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Original article

Assessing the potential use of chitosan scaffolds for the sustained localized delivery of vitamin D

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ABSTRACT

Vitamin D is a commonly used bone modulator in regenerative medicine. Several modalities have been explored for the delivery of vitamin D including nanoparticles and scaffold. The present study aimed to assess the potential use of a bio-degradable chitosan scaffold for the delivery of vitamin D. The objectives included fabrication of a bio-degradable chitosan scaffold, integration of vitamin D into the scaffold, characterization of the vitamin D integrated scaffold. Characterization was carried out using, X-ray diffraction, Fourier transform infrared spectroscopy, and differential scanning calorimetry. The structure of the scaffold was assessed by scanning electron microscopy. The scaffold was placed in phosphate buffer saline and the release duration of vitamin D was observed using UV spectrophotometry. Dental pulp mesenchymal stem cells were added to the scaffold to study the scaffold associated toxicity and the functionality of the scaffold released vitamin D. The vitamin D release period from the scaffold was estimated to be for 80 hrs. MTT assay of the stem cells was comparable to that of the control group (stem cells cultured in media) inferring that the scaffold is not toxic towards the stem cells. The positive alizarin red S staining, a higher expression of alkaline phosphatase, osteocalcin, and RunX2 confirmed the functional capability (osteogenic differentiation of the stem cells) of the released vitamin D. Based on the data from the present study, it can be inferred that chitosan scaffold can be used for the sustained delivery of functional vitamin D for 3–5 days.

1. Introduction

Bone modulators are used to enhance the rate of bone formation in the field of regenerative medicine. One of the most commonly used bone modulators is vitamin D (Bosetti et al., 2017; Fügl et al., 2015; Hong et al., 2015; Ikeda and Ogata, 2000; Kim et al., 2018). The practical limitation of using bone modulators is due to the lack of optimal delivery for a sustained period. In most cases, the delivery period is limited due to an uncontrolled rapid delivery rate. High doses being delivered over a short period would be both toxic and relatively ineffective. In the past two decades, material science has evolved rapidly leading to the fabrication of several natural and synthetic materials (nanoparticles, scaffolds,

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etc) with a wide range of biological applications including drug delivery (Bernkop-Schnürch and Dünnhaupt, 2012; Bi et al., 2016; Chaudhary and Garg, 2015; Foox and Zilberman, 2015; Garg et al., 2012; Hu et al., 2013; Huang et al., 2017; Li et al., 2016; Ragelle et al., 2017; Zhang and Huang, 2017). The most vital property of an effective delivery vehicle is biocompatibility with the host. Thus, when introduced into the tissue, the vehicle must either not possess any antigenic potential or must be capable of masking the antigenic stimuli from the host immune system. In addition to biocompatibility, the nature of the interaction between the agent and its delivery vehicle is of utmost importance. The vehicle must be inert to the agent it is carrying, such that there is either no active interaction with the agent or the interaction must not alter the agent properties significantly (Bohr et al., 2015; Heidegger et al., 2016; Timin et al., 2018). Although several biocompatible delivery vehicles including varying scaffold design and materials have been formulated in the past, most have had significant limitations in controlling the delivery dosage and period. Lack of coherent integration between the vehicle and the agent often leads to the rapid release of the agent increasing the risk of toxicity and limiting the effect to a relatively shorter period. Thus, the delivery vehicle must be chosen and fabricated such that it is capable of integrating coherently with the agent allowing an optimally controlled release over a sustained period. One of the most commonly explored delivery vehicles was the collagen scaffolds soaked in growth factor (GF) solution before implantation (Friess, 1999; Hollinger et al., 1998). Unfortunately, the poor GF release characteristics and the short half-life of GFs in situ were challenging to maintain the therapeutic release profile (Carragee et al., 2011). 3D printed polycaprolactone (PCL) scaffolds coated with gold nanoparticles showed promising osteogenesis aiding in bone remodeling and healing bone defects (Lee et al., 2018b). However, the controlled release of GFs was not well explored. Thus, the present study was formulated to assess the use of bio-degradable chitosan scaffold for the sustained delivery of vitamin D. Several properties including the vitamin D release duration, functionality, and the associated toxicity were recorded.

2. Materials and Methods:

The bone modulator vitamin D and the biodegradable chitosan powder were procured from Biobench Solutions and Dodal Enterprises Pune, India. Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the materials.

Determination of Infrared (IR) Absorption spectrum of vitamin D (solid sample): Vitamin D along with potassium bromide's dry samples positioned in a sample holder was assessed using the potassium bromide dispersion technique to record their IR absorption spectrum. FTIR spectrophotometer was utilized to record the sample's infrared spectrum. For comparison, the raw chitosan was characterized using the FTIR technique.

Scaffold Preparation: Vitamin D 10 mg was dissolved in ethanol 100 ml to obtain a standard solution with a 100ug/ml concentration. It was further diluted in distilled water to get solutions with concentrations of 10, 20, 30, 40, 50, 60 μg/ml. A UV visible spectrophotometer was used at 262 nm to measure the absorbance of the solution. A solution containing 1% acetic acid along with 1gm of chitosan and 25 mg of vitamin D was made. The crosslinker sodium tri-phosphate (TPP) was added to the above solution. The scaffolds were lyophilized at a temperature of −20° C, at a controlled chamber pressure (0.4 torrs), and a shelf temperature (30° C). The prepared scaffolds were cut in the form of discs.

Differential Scanning Colorimetry (DSC): The material's thermal characteristics were assessed using the DSC. The purpose of such a measurement was to estimate the potential relationship between the substance's physical properties and the temperature. DSC is considered the optimal assessment tool for estimating the enthalpy related to the process of interest. The DSC study was carried out by using Mettler-Toledo DSC-I (USA) for lyophilized blank chitosan scaffold and chitosan scaffold incorporated with vitamin D. To assess the samples, thermal analysis was carried out by heating the samples from a low 40 °C to 300 °C. The rate of heat acceleration was 10 °C/min. Nitrogen gas was purged at a 50–70 ml/min rate all through the procedure to maintain the inert atmosphere. To ensure the accuracy of the results, the samples (1–4 mg) were heated on a crimped aluminum pan.

X-ray diffraction (XRD): To identify the phase of a crystalline material, a fast analytical method such as X-ray powder diffraction (XRD) was employed. The dimensions of the unit cell were also determined by the XRD. To analyze a material, first, it was grounded finely and homogenized, following which the mean bulk composition was measured. The XRD study was carried out for blank chitosan scaffolds and scaffolds incorporated with vitamin D, using Rigaku, Miniflex-600 (Europe). The Bragg equation, $n\lambda = 2d\sin\theta$ where n is an integer, λ is the characteristic wavelength of the X-rays impinging on the crystallized sample, d is the interplanar spacing between rows of atoms, and θ is the angle of the X-ray beam concerning these planes was satisfied. A set of diffracted intensities and the angles at which they are observed were recorded. A chemical fingerprint was obtained which was compared to the diffraction patterns of a known database.

Scanning electron microscopy (SEM): SEM measured the scaffold's external morphology. Lyophilized scaffolds were adhered to an aluminum stub using a double adhesive tape to prepare the samples. Afterward, the stubs containing the coated samples were placed in the SEM (FEI Nova Nano SEM 450, India) chamber. The scanning of the samples was carried out randomly, and at a 10 kV acceleration voltage, the photomicrographs were shot.

Drug release experiments: The scaffold material was placed in a phosphate buffer saline for 24 h (h), 48 h, and 72 h to allow the bone modulator to diffuse out of the scaffold material. The phosphate buffer saline solution was then analyzed spectrophotometrically under a UV analyzer.

Cytotoxicity assay (MTT- Tetrazolium dye assays): The metabolic function of the cells was assessed by the colorimetric MTT assay. Agents that possess medicinal value or toxic properties are assessed using the MTT assay to determine the degree of loss in cell viability (cytotoxicity) or the change from a proliferating into a quiescence state (cytostatic effect). The total viable cells are reflected by the NAD(P)H-dependent cellular oxidoreductase enzymes.

Cell culture: Healthy human permanent tooth extracted for orthodontic purposes from patients between 18 and 25 years of age was used to isolate the DPMSCs. The isolated DPMSCs were made to adhere to plastic tissue culture dishes after enzyme digestion. Cells were cultured in Minimum Essential Medium-α (Gibco Co., Carlsbad, CA, USA). The media had a 10% fetal bovine serum (FBS) supplementation. 1% penicillin/streptomycin (Gibco Co.) was also present in the medium as a supplement. Replacement of the media was carried out at a regular interval of 2 days. The media was replaced every 2 days. Cells were detached from the floor of the well plate when passaging the DPMSC by using the 0.05% trypsin solution (Gibco Co.). Passage 2 was used in the present study. 5×10^4 cells of DPMSCs were cultured on prepared scaffolds for 14 days. At regular 2 day intervals, the media was replaced.

Alizarin Red S staining: Scaffold incorporated DPSC's ability for differentiation into the osteogenic lineage was assessed using the Alizarin red S. On day 7 and day 14, the staining was performed. The media of the DPSCs seeded scaffold was removed and subjected to PBS washing thrice. Over this cleaned scaffold, the 50 μl

solution of Alizarin Red S was placed. Following a time interval of 2 min 30 sec, the scaffold was subjected to PBS washing until any residual solutions of stains were removed. Canon Power shot G6 camera (Tokyo, Japan) was used to photograph the stained scaffold.

RT PCR: Expressions of alkaline phosphatase, osteocalcin, and RunX2 were evaluated to ensure that vitamin D is functional. Briefly, the cells were pelleted down and the total RNA was extracted by using the Trizol method. RNA (1 μg) was reversely transcribed by using a cDNA synthesis kit according to the manufacturer's guidelines. Quantitative analysis of genes of interest was carried out using SYBR Green PCR master mix on a Real-Time PCR system. Expressions of target genes (ALP, OCN, and RUNX2) were normalized to GAPDH by using the ΔΔCt method. The cycle threshold (CT) values for each gene were corrected by using the mean CT value. RT-PCR data were quantified by using the 2–ΔΔCt method and were presented as relative gene expression normalized to the average CT for the GAPDH gene.

3. Results:

FTIR revealed that the functional groups of chitosan and vitamin D include amine and carboxyl (NH and CO) groups (Fig. 1A). These functional groups remained intact in both blank chitosan and vitamin D incorporated chitosan scaffolds. Differential scanning calorimetry (DSC) reveals the endothermic reactions occurring in the scaffolds (Fig. 1B). The graph depicts that at 80 °C both blank chitosan scaffolds and chitosan scaffolds incorporated with vitamin D showed water evaporation. However, the peak representing endothermic reactions in DSC is not very sharp because the polymers don't show sharp melting points unlike compounds like paracetamol, etc. Hence the evaporation of water is seen between 85 and 100 °C. Both blank chitosan scaffolds and chitosan scaffolds incorporated with vitamin D showed degradation at around 270 °C where the exothermic reaction started.

X-Ray Diffraction helps in identifying the crystalline nature of the compound polymer formed. In X-ray diffraction, chitosan gives sharp peaks at 20 and 30 which are characteristic of the polymer (Fig. 1C). Blank chitosan scaffolds gave characteristic peaks of chitosan. Chitosan scaffolds incorporated with vitamin D showed diminished peaks in the same region however, the peaks can still be appreciated. This shows that Vit D incorporated chitosan scaffolds are semi-crystalline but not amorphous.

Scanning Electron Microscopy revealed the porous nature of both blank and vitamin D incorporated chitosan scaffolds (Fig. 2A). SEM also revealed the growth of DPMSCs cultured on the scaffolds, indicating that the scaffolds (with and without vitamin D) provide a favorable environment for DPMSC cell growth. Traces of vitamin D were detected by UV spectrophotometry in the phosphate buffer saline until 73 h of the experiment indicating a relatively sustained release period (Fig. 2B).

Cytotoxicity assay revealed that both blank and vitamin D incorporated chitosan scaffolds cultured with the DPMSCs were as biocompatible as the control group (only DPMSCs in the culture medium) (Fig. 2C).

Alizarin Red S staining suggested bone formation over the DPMSCs co-cultured vitamin D incorporated chitosan scaffolds until 14 days of the experiment (Fig. 2D). The mineralization was attributed to being the result of the DPMSCs differentiating into osteoblasts. The bone formation over the scaffolds was confirmed by the higher gene expression of alkaline phosphatase, osteocalcin, and RunX2 in the DPMSCs co-cultured vitamin D incorporated chitosan scaffolds in comparison to the blank chitosan scaffolds (Fig. 3 A, B, C).

4. Discussion:

Tissue engineering is a multidisciplinary field combining conventional biology, medicine, engineering, and chemistry (Fisher and Mauck, 2013; Fodor, 2003; Park et al., 2018; Salgado et al., 2013). The primary goal of tissue engineering was to provide graft material to aid in the rehabilitation of a deficit site. The applications of tissue engineering have evolved in the past two decades to involve several novel applications including drug delivery. Several modalities have been engineered for targeted drug delivery

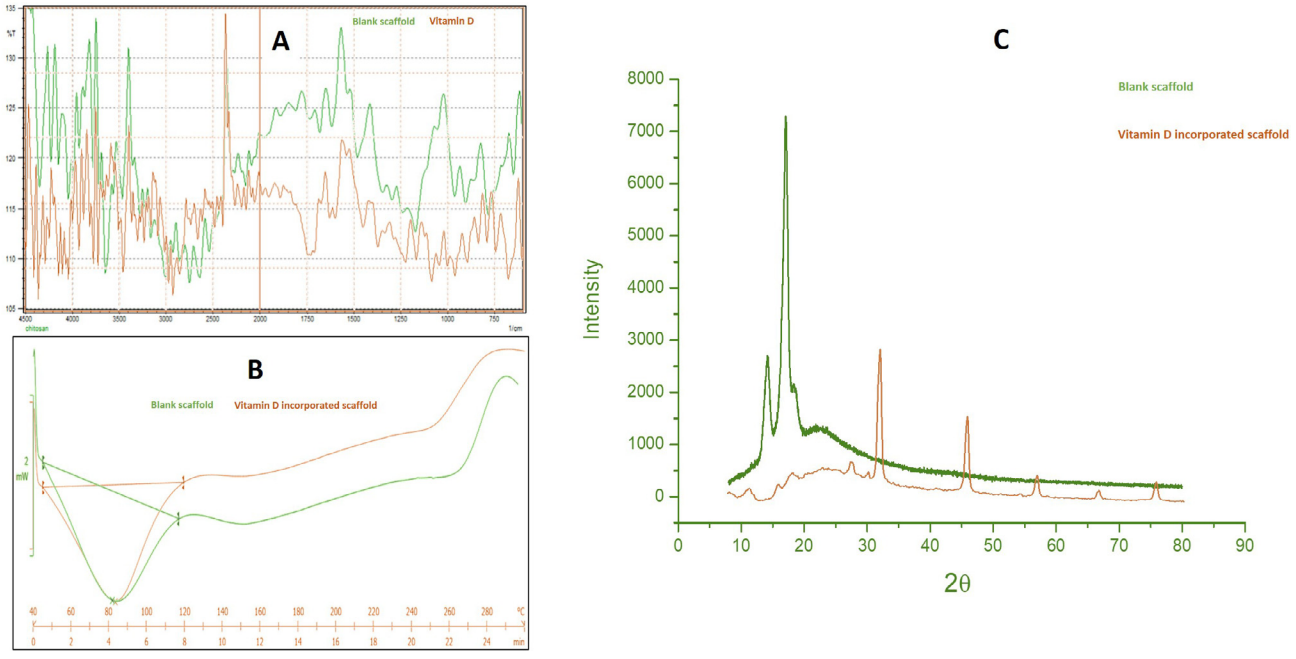

Fig. 1. A- FTIR results showing intact functional groups of chitosan and vitamin D in scaffolds; B- DSC showing the endothermic reaction of both blank and vitamin D incorporated chitosan scaffolds between 80 and 100C; C- Diminished but still appreciable peaks of vitamin D incorporated chitosan scaffolds between 20 and 30 showing the scaffold to be semi-crystalline.

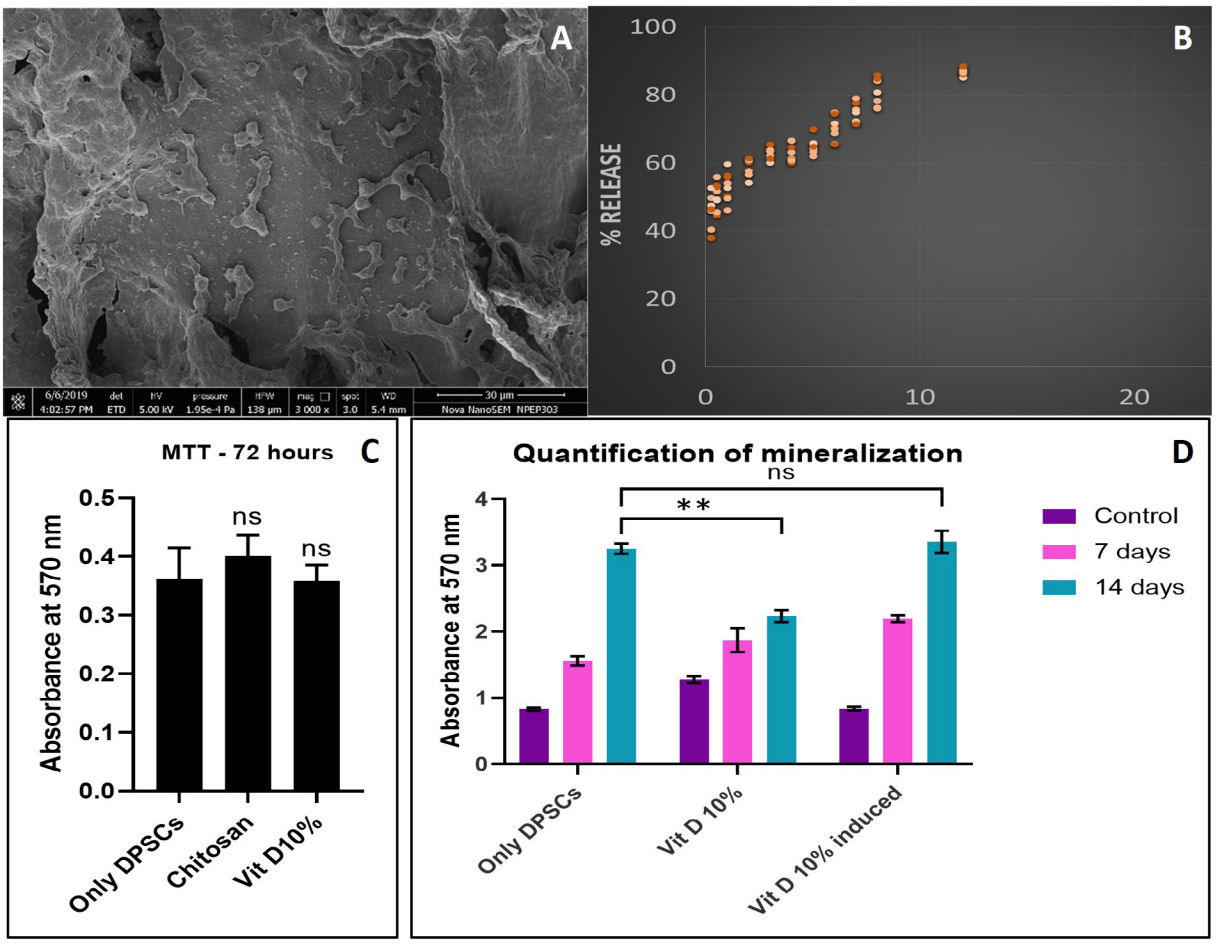

Fig. 2. A- SEM showing cell growth over the porous Vit D incorporated Chitosan scaffolds; B- Traces of Vit D release was detected by the UV Spectrophotometer for 73 h; C- MTT assay shows that the vitamin D incorporated scaffold had biocompatibility comparable to that of blank scaffolds and the control group (only DPMSCs); D- Alizarin Red Assay: Calcification deposits over the vitamin D incorporated scaffolds showed a marked increase compared to control.

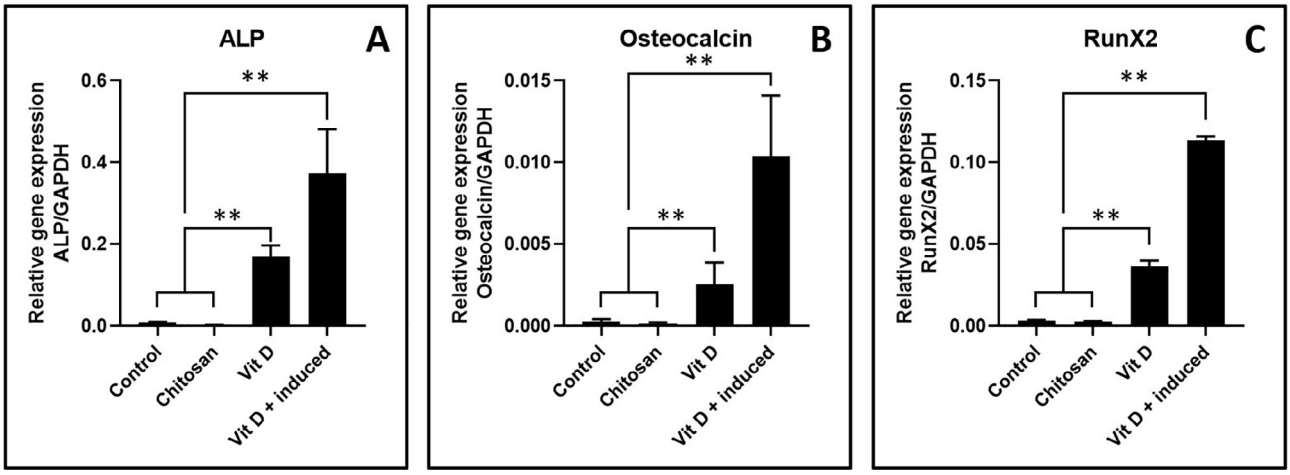

Fig. 3. A- Alkaline phosphatase gene expression; B- Osteocalcin gene expression; C- Runx2 gene expression.

including nanoparticles, and a wide variety of scaffolds (Bernkop-Schnürch and Dünnhaupt, 2012; Gupta et al., 2018; Jaisinghani et al., 2018; Li et al., 2016). Among scaffolds, much importance is given to bio-degradable materials including chitosan (Bernkop-Schnürch and Dünnhaupt, 2012; Chaudhary and Garg, 2015; Hu et al., 2013; Huang et al., 2017; Li et al., 2016). The major limitation of using scaffold for targeted delivery was the inability to control the release period and dosage. Inadequate integration of the agent to the scaffold often leads to disintegration (Gupta et al., 2019) or a short rapid release. Thus, researchers have focused on formulating

scaffold with customized properties depending on the characteristics of the agent. Successful integration between the scaffold and the agent must allow the optimal delivery of the agent for a sustained period. The present study is one such attempt to use a bio-degradable chitosan scaffold to deliver vitamin D for a sustained period.

Vitamin D is a well-known bone modulator, often used to enhance the osteogenic potential, in the field of regenerative medicine (Bosetti et al., 2017; Fügl et al., 2015; Hong et al., 2015; Ikeda and Ogata, 2000; Kim et al., 2018). In the present study, vitamin D was used as the agent being delivered. Vitamin D was integrated into a bio-degradable chitosan scaffold. The release of vitamin D from the scaffold was evaluated using UV spectrophotometry, which detected the presence of vitamin D being delivered from the chitosan scaffold for 3–5 days. In addition to a sustained delivery period, it is also essential to determine if the delivery vehicle has altered the functional capability of its agent. Thus, the functional capability of vitamin D being released from the scaffold was assessed by culturing DPMSCs on the vitamin D integrated scaffolds. The ability of vitamin D to induce osteogenic potential in the DPMSCs on the scaffold were recorded. Alizarin red staining showed the presence of mineralization throughout 3–5 days. Also, increased expression of mineralization markers including alkaline phosphatase, osteocalcin (a calcium-binding protein found in both bone and dentin), and RunX2 (key transcription factor associated with osteoblastic differentiation) were noted, confirming the functional ability of the vitamin D being released from the scaffold.

In addition to drug release properties, another vital property to be analyzed in a delivery vehicle is its associated cytotoxicity. Apart from assessing the functional ability of vitamin D, the DPMSCs cultured on the chitosan scaffolds also served as a module for studying the cytotoxicity of the delivery vehicle. SEM revealed the presence of DPMSCs on the scaffold through the study period (3–5 days). The MTT assay (IC50 = 60 μg/ml) showed DPMSC's metabolic activity in the chitosan integrated scaffold to be comparable to that of the control sample (only DPMSCs in the culture media) indicating an active cell population.

Previous research has revealed that apart from scaffolds, gold nanoparticles (GNP) have been used to deliver vitamin D (Ashfaq et al., 2017; Daraee et al., 2016; Kong et al., 2017; Nah et al., 2019; Tamarkin and Kingston, 2017). The natural affinity of GNP to conjugate with functional groups including amines, thiols, and phosphines allows it to be an ideal carrier. Nah et al (Nah et al., 2019) assessed the potential use of GNP for the localized delivery of vitamin D. The GNP-mediated delivery of vitamin D was optimal as indicated by the successful induction of osteogenic differentiation in the human adipose-derived stem cells. Despite optimal delivery, the GNP-associated toxicity limits its applications (Alberts and Garcia, 1997; Chang, 2010; Hussain et al., 2005; Rutberg et al., 2008).

Scaffolds have been advocated as a relatively safer modality for delivering agents, provided that the scaffold materials used are bio-compatible and is well integrated with the agent to be delivered. Vu et al (Vu and Bose, 2020) used tricalcium phosphate (TCP) scaffolds to deliver vitamin D3. The open porous structure of the TCP scaffolds provided a greater surface area, allowing the optimal release of vitamin D3. Similar to TCP scaffolds, the chitosan scaffold fabricated in the present study was shown to have a porous structure in the SEM analysis, which could have aided in the sustained delivery of the vitamin D. Based on the success of scaffold mediated delivery and the affinity of GNP for conjugations, Lee et al (D. Lee et al., 2018a) combined the GNP with poly (lactide-co-glycolide) nanofibrous scaffolds which enhanced the overall osteogenic effect. Like Lee et al, future studies could potentially combine bio-degradable chitosan scaffolds with GNP for enhancing the delivery of vitamin D beyond 3–5 days reported in the present study.

5. Conclusion:

Based on the observations made above, it can be inferred that the chitosan scaffold is capable of delivering functional vitamin D for 3–5 days without any significantly associated cytotoxicity. The translational value of the chitosan scaffold mediated delivery would depend on the results of in-vivo animal-model studies. Further modifications including the addition of other osteogenic factors, incorporation of GNP's could be implemented to potentially enhance the overall delivery period of vitamin D. The major practical difficulty in implementing any modifications would be during the integration of the additional factors with the scaffold material. Successful integration would enhance the delivery period, enabling sustained effect, without increasing the risk of cytotoxicity.

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None

Declaration of Competing Interest

The results of the study have been filed as a patent (Application number – 202021041322)

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