds obviously reduced the pore size to 35.90 μm, 60.86 μm, 67.69 μm, 49.41 μm and 63.60 μm respectively compared to TMM0 scaffold. The microporous size showed increasing trend as addition of TMM powders increased from 1 to 5 wt%. However, the pore size of TMM4 scaffold did not follow this increasing trend as the pore size reduced to 49.41 μm. The FESEM image (see figure 1 (e)) have confirmed the behaviour of TMM4 scaffold. The image showed smaller pore size with thickened interconnectivity walls compared to TMM2, TMM3 and TMM5 scaffolds. It might be due to irregular pore size distribution produced using the freeze-drying method. The nucleation and growth of ice crystals at a certain freezing rate are important in producing desired shape and size of pores by considering the thickness of the scaffold [14].

Previous studies determined the minimum requirement pore size suitable for artificial 3D scaffolds for bone regeneration lies in between 100 – 300 μm [15]. Apart of that, other reports mentioned pore size between 75 – 100 μm was a minimum requirement for bone regeneration with optimum values ranged between 100 – 135 μm [16]. As the results exhibited pore size less than 75 μm, the outcome from this study expected the reduction of pore size to micropores can effectively increase surface area and enhanced permeability of scaffolds. Hence, both positive effects contributed to production of more proteins adsorption sites which help to improve cell bioactivity such as cell attachment, proliferation and especially differentiation which rapidly increase degradation of scaffolds [17].
Figure 1. FESEM images of TMM-CTNTs scaffolds (a) TMM0 (b) TMM1 (c) TMM2 (d) TMM3 (e) TMM4 and (f) TMM5.
The porosity TMM0, TMM1, TMM2, TMM3, TMM4 and TMM5 scaffolds was 83.40 %, 87.10 %, 84.10 %, 81.67 %, 82.97 % and 79.93 % respectively (as shown in table 3). From the result, addition of TMM powders at different wt% did not influence porosity of scaffold samples. The increment of TMM wt% did not show increasing or decreasing trend of porosity. Addition of TMM powders at 5 wt% showed much reduced porosity of 79.93 %. Despite of that, the porosity of TMM-CTNTs scaffolds at varied TMM wt% was in the range of optimum requirement for bone tissue regeneration which was reported in between of 30 – 90 % [18].

Table 3. Porosity of scaffolds at varied wt% TMM.

| Scaffold Type | Weight percent of TMM powder (wt%) | Average Porosity (%) |
|---------------|-----------------------------------|----------------------|
| TMM0          | 0                                 | 83.40                |
| TMM1          | 1                                 | 87.10                |
| TMM2          | 2                                 | 84.10                |
| TMM3          | 3                                 | 81.67                |
| TMM4          | 4                                 | 82.97                |
| TMM5          | 5                                 | 79.93                |

Fabrication of scaffolds using freeze-drying method generated high porous 3D scaffolds with wide interconnected network of pore structure that works well with degradable and temperature sensitive materials like chitosan as reported in previous studies [19-20]. Freeze drying was proven to preserve the biocompatibility of chitosan scaffolds compared to other conventional drying methods like oven drying [21]. Thus, these scaffolds template were expected to be osteoconductive as it will support osteoblastic cell adhesion, proliferation and mimic the function of extracellular matrix before it degraded and replaced with new regenerated bone tissue.

The compressive modulus of TMM0, TMM1, TMM2, TMM3, TMM4 and TMM5 scaffolds showed 0.012 MPa, 0.009 MPa, 0.029 MPa, 0.013 MPa, 0.01 MPa and 0.038 MPa respectively as shown in figure 2. The incorporation of TMM into CTNTs scaffolds did not tremendously increase the compressive modulus. Overall, the compressive modulus for TMM-CTNTs scaffolds was very low. Besides, the compressive modulus of TMM0 scaffolds did not show any significant difference with TMM1, TMM3 and TMM4. The introduction of TMM in scaffolds changed compressive modulus inconsistently. However, the addition of TMM at the highest wt% (TMM5) obviously enhanced the compressive modulus to 0.038 MPa. Thus, increased wt% of TMM up to 5 %, improved the mechanical properties of scaffolds.

Ideally, increased porosity of scaffolds decreased their mechanical properties and vice versa. Based on table 3, TMM1 scaffolds with the highest porosity showed the lowest compressive modulus among scaffolds. Meanwhile, TMM5 scaffolds that exhibited the lowest porosity showed the highest...
compressive modulus. Thus, the results of compressive modulus and porosity were in negative correlation to each other. The compressive modulus of TMM2 scaffold exhibited greater increment compared to TMM3 and TMM4. Meanwhile, the porosity of TMM2 showed a higher value compared with TMM3 and TMM4. It was expected that higher in porosity showed lower compressive modulus. This unusual behavior might due to the agglomeration of TMM powder which acts as a filler to strengthen C-TNT scaffolds. Further investigation on the proportional relationship between porosity and compressive modulus of scaffolds could be confirmed by determining the density and linear shrinkage properties due to their unusual mechanical behavior as reported by Kim et al. [22].

Figure 2. The compressive modulus of TMM-CTNTs scaffolds at varied wt% of TMM.

Figure 3 shows a study of swelling ratio of TMM-CTNTs scaffolds at varied weight percent of TMM. The swelling ratio of TMM0, TMM1, TMM2, TMM3, TMM4 and TMM5 scaffolds was 407.4 %, 373.1 %, 401.8 %, 388.4 %, 386.5% and 371.4 % respectively. In figure 3, introduction of TMM reduced swelling ratio of TMM-CTNTs scaffolds. This was due to the network between chitosan, TNT and TMM forming much thicker pore wall at higher TMM wt%. As a result, the penetration of water into scaffolds was also reduced.
Hence, there was no correlation between wt% of TMM and swelling ratio even theoretically, scaffolds with larger swelling ratio help cells infusion into the scaffolds for better cell attachment and growth. However, the swelling ratio for all scaffolds showed higher water absorption capacity which was more than 200 % which possessed optimal structure of porous scaffolds for cells to be cultured as reported [23-24]. All scaffolds samples showed high percentage of porosity that revealed their hydrophilic behaviour which allows water to be absorb in scaffolds and offered space for adhesion and proliferation [25].

The degradation performance of TMM-CTNTs scaffolds at different TMM powders weight percent (wt%) after 28 days for each scaffold was evaluated. The degradation ratio (%) of TMM0, TMM2, TMM3, TMM4 and TMM5 scaffolds was 0.959 %, 2.843 %, 1.017 %, 1.390 %, 1.845 % and 2.385 % respectively and illustrated in figure 4. From the results, TMM5 showed highest degradation rate compared to other scaffolds. This showed additional of TMM in scaffolds increased the degradation rate of scaffolds and enhanced the formation of new bone regeneration.

Figure 3. The swelling ratio (%) of TMM-CTNTs scaffolds at varied wt% of TMM.
Figure 4. Degradation ratio (%) of TMM-CTNTs scaffolds at varied wt% of TMM.

The FTIR spectra of several important functional groups present in TMM-CTNTs scaffolds at different wt% of TMM are shown in figure 5. TMM was used as a control to confirm the present of β-glucan, the bioactive compound in TMM for inflammation properties in each scaffold. In figure 5, the broad band was visibly seen at 3400 cm\(^{-1}\) on the spectra which was attributed to OH stretching vibrations [26]. Another small peak next to –OH group was –CH\(_2\) asymmetric stretching group at 2879 cm\(^{-1}\). The peak at 665 to 668 cm\(^{-1}\) represented Ti-O-Ti stretching vibration [27]. Another small peak at 1635 to 1640 cm\(^{-1}\) showed Ti-OH bending vibration due to the absorption of H\(_2\)O [28]. The presence of β-glucan was confirmed in TMM1 to TMM5 scaffolds at spectral range of 1077 to 1080 cm\(^{-1}\) under C-O \(\alpha\)-glycosidic bond and C-O-C asymmetric stretching of glycosidic linkage respectively [30]. This finding confirmed that freeze drying preserved the characteristic compounds of TMM which would later ensure the in vitro efficacy of the scaffolds.
Figure 5. FTIR spectra of TMM-CTNTs scaffolds at varied wt% of TMM.

3.2. Enhanced cell proliferation on TMM-CTNTs scaffolds

Figure 6 showed the cell proliferation of the cells seeded on the scaffolds analysed using MTT assay at 3rd, 7th and 14th days of incubation period. The cell growth of MG63 on CTNTs-TMM scaffolds was measured in terms of optical density (OD) over the positive control. The positive control was referred to cells directly seeded on well without scaffolds. The OD values for all scaffolds showed significantly differences (p<0.05) at 3rd, 7th and 14th days of incubation. The values of OD is presented as means ± S.D (N=3) analyzed using one-way ANOVA analysis.
Figure 6. MG63 cells on TMM-CTNTs scaffolds at different weight percent of TMM and polystyrene as positive control evaluated by MTT assays at 3rd, 7th and 14th days of incubation period.

The OD values on 3rd day of incubation revealed TMM0 scaffolds with higher (p<0.05) OD values compared to other scaffolds. Furthermore, the proliferation of MG63 showed declined OD values with the wt% of TMM. In addition, the OD values of cells cultured on all TMM scaffolds were significantly higher (p<0.05) than OD value of cells on positive control. The proliferation of cells seeded on TMM0 scaffolds was significantly lower (p<0.05) compared to OD values of TMM5 scaffolds at 7th days of incubation. TMM5 scaffolds promoted high (p<0.05) proliferation of MG63 followed by TMM4 scaffolds. Despite of that, TMM4 and TMM5 scaffolds exhibited slightly small difference in OD values in between 3rd and 7th days of treatment compared to other scaffolds. This pattern of proliferation could not be understood very well. However, introduction of TMM gradually dropped the proliferation compared to scaffolds without additional of TMM powders. Such proliferation pattern also could be observed on TMM2 and TMM3 scaffolds on 14th day of incubation. The proliferation of MG63 on all scaffolds decreased at this incubation period especially TMM0 promoted the lowest (p<0.05) proliferation rate. Besides that, the OD values of all TMM scaffolds on 7th and 14th day of incubation were significantly lower (p<0.05) compared to cells seeded on positive control.

Based on MTT results, the additions of TMM in CTNTs scaffolds seem to reduce the in vitro proliferation of MG63. This can be clearly seen on early stage at 3rd day of incubation. The osteoblastic proliferation became low as the weight percent of TMM increased. Proliferation of cells could be affected by porosity and pore size and these contributed as factors why the proliferation rate became slow at early stages. Porosity result showed decreased pattern which is directly proportional with scaffolds pore size that reduced to less than 100 μm. Scaffolds with higher porosity enhances cell ingrowth, provides more protein sites and promotes nutrient and oxygen exchange [31]. Corresponding to this argument, this could be a reasonable explanation for the proliferation of MG63 on TMM0 scaffolds at the beginning which was higher than cells on other scaffolds containing TMM. TMM0 got higher porosity and pore size which positively affected the cell growth capability. Furthermore, TiO2 was good filler for biodegradable material with high surface area that enhanced adhesion and proliferation of cell growth [26]. However, the proliferation of MG63 on TMM0 scaffolds drastically reduced on 7th and 14th days of incubation due to rapid migration of cells into TMM0 scaffolds template.
The cells grown on TMM5 scaffolds seemed to need adaptation period on 3rd day of incubation before showed the highest proliferation at 7th days of incubation. This might be due to the addition of TMM at the highest weight percent slowly promotes cells growth which prolonged the duration of proliferation. TMM5 scaffolds presented as the best scaffolds in terms of enhancing cell growth compared with other scaffolds on 7th days of incubation.

4. Conclusion
In conclusion, the reinforcement of TMM in C-TNTs scaffolds improved porous structure, mechanical strength and biocompatibility properties which fulfills the physical requirement of artificial 3D templates for bone regeneration. The cell proliferation through MTT assay test revealed addition of TMM slowly promoted cell growth which prolonged the duration of proliferation. Notably, TMM5 scaffold with 5 wt% of TMM presented as the best scaffolds compared with other scaffolds on 7th days of incubation. Hence, further study on adhesion and differentiation should be carried out to better realize the in vitro biocompatibility of TMM-CTNTs scaffolds.

References
[1] Williams D F 2008 Biomaterials 29 2941–53
[2] Lim S S, Chai C Y and Loh H-S 2017 Mater. Sci. Eng. C. 76 144–52
[3] Subramanian A and Lin H 2005 J. Biomed. Mater. Res. 75A 742–53
[4] Tuzlakoglu K and Reis R L 2008 Handbook of Natural-Based Polymers for Biomedical Application vol 1, ed R L Reis, N M Neives and J F Mano,(Cambridge:Elsevier) pp 357–73
[5] Levengood S K L and Zhang M 2014 Eng. 3161–84
[6] Madihally S V and Matthew H W T 1999 Biomaterials 20 1133–42
[7] Jana S, Florczyk S J, Leung M and Zhang M 2012 J. Mater. Chem. 22 6291–9
[8] Bjursten L M, Rasmusson L, Oh S, Smith G C, Brammer K S and Jin S 2010 J. Biomed. Mater. Res. - Part A. 92 1218–24
[9] Cheng Y, Yang H, Yang Y, Huang J, Wu K and Chen Z 2018 J. Mater. Chem. B 6 1862–86
[10] Johoathan M, Gan S H, Ezumi M F W, Faezahtul A H, Nurul A A 2016 BMC Complement. Altern. Med. 16 1–13
[11] Eik L F, Naidu M, David P, Wong K H, Dunne N and Li X 2012 Evidence-based Complement. Altern. Med. 2012
[12] Yap Y H, Tan N, Fung S, Aziz AA, Tan C and Ng S 2013 J. Sci. Food Agric. 93 2945–52
[13] Chen Q, Zhou W, Du G H and Peng L M 2002 Adv. Mater. 14 1208–11
[14] Pezeshki M M, Mirzadeh H and Zandi M 2012 Iran. Polym. J. 21 191–200
[15] Bružauskaitė I, Bironaitė D, Bagdonas E and Bernotièienè E 2016 Cytotechnology 68 355–69
[16] Murphy C M and O’Brien F J 2010 Cell Adh. Migr. 4 377–81
[17] Zhang K, Fan Y, Dunne N and Li X 2018 Regen. Biomater. 5 115–24
[18] Chen Y, Frith J E, Dehghan-Manshadi A, Attar H, Kent D and Soro N D M 2017 J. Mech. Behav. Biomed. Mater. 75 169–74
[19] Sultana N and Wang M 2008 J. Mater. Sc. 19 2555-61
[20] Sun T, Khan T H and Sultana N 2014 J. Nanomater. 2014 194680
[21] Román J, Cabanas M V, Peña J and Vallet-Regi M 2011 Sci. Technol. Adv. Mater. 12 45003
[22] Kim T-R, Kim M-S, Goh T S, Lee J S, Kim Y H and Yoon S-Y 2019 Appl. Sci. 9 1965
[23] Eves P C, Beck A G, Shard A G and Mac-Neil S 2005 Biomaterials 26 7068–81
[24] Wang H M, Chou Y T, Wen Z H, Wang Z R, Chen C H and Ho M-L 2013 PLoS One 8 2–12
[25] Radwan-Pragłowska J, Janus Ł, Piątkowski M, Bogdał D and Matysek D 2020 Polymers (Basel) 12
[26] Kumar P. 2018 Int. J. Biomater. 2018 6576157
[27] Pan H, Wang X D, Xiao S, Yu L G and Zhang Z J 2013 Indian J. Eng. Mater. Sci. 20 561–7
[28] León A, Reuquen P, Garin C, Segura R, Vargas P and Zapata P 2017 Appl. Sci. 7 1–9
[29] Chen X, Liu X, Sheng D, Huang D, Li W, Wang X 2012 Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 97 667–72
[30] Ma Y, He H, Wu J, Wang C, Chao K and Huang 2018 Sci. Rep. 8
[31] Loh Q L and Choong C 2013 Tissue Eng. Part B Rev. 19 485–502
Acknowledgements

Authors would like to express our sincere gratitude to the Fundamental Research Grant Scheme from the Ministry of Education (FRGS/1/2020/TK0/UNIM/02/9) for funding support in our research work. Special acknowledgement to Ms Khairani Hasuna Jaapar on technical support for FESEM characterization.