Chitosan-TiO₂ nanotubes scaffolds for proliferation and early differentiation of MG63 by functionalization with fetal bovine serum

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Abstract. Scaffolds have been used as alternative biomaterials to overcome physiological bone disorders. Production of scaffolds has been challenging to fulfil the following criteria: biodegradability, mechanical sustainability, and biocompatibility. For cellular interaction, protein adsorbed on scaffold surface is important for osteoblastic activities. This study aimed to functionalize chitosan-TiO₂ nanotubes scaffolds with fetal bovine serum and investigate in vitro efficacy of such scaffolds with fetal bovine serum. Chitosan-TiO₂ nanotubes scaffolds were prepared via direct blending and lyophilization. They were then functionalized with fetal bovine serum via adsorption for 4, 8, 12 and 24 h. The in vitro efficacy of the functionalized scaffolds was evaluated using MG63 cells. The adsorption of fetal bovine serum onto the scaffolds was complex where saturation of adsorption was hardly attained. The in vitro efficacy of scaffolds with adsorbed fetal bovine serum was higher than that of those without fetal bovine serum by promoting better osteoblastic functions. Notably, the scaffolds functionalized for 4 h enhanced cell adhesion and proliferation on 7 day suggesting good regulation of osteoblastic binding and proliferation. ALP protein was expressed on 26 day in all functionalized scaffolds. Chitosan-TiO₂ nanotubes scaffolds with adsorbed fetal bovine serum can be a potential regenerative material for bone regeneration.

1. Introduction

A huge number of people suffered from bone diseases like osteomalacia, osteogenesis imperfecta and Paget’s disease. In most cases, the removal of defective bones is inevitable and results in bone void or loss. This greatly affects the functionality of the patients. The current replacement of bones is very troublesome of several revisions of surgeries which are time consuming and cause finance burden to patients. Therefore, bone tissue engineering as a therapeutic method has gradually gained attention in treating bone diseases and disorders. To achieve guided bone regeneration, three-dimensional (3D) scaffolds serve as temporary support to stimulate bone growth. These scaffolds have often been fabricated with improved physicochemical properties, biocompatibility and mechanical properties.

Scaffolds with bioinspired features are very critical for cellular functions like proliferation, differentiation and mineralization. The ultimate faith of scaffolds is to get replaced by new bone tissue.
Ideally, the degradation rate of scaffolds matches the bone growth rate. To make scaffolds degradable, natural polymers like chitosan have been extensively researched in biomedical aspects [1-2]. Chitosan possesses high hydrophilicity which is responsible for its exceptionally high swelling behaviour. This feature facilitates the anchorage of bone cells on the scaffolds which is pre-requisite of subsequent osteoblastic activities [3]. However, chitosan cannot be used alone in guiding bone growth due to its poor mechanical strength and low bone-bonding ability [4]. Hence, the reinforcement of chitosan with fillers especially nanoparticles becomes an imperative step.

In recent decades, nanoparticles evidently promoted enhanced osteoblastic functions due to their close resemblance to the building blocks of bone [5–6]. Notably, nanohydroxyapatite and TiO2 nanotubes showed better interactions with the host cells after their incorporation into chitosan-based scaffolds [4,7,8]. These studies affirmed the excellent osteoconductivity and mechanical stability of nanoparticles by promoting cellular adhesion and proliferation via enhanced adsorption of proteins and metal ions [4,9]. In particular, the high adsorption affinity of chitosan-TiO2 nanotubes scaffolds towards calcium ions was proven to elevate the in vitro efficacy of the scaffolds [4].

Upon the implantation of scaffolds in host tissue, this is quickly followed by plasma proteins adsorption to the scaffolds [10]. The deposition of plasma proteins on scaffolds regulates the efficacy of the biomaterials. This can also be perceived as an in-situ functionalization of the scaffolds. Protein adsorption is a dynamic process, as serum proteins arrive and leave the surface of biomaterials in a time-dependent manner [11]. Besides, the adsorption behaviour of plasma proteins depends on the type of scaffolds [12]. With high adsorption affinity towards calcium ions, chitosan-TiO2 nanotubes scaffolds were expected to facilitate sites for the adsorption of plasma proteins. This study aimed to develop bioinspired scaffolds by functionalizing chitosan-TiO2 nanotubes scaffolds with fetal bovine serum (FBS) via adsorption over 24 h. Subsequently, the in vitro efficacy of functionalized scaffolds was verified using MG63 osteoblast-like cell for adhesion, proliferation, and early differentiation. Finally, the expression of housekeeping protein in osteoblast differentiation was examined by using a dot-blot semi-quantitative analysis. The correlation between the adsorption duration of proteins and in vitro efficacy of the new scaffolds would then be elucidated.

2. Methods

2.1. Fabrication of Chitosan-TiO2 nanotubes scaffolds

Synthesis of the TiO2 nanotubes was attempted by employing the method developed by Chen et al. (2002). Two grams of TiO2 (Sigma Aldrich, USA) was mixed with 40 mL of 10 M NaOH (Sigma Aldrich, USA) in a Teflon container for 5 min. The Teflon container containing mixture of TiO2 and NaOH was screwed tightly in a stainless-steel reactor and heated to 150 °C for 72 h. Under hydrothermal condition (at 150 °C), the corrosiveness of NaOH was elevated. As a result, NaOH delaminated the TiO2 in micron into nanosheets. All these nanosheets were subsequently rolled into nanotubes by the movement of NaOH. After 72 h, white powder was collected from the reactor and filtered with distilled water and 0.1 M HCl (Merck, Germany). The washing was completed when a neutral pH of the powder was achieved. Finally, the powder was dried at 50 °C for 6 h prior to the grinding and 5-h of calcination at 400 °C to increase the crystallinity of the powder.

Subsequently, the chitosan powder was dissolved in 0.2 M of acetic acid (Merck, Germany) and stirred homogenously for 2 h. Approximately 16 weight percent (wt%) of TiO2 nanotubes which was previously synthesized and chitosan solution were agitated using a magnetic stirrer for 5 h. One mL of the mixture was transferred to each well of a 24-well plate. The same plate was frozen at -20 °C for 24 h. It was then freeze dried at -40 °C and 0.12 mbar for 24 h. Freeze-dried scaffolds were rehydrated with different concentration of ethanol starting with 1-h immersion in absolute ethanol. This was then followed by immersion in 70% ethanol and 50% ethanol for 30 min respectively. This rehydration ensured sterilization of the scaffolds and also gradually introduced water into the scaffolds. After rehydration, scaffolds were dehydrated in a desiccator prior to protein adsorption and in vitro tests.
2.2. Functionalization of Scaffolds via Adsorption of FBS
The mass of the scaffolds was measured. Then, scaffolds were completely immersed in phosphate buffered saline (PBS) overnight before the functionalization with FBS. After 24 h of incubation with PBS at room temperature, the solution was removed. In the functionalization, chitosan-TiO₂ nanotubes scaffolds were immersed in 2 mL of FBS at different time intervals (4, 8, 12 and 24 h). The incubation was carried out at 37 °C. After each time interval of incubation, 1 mL of FBS was taken to measure the concentration of the protein remnants after the adsorption by the scaffolds. The remaining FBS was removed and scaffolds were gently washed with PBS for three times to remove free and loosely bound proteins. The Bradford assay was used to quantify the amount of adsorbed proteins and the initial concentration of protein in FBS was also determined. The remaining FBS was diluted by 4000 times with PBS and the diluted solution was mixed with Bradford reagent (Bio-Rad Laboratories) at 1:1 ratio in a 96-well plate. The absorbance of the diluted FBS solution was measured at 595 nm using Varioskan Flash multimode reader (Thermo Scientific, USA). The amount of FBS adsorbed (qₑ) was calculated using equation (1).

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qₑ = \frac{(C₀-Cₑ)V}{w}
\]

Where qₑ was the amount of protein adsorbed per gram of scaffold (mg/g), C₀ was initial concentration of protein in FBS, Cₑ was the concentration of FBS after adsorption (mg/mL) at different time intervals, V was the volume of FBS in each well (2 mL) and w is the mass of scaffolds (g).

2.3. Cell Culture
MG63 cells (ATCC, non-USA) were cultured in a 75-mL cell flask with minimum essential medium (MEM) supplemented with 10% FBS, 100 mM of sodium pyruvate, 1% penicillin-streptomycin, and 1% sodium bicarbonate. The cell flask was incubated at 37 °C with 5% CO₂. The culture medium was changed every 3 days and harvested prior seeding on the scaffolds.

2.4. Fluorescein Diacetate (FDA) and Propidium Iodide (PI) Staining
MG63 cells (3x10⁴ cells/well) were seeded on functionalized the chitosan-TiO₂ nanotubes scaffolds and incubated for 4 h. After 4 h, 5 µg/ml of FDA solution and 1 µg/ml of PI solution were added to observe the survival and death of cells under a florescence microscope (Nikon, Japan).

2.5. Proliferation Assay
MG63 cells (3x10⁴ cells/well) were seeded on the functionalized scaffolds and incubated in complete media for 3, 5, and 7 days. MG63 cultured directly on well served as control. After each incubation period, complete media was discarded and replaced with pure MEM in each well. Subsequently, 5 mg/mL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and incubated for 4 h at 37 °C until the reduction of the yellow tetrazole (MTT solution) to purple formazan by the living cells. After 4 h, dimethyl sulfoxide (DMSO, 1.0 mL/well) was added and mixed with purple crystal homogeneously. The absorbance of DMSO-formazan solution was measured and recorded at 570 nm using Varioskan Flash multimode reader. The reading was recorded three times for each scaffold. The viability of MG63 was determined by using equation (2).

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Cell \ viability \ (%) = \frac{OD_{scaffold}}{OD_{control}} \times 100\%
\]

Where ODscaffold was the optical density of MG63 cultured on the scaffolds functionalized for different time intervals and ODcontrol was the optical density of MG63 on cultured on well.

2.6. Alkaline Phosphatase (ALP) Activity Assay
Early differentiation of MG63 was analysed by using alkaline phosphatase (ALP) at 7, 14, 21, and 26 days. The cells and scaffolds were washed with PBS for three times and homogenized with cell lysis solution (Pro-Prep, protein extraction solution). The mixtures were centrifuged at 10,000 rpm at 4 °C
for 10 min. Approximately 100 µL of supernatant was collected and incubated with P-nitrophenyl-phosphate liquid substrate at 1:1 volume ratio in 96-well plate for 2 h. The incubation was carried out at 37 °C. In the presence of alkaline phosphatase, the liquid substrate changes its color from transparent to pale yellow. After 2 h of incubation, the absorbance of the solution was measured at 405 nm using Varioskan Flash multimode reader. The remaining supernatants were stored at -80 °C for dot blot analysis.

2.7. Expression of Housekeeping Proteins (GAPDH and ALP)

The supernatant from ALP assay was used for the expression of housekeeping protein such as GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and ALP (Alkaline Phosphatase). Polyvinylidene fluoride (PVDF) membrane was used for the dot blot analysis. The grid lines were drawn on the membrane for the supernatant to load. Approximately 10 µg (10 µL) of supernatant was loaded on the membrane and air dried. Then, the membrane was soaked with blocking buffer (PBS-Tween containing 4% milk) in a petri dish and shaken at 60 rpm horizontally at room temperature for 1 h. The blots were incubated in 1 µg/mL of mouse monoclonal anti-GAPDH (Abcam, UK) or 0.5 mg/mL of anti-alkaline phosphatase antibody (ABcam, UK) at 1:10000 dilution in blocking buffer and shaken horizontally at 60-70 rpm for 2 h. Then, the membranes were washed with 10 mL of PBS-Tween 3 times for 5 min. About 2 mg/mL of rabbit polyclonal anti-mouse IgG Horseradish Peroxidase conjugated antibody (ABcam, UK) was diluted at 1:10000 in PBS-Tween and used as secondary antibody incubation for 2 h. After washing with PBS-Tween for 4 times, 1 mL of tetramethylbenzidine (TMB) substrate was added to observe the band. The relative protein bands were analysed with QualityOne GS-800 (Biorad, United States).

2.8. Statistical Analysis

Two-way ANOVA was used to analyse MTT and ALP assays. The dot blot analysis and ALP assay were analysed with Pearson correlation for comparison of relationship.

3. Results

3.1. High Adsorption Affinity of Chitosan-TiO2 Nanotubes Scaffolds towards FBS

Chitosan-TiO2 nanotubes scaffolds were incubated in FBS at different time intervals and the amount of adsorbed proteins by the scaffolds was quantified. As shown in figure 1, the amount of adsorbed proteins (q_e) after 4 and 8 h of incubation was approximately 300 mg/g of scaffold. However, q_e reduced slightly to 277 mg/g after the incubation was increased to 12 h. This was explained by the replacement of heavier proteins like fibronectin (FN) by lighter proteins like albumin at the incubation period of 12 h. Ultimately, 24 h of incubation marked the highest amount of protein adsorbed by the scaffold at 442 mg. There was no sign of saturation was observed in these scaffolds due to the dynamic nature of protein adsorption and desorption. The high affinity of chitosan-TiO2 nanotubes scaffolds towards FBS proteins was evidently demonstrated in this study.
3.2. Cell Adhesion on Chitosan-TiO2 nanotubes Scaffolds

FDA and PI reagents were used to observe the viability and death of MG63 on the functionalized scaffolds respectively after 4 h of incubation. FDA is converted by intracellular esterases into fluorescein with green fluorescence which represents for living cells. On the other hand, PI interacts with nucleotides to emit red fluorescence which represents for dead cells. More FDA stained cells were observed in chitosan-TiO2 nanotubes scaffolds functionalized with FBS for 4 h as shown in figure 2(a). This promising result was also coupled with less PI stained cells on the same scaffold (in figure 2(b)). This suggested that the adsorbed proteins at this time interval were likely to be FN (cell adhesive protein) which facilitated the adhesion of bone cells. Similar outcome was observed in cells cultured directly on well (in figure 2(e)). However, more PI stained cells were detected in scaffolds functionalised for 12 h. The amount of adsorbed protein at 12 h was slightly less than that of at 4 h but the proteins adsorbed at 12 h caused more death of MG63. The scaffolds without adsorbed FBS also caused cell death by showing more PI stained cells.

![Figure 1](image_url)  
**Figure 1.** The amount of adsorbed proteins ($q_e$) by chitosan-TiO2 nanotubes scaffolds at different time intervals.
3.3. High cell viability on FBS-functionalize scaffolds

MTT is a colorimetric assay for quantifying cell viability via the reduction of MTT dye (tetrazolium) into formazan (purple colour) by cellular oxidoreductase enzymes in living cells. The cell viability of MG63 cultured on the functionalized chitosan-TiO₂ nanotubes scaffolds after 3, 5 and 7 days was evaluated and MG63 cultured directly on well was control. In Figure 3, the cell viability of MG63 cultured on the functionalised scaffolds ranged roughly from 120% to 180% which was higher than the control. This suggested that the adsorbed proteins promoted cell proliferation of MG63 more effectively. In general, the proliferation of MG63 cultured on the functionalized scaffolds increased with incubation periods. The scaffolds functionalized with FBS for 4 h gave the highest proliferation at day 7 compared to the remaining scaffolds as well as the controls (*p<0.05).
Figure 3. Histogram showing proliferation of MG63 cells seeded on functionalized chitosan- TiO$_2$ nanotubes scaffolds after 3, 5, and 7 days of incubation in terms of cell viability. The error bars represent mean ± SD. These experiments were repeated in triplicate (*p<0.05).
3.4. High ALP Activity on Day 26
ALP was used as an early detection of cellular differentiation markers on MG63 cells and was measured with P-nitrophenyl-phosphate liquid substrate on 7, 14, 21 and 26 days. As shown in figure 4, ALP activity of MG63 cells grown on chitosan-TiO$_2$ nanotubes scaffolds gave the highest significant on 26 day than the controls (**p<0.01).

Figure 4. Histogram showing ALP activity of MG63 cells on functionalized chitosan-TiO$_2$ nanotubes scaffolds at 4 different time intervals after 7, 14, 21 and 26 day of incubation period (**p<0.01).
3.5. Expression of Housekeeping Proteins of GAPDH and ALP
GAPDH is one of the housekeeping proteins expressed in all types of cells and thus it can be used as a control for the expression of ALP protein. ALP protein plays an important role in bone differentiation. The functionalized chitosan-TiO₂ nanotubes scaffolds gave differentiation on 26 day and it may be due to ALP protein expression. Therefore, the dot blot analysis was performed to confirm the level of ALP expression on 26 day. It was found that there was positive correlation between ALP enzyme assay and dot blot analysis (figure 5, p<0.05).

![Figure 5](image_url)

**Figure 5.** (a) Dot blot analysis of housekeeping proteins and (b) percentage of ALP activity comparison with GAPDH expression on 26 day of incubation. (p<0.05, correlation with ALP assay on 26 day).
4. Discussion
In this study, adsorption is a dynamic process between nanoscale surface of the scaffolds and FBS proteins. This is associated with attractive and repulsive forces by local changes of the scaffold surface which can cause changes in density and orientation of adsorbed proteins [13]. It is clear to notice that the amount of adsorbed proteins increases with the increase of adsorption duration except the adsorption for 12 h. The proteins undergo orientation for more contact with the surface of scaffolds by removing some of existing binding proteins [14], which causes the reduction of protein adsorption after 12 h as shown figure 1. The chitosan-TiO$_2$ nanotubes scaffolds are still not saturated with FBS proteins even after 24 h of adsorption, as there is no plateau observed in figure 1. A plateau is an indicative of scaffolds can no longer uptake any protein. The structural rearrangement of FBS proteins is responsible for accommodating more sites for the subsequently adsorbed proteins [14]. This explains the high adsorption affinity of the scaffolds towards FBS proteins.

In the FDA staining, scaffolds functionalised with FBS for 4 h gave the highest cellular adhesion compared to the other functionalized ones. These results highlighted the role of adsorbed proteins on the modulation of cellular adhesion. This suggests that majority of the adsorbed protein is FN after 4 h of adsorption. FN as a cell adhesive protein regulates the adhesion of bone cells like MG63. As the adsorption duration is prolonged to another 4-8 h, FN which is initially adsorbed by the scaffolds is likely to be displaced by other FBS proteins like albumin [15]. The displacement of FN by other FBS proteins contributed to lower adhesion of MG63 on scaffolds functionalised for 8 and 12 h. Even though the scaffolds without functionalization also show high adhesion of MG63 in figure 2(g), it is also followed by high cell death as shown in figure 2(h). This again affirms the importance of adsorbed proteins in facilitating osteoblastic adhesion. As an adherent type of cell line, MG63 seeded directly on well is also stained with FDA the most. The type of protein bind to the scaffolds should be analysed by using Western blot analysis or recombinant fragments of FN [16]. This would help to identify the actual type of adsorbed protein by the scaffolds.

In MTT assay, protein adsorbed for 4 h gave the highest proliferation on 7 day (figure 3). In general, the cell viability of MG63 on the functionalized scaffolds is higher than that of control. Since cell adhesion is dynamic process with initial cell-protein contact prior to long term adhesion for cellular responses [17]. From the response of MG63, FN was probably activated after being adsorbed on the surface of scaffolds for cellular regulation [15]. Other scaffolds functionalized for more than 4 h exhibited slightly lower cell viability due to the desorption process of FN [17]. It can also be due to albumin that can co-adsorb with FN for competition and replacing FN and resulted in low cellular response [15]. Further extension of MTT assay should be done to examine more proliferation profile to compare with the events of ALP assay.

ALP activity was significantly expressed on 26 day (figure 4). In chitosan-based scaffolds, ALP activity was seen on 14-21 days for osteosarcoma cell [3]. Interestingly, all functionalized scaffolds gave differentiation on 26 day and was even higher than the control which has no change (figure 4). This not only signified the presence of adsorbed proteins as effective biological cues for the scaffolds, but also 3D structure of the scaffolds is the key feature of promoting the early differentiation of MG63. This finding is corroborated with a previous study conducted by Lim and co-workers (2017). They also demonstrated the significance of 3D structure of scaffolds in promoting early differentiation of MG63. Moreover, dot blot analysis confirmed that it was caused by ALP protein expression (figure 5(a)-(b)). Therefore, chitosan-TiO$_2$ nanotubes scaffolds functionalized with FBS were able to promote MG63 cell adhesion, proliferation as well as differentiation.

5. Conclusion
In conclusion, the chitosan-TiO$_2$ nanotubes scaffolds showed high adsorption affinity toward FBS over 24 h. Chitosan-TiO$_2$ nanotubes scaffolds functionalized with FBS for 4 h promoted the highest osteoblastic adhesion which was then coupled with the highest proliferation at day 7. This suggested that the type of FBS protein adsorbed by the scaffolds functionalized for 4 h was mainly FN, the cell adhesive protein. Surprisingly, high ALP activity and ALP protein were demonstrated on 26 day in
MG63 cultured on functionalized scaffolds. Hence, chitosan-TiO$_2$ nanotubes scaffolds with adsorbed FBS proteins can be an alternative regenerative material in bone tissue engineering.

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