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Development and in vitro evaluation of Fucoidan Injectable Hydrogel Containing gelatine and chitosan in bone regeneration

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ABSTRACT

Past two decades have witnessed tremendous innovations in the development of artificial bone substitutes. In this study, an attempt was made to fabricate injectable chitosan containing fucoidan hydrogel to prove its efficiency in bone regeneration. The hydrogel was prepared using sol-gel method and was subjected to various characterisation like physical, chemical and biological to understand its nature in the formation of new bone. The gelation time was found to be 6 min at pH 6. The porosity and water uptake of the prepared hydrogel showed good efficacy. A decrease in cell number results in a concomitant change in the amount of formazan formed, which indicates the degree of effects caused by the test material. *I*n vitro studies using the MG-63 osteoblasts, cell line revealed hydrogel to be biocompatible, biodegradable and showed slow and sustained drug release, increased cell proliferation and enhanced alkaline phosphatase secretion. Hydrogel proved that calcium deposit by converting bright orange-red in mineralisation assay to be greater in the Chi-fucoidan hydrogel. The ALP activity of chitosan containing fucoidan hydrogel was high in comparison to control and constant. Hence, we suggest that Chi-fucoidan will be a promising biomaterial for bone tissue regeneration.

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INTRODUCTION

Bone is a dynamic connective tissue that protects internal organs, helps in locomotion as well as maintaining homeostasis. Bone defects are a common finding in various systemic and dental disorders. During trauma to the bone, natural healing is disturbed, resulting in functional and structural oddness (Saravanan *et al.*, 2019). Tissue engineering is the heart of regenerative medicine and is the fastest emerging biomedical field which offers treatment for damaged bone tissues (Suzuki and Babcock-Goodmans, 1989). Regeneration of the lost tooth-supporting structures is mainly dependent on the interplay among scaffold, cells, and bioactive cues. Regeneration in osseous defects is possible by different grafts, materials, barrier membranes and bone substitutes. All regenerative material recorded in the literature has known advantages with certain limitations like additional surgery,

inadequate bone supply, inappropriate biodegradation, immune response and low tissue compatibility. Hence these limitations have evoked an interest in the development of artificial biomaterials (İkinci *et al.*, 2002).

Natural polymers have gained significant interest in the field of biomaterials as a result of their biological properties. Preformed scaffolds or hydrogels are primary components of bone tissue engineering. Scaffolds have been widely researched and proved as a promising bone regenerative material; however, certain limitations like poor integration, limited penetration and cost have made way for the use of injectable hydrogels in bone regeneration.

Hydrogels are three-dimensional hydrophilic polymer chains which exhibit excellent mechanical strength, maximum penetration and mimics extracellular matrix (Saravanan *et al.*, 2019).

Chitosan is a natural polymer is considered as a boon to regenerative dentistry because of its excellent biological properties like non-toxic, anti-inflammatory, biocompatible, biodegradable and bio-adhesion. Chitosan is believed to activate osteoblasts and increases osteoconductivity and neovascularisation leading to increased bone growth (Akıncıbay *et al.*, 2007) Chitosan being very flexible can be transformed in many shapes which will adapt in the tissues, chitosan has good structural properties with good pore size and volume hence instrumental in tissue engineering, drug delivery and wound healing. Though having many advantages, chitosan lacks bioactivity and degradation to enhance these properties. It has been crosslinked with synthetic polymers and biocomposites such as alginate, gelatine, hyaluronic acid and growth factors to name a few. Nanocomposite containing chitosan shows excellent cellular proliferation and mineralisation. Chitosan possesses osteoconductive and osteoinductive properties but lacks osteogenic potential in new bone formation, to attain the osteogenic property in bone, chitosan nanocomposite containing fucoidan was developed in this study (Klokkevold *et al.*, 1996).

In the present day, seaweeds and their products have acquired the market and the demand for these products is believed to expand continuously over the years. To our knowledge, there are almost 221 seaweed species worldwide accounting for varied applications in multiple sectors. In India, brown seaweeds are the largest species harvested from the natural beds in both northern and southern parts of the country. Gulf of Mannar, located on the Southeast coast of India, is being flourished with brown seaweeds, mainly belonging to the group Fucales (Li *et al.*, 2009b).

Fucoidan, a marine brown seaweed, is a sulphated polysaccharide that contains L-fucose and sulfate. It can increase the level of alkaline phosphatase (ALP), type-1 collagen expression, osteocalcin and BMP-2 and even helps in mineral deposition associated with bone mineralisation (Cho *et al.*, 2009).

Studies have been reported that in human adipose-derived stem cells, the expression of ALP, type-1 collagen, Runt-related transcription factor 2 (Runx-2), osteopontin and osteocalcin was enhanced by fucoidan treatment. Osteogenic differentiation in human amniotic fluid stem cells was also promoted, suggesting that it is a potential candidate for bone tissue regeneration (Crouch *et al.*, 1993).

(Sezer *et al.*, 2008, 2005) have confirmed the efficacy of chitosan – fucoidan hydrogel in animals for wound healing and concluded that the regeneration was observed on the dermal papillary formation and the closure of the wound was faster in fucoidan-chitosan hydrogels after 14 days treatment. Similarly, a study by Venkatesan *et al.* (2014) has compared chitosan alginate- fucoidan scaffold as a promising material for bone regeneration.

Though few studies have already reported the efficacy of chitosan – fucoidan polymer in wound healing and bone regeneration, the test species vary from the study being conducted. Previous studies have proved the efficacy of fucoidan vesiculous, whereas in the present study fucoidan (sargassum wightii) is being experimented as a bone regenerative material and this study intends to assess the biological properties in its proper form (without the addition of any crosslinkers).

Hence, considering the biocompatibility, biodegradation, antibacterial nature, film-forming ability and induction of osteogenic differentiation by fucoidan and chitosan, we aimed to report the synthesis and characterisation of the newly developed Chitosan biocomposite containing fucoidan for bone tissue engineering.

Objectives

To develop a thermosensitive injectable hydrogel containing chitosan, gelatine and fucoidan.

To characterise the microstructural, physical and biological properties of the hydrogel.

MATERIALS AND METHODS

The chitosan was obtained from Everest biotech pharma, Bangalore. All the cell lines were purchased from ATCC.

Preparation of hydrogel containing chitosan and fucoidan

Chitosan was used after purification by dissolution-precipitation and dialysis and re acetylation up to an 85% degree. chitosan nanocomposite was used to produce gels at 37°C following simple neutralisation with sodium hydroxide, although with very slow gelation and a weak increase in viscosity to produce a stable formulation for a medical application (Qiu and Park, 2001).

Fucoidan was used after purification from brown seaweed species; *Sargassum wightii*, seaweeds were collected and dried overnight and placed in an oven to remove moisture. Dried seaweeds were milled and strained.

Extraction

Crude extract was prepared by mixing 1 mg of seaweed with 10 mL ethanol solvent and stored for two days, centrifuged 10000 for 15 mins.

Fucoidan extraction

20 mg powder treated with ethanol and stirred for 12 hours and centrifuged for 20 mins (Yang *et al.*, 2008).

Hydrogel preparation

Fucoidan (1 g) was added to 100 mL of water and stirred for two h, 500 mg of chitosan was added and dissolved. Later 500 mg of gelatine was added and dissolved at 40^0C at 2500 rpm in a magnetic stirrer and cooled at room temperature at 25^{o}C. The gel solution was continuously stirred for 2 hours until it attains homogeneity, 0.1% methylparaben was added to the gel as a preservative. Chitosan nano gel was sterilised by autoclaving (121^{o}C for 15mins).

Characterisation Methods

Scanning Electron Microscopy

SEM assessed surface morphology and pore structure of the developed hydrogel. With the help of a scalpel, a section of hydrogel was segmented. Samples were prepared by mounting on aluminium stubs, and samples were cut precisely to small pieces and fixed on carbon tapes. Then the samples were dried using vacuum and gold-coated and subjected to SEM analysis.

X-ray diffraction analysis

The crystalline nature was studied by grounding the samples to powder form in liquid nitrogen. The powdered hydrogels were analysed by XRD with a 2θ angle of 5°–80° at a speed of 2° min^{-1} using an analytical XPERT PRO powder diffractometer operating at a voltage of 40 kV (Cu Kα radiation).

Fourier transform infrared spectroscopy

FTIR analysed the intermolecular chemical interactions between the various functional groups within the components present in the chitosan fucoidan hydrogel. (Li *et al.*, 2009a; Mao *et al.*, 2003)

Gelation Time

Hydrogel (5 mL) was taken in a beaker. The gel was heated at 60^{o}C. After the complete dissolution of gel, it was allowed for solidification at room temperature. The time for complete solidification of hydrogel was recorded.

Swelling Index

The swelling test was performed in PBS (pH 6) at 37^{o}C. The dry weight of the hydrogel was considered as W_d. Hydrogels were immersed in buffered saline for different time durations (1, 6, 12, 24) hours at 37^{o}C. After incubation, the hydrogels were washed using deionised water and blot dried using filter paper. The wet weight was reported as WW, and the ratio was calculated.

Wet weight (W_w) – Dry weight (W_d)

$$\% \text{ swelling } = \frac{}{\text{Wet weight } (W_w)} \times 100$$

Cytotoxicity studies

For cytotoxicity studies, 0.5%, 1% and 1.5% concentrations were prepared from the sample stock using DMEM plain media. DMEM culture media was used with other chemicals to culture cells in an atmosphere of 5% CO_2 at 37^{o}C until confluent. For standard 2%, 3% and 4% concentrations were prepared using DMEM plain media. Cells were separated using a dissociation solution, and the viability of the cells was checked. Cells (50,000 cell/wall) were suspended in a 96 well plate, each well was filled with 100 μL of cells and incubated for 24 hrs at 37^{o}C in 5 % CO_2 incubator. At the end of 24 hours, the supernatant was removed and washed. Again, different concentrations of drug were tested by following the same procedure, the supernatant was removed, and the formation of formazan was measured with the help of microplate reader. The amount of drug needed to inhibit cell growth was calculated from each cell line.

Inhibition Calculation

%Inhibition $=$ (OD of sample / OD of Control) $\times$ 100.

Dissolution studies

Drug containing 0.5% chitosan, 1% fucoidan without agarose was used for HPLC. A thin film of hydrogel formulation was prepared by pouring to plate allowed to dry. Artificial saliva was prepared, and 5mL was added to the plate, and 100uL samples

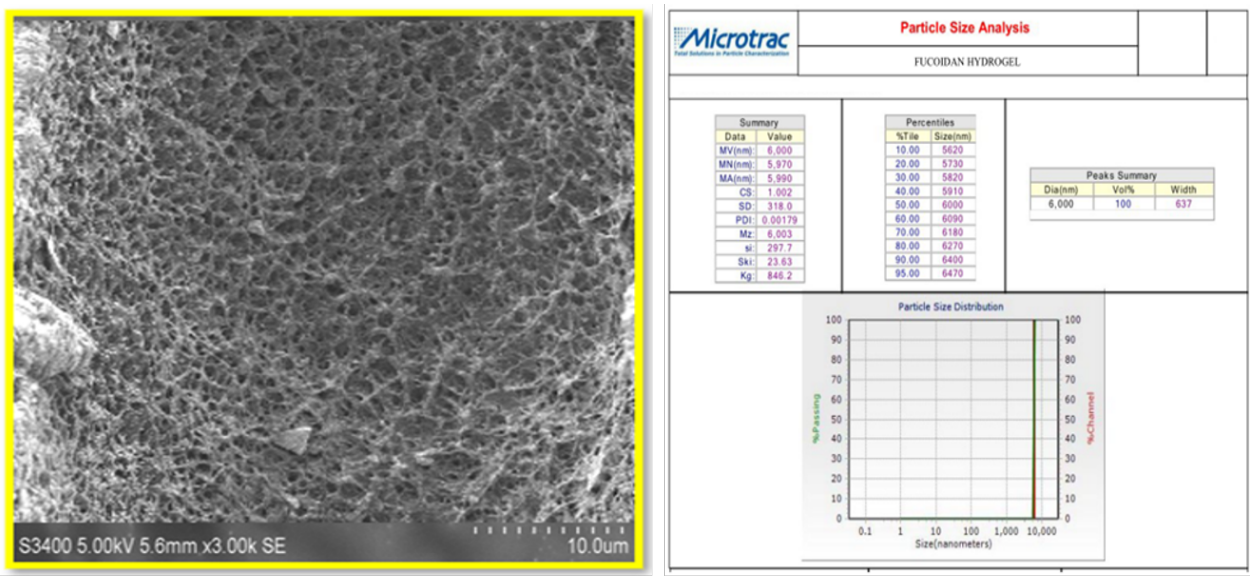
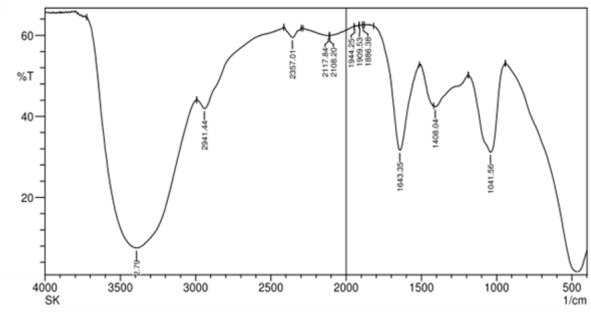


Figure 1: A) SEM Image of a hydrogel. B) SEM analysisof hydrogel depicting particle size

at different timings 0min, 15min, 30 min, 1h, 2h, 3h, 4h, 6h and 24h were collected and used for HPLC analysis. The retention time of samples was matched with that of each standard, and the concentration of the respective standard was calculated using the formula.

$$\text{Conc. } (\%) = (\text{Sample area}) / (\text{Standard area})$$



No.	Peak	Intensity
1	459.06	1.537
2	1041.56	31.168
3	1408.04	42.367
4	1643.35	31.68
5	1886.38	62.577
6	1909.53	62.485
7	1944.25	62.195
8	2108.2	59.987
9	2117.84	59.969
10	2357.01	59.494
11	2941.44	41.96
12	3392.79	7.554

Figure 2: FTIR - Fourier transform infrared spectrum of chitosan- fucoidan

Osteoblast Re-mineralization assay

Production of calcium deposits through inducing osteoblasts is mineralisation. Calcium deposits formed indicates the formation of bone, and this is notified through the bright orange-red using Alizarin Red Stain. Hydrogels were immersed in Culture 1 x106 cells in 5 P35 dishes containing 2

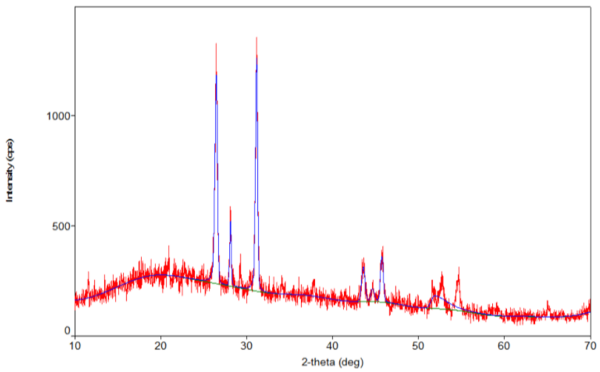


Figure 3: XRD Analysis of hydrogel

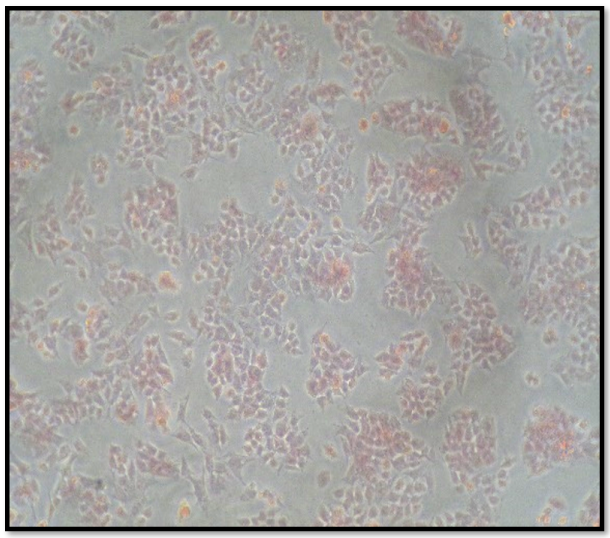


Figure 4: Mineralisation assay of hydrogel (control)

Table 1: In vitro cytotoxicity behaviour of chi – fucoidan hydrogel

Compound name	Conc. %	OD at 590nm	% Inhibition
Control	0	0.912	0.00
	0.1	0.900	1.32
	0.2	0.844	7.46
Chitosan	0.3	0.796	12.72
	0.4	0.698	23.46
	0.5	0.613	32.79
	0.5	0.860	5.70
	1	0.810	11.18
Fucoidan	1.5	0.743	18.53
	2	0.681	25.33
	2.5	0.622	31.78
	3	0.512	43.86

Table 2: Results of biocompatibility

Formulations	Fucoidan %	Chitosan %	OD - 590nm	Cytotoxicity %
1	4.5	2	0.419	54.06
2	3	1	0.429	52.96
3	1.5	0.5	0.659	27.74
4	1	0.25	0.832	8.77
5	0.5	0.5	0.751	17.65

Table 3: In-vitro drug release properties of ch – fucoidan hydrogel at different time intervals

Compound	RT	Area	Concentration (%) in 100 ul	Concentration (%) in 5 mL
Drug	2.143	1281.482		
0 min	-	-	-	-
15 min	2.173	40.261	0.031	1.571
30 min	2.167	54.1	0.042	2.111
1 h	2.15	69.646	0.054	2.717
2 h	2.157	285.448	0.223	11.137
3 h	2.153	382.414	0.298	14.921
4 h	2.147	406.387	0.317	15.856
6 h	2.15	594.786	0.464	23.207
24 h	2.15	700.081	0.546	27.315
Cumulative drug released				98.84 %

mL of complete DMEM media. After 24h of incubation, cells are treated with 1.5% Fucoidan, 0.5% Chitosan, 0.5% Chitosan+ 1% Fucoidan, 0.25% Chitosan + 0.5% Fucoidan and Control in 2 mL / well of serum-free DMEM media and incubated for 24 hr. Every third day, media was changed with test samples for 21 days. After 21 days, the cell monolayer with 1X PBS was washed without disrupting the cell monolayer. Following careful aspiration, the buffer was discarded, and the cells were fixed using formalin and incubated for 30 mins and washed with distilled water, then discard the water and add Alizarin Red S stain to cover the cell monolayer and incubate the cells in the dark for 45mins at RT. Following incubation aspirate the stain and wash the cell layer using distilled water for four times and subject for microscopic analysis to appreciate the calcium deposits. (Stein and Lian, 1993; Kasperk *et al.*, 1995; Changotade *et al.*, 2008).

Alkaline phosphatase activity

MG63 cell culture was dissociated using 10% Foetal bovine serum. In a 96 well microtiter plate, each of the wells was immersed with 100 μL of cell sus-

Table 4: ALP activity of hydrogel

Sample	Conc. μg/ml	AT 1 MIN Absorbance @ 405nm	AT 3 MIN Absorbance @ 405nm	ALP Activity
Control	0	0.324	0.325	0.91
Hydrogel	0.5%	0.806	0.859	48.05
Sample name	Conc. μM	AT 1 MIN Absorbance @ 405 nm	AT 3 MIN Absorbance @ 405 nm	ALP Activity
Reso Sod	3.125	0.291	0.298	6.62
	6.25	0.325	0.338	11.80
	12.5	0.474	0.498	21.55
	25	0.532	0.560	25.02
	50	0.612	0.648	32.37
	100	0.785	0.835	45.33

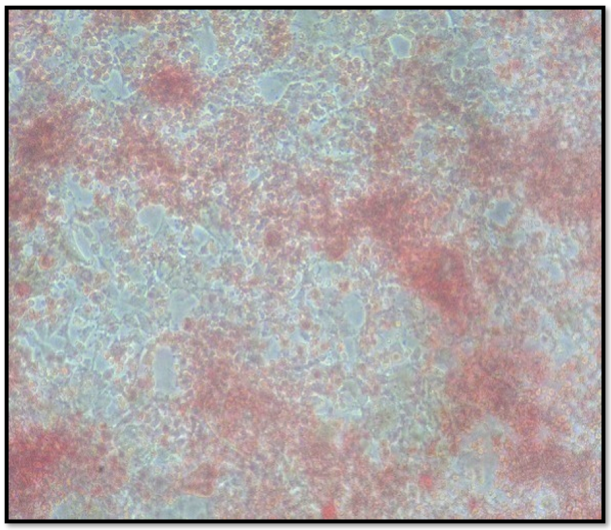


Figure 5: Mineralisation assay of hydrogel (1.5% fucoidan)

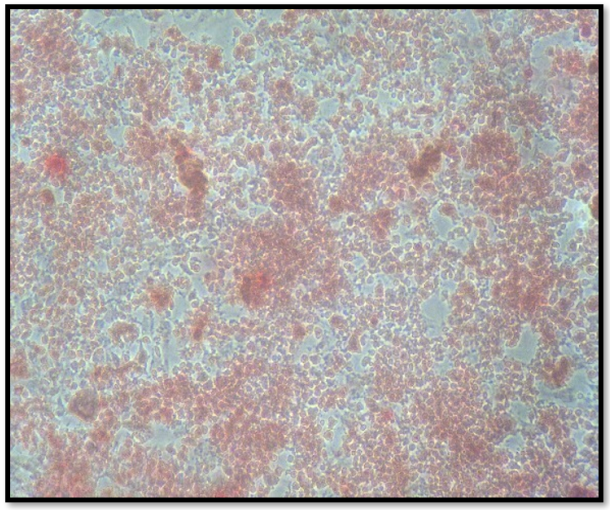


Figure 6: Mineralisation assay of hydrogel (0.5% chitosan)

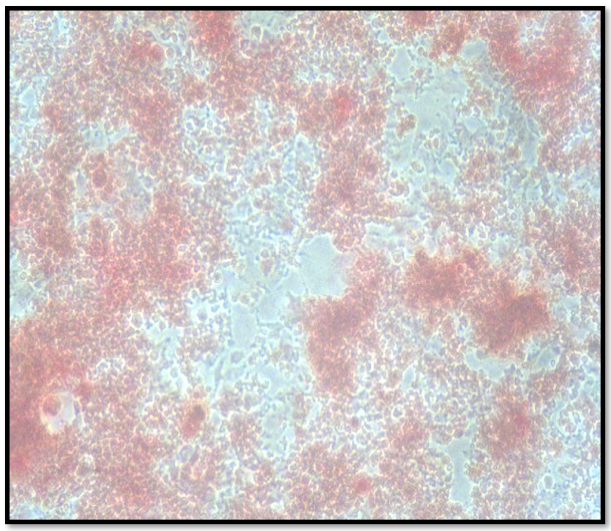


Figure 7: Mineralisation assay of hydrogel (0.25% chitosan and 0.5% fucoidan)

pension and incubated for 24 h. At the end of 24 h monolayer was formed which was discarded and washed. Different test concentrations (100 μL) of test drugs were added in well plates and incubated at 37^{o}C for 24h in 5% CO_2 atmosphere. After incubation to the test solutions 1000μL working reagent is added. OD is taken at 405nm every 1min for 3-4 times. ALP activity is calculated later with the formula (Park *et al.*, 2012).

$$\text{ALP activity } (\text{IU/L}) \triangle \text{A/ min} * 2720.$$

RESULTS

In the present study, the hydrogel was prepared by sol-gel method. Chitosan containing fucoidan hydrogel was light brown and uniformly dispersed, no flakes were seen in the final hydrogel.

Gelation Time and Viscosity

The gelation time was found to be 6min at pH 6 and viscosity of hydrogel was 1 cps.

Physicochemical characterisation

Scanning electron microscopy revealed that the hydrogel had porous structure indicating micro and macro-sized pores which were uniform and well interconnected. The particle diameter was in the range of 6000nm with a width of 637, which is suitable for penetration of the nutrient transport, thus yielding successful cell adhesion, penetration to achieve regeneration. (Figure 1)

Fourier transform infrared spectroscopy

Spectra of chitosan and fucoidan in hydrogels showed absorption band at 1041.56 indicative of sulphate group, 1409.04 – carboxyl group, 2941.44 – sodium hydroxide stretching and 3392.79 – hydroxyl group. (Figure 2)

X-ray diffraction analysis

Hydrogel presented peaks at 280 – 300°Θ indicating the semi-crystalline nature of the polymer. (Figure 3)

In-vitro cytotoxicity behaviour

This is the decisive and fundamental criterion to be considered in tissue engineering. Toxicity of the prepared hydrogel needs to be considered before application in humans. There are many tests to gauge the toxicity like MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium), WSTs (Water Soluble Tetrazolium salts) and LDH (Lactate dehydrogenase) tests. MTT means tetrazolium dye used to find out cell toxicity, cellular functions and viability. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide).

If the cell is viable, mitochondria are healthy and maintain all cellular and enzymatic functions, the enzyme NADPH dependent oxidoreductase produced by mitochondria converts tetrazolium dye to purple coloured compound formazan. The resulting purple solution is spectrophotometrically measured. Formazan produced indicates the cell viability, if not produced, then the cell is non-viable.

An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material. Hydrogel showed biocompatibility and non-cytotoxic in nature, and the cell viability is shown in Table 1 and Table 2.

Dissolution studies

At different time of intervals, the drug is found to release in increasing order with increase time of incubation. At 15min it is found to release 1.57% in 5mL. After 24h incubation, 27.32% drug was released in 5ml. Drug release was slow and sustained. The drug concentration at different interval is listed in Table 3.

Remineralisation assay

The MG-63 cell lines treated with sample fucoidan (Figure 5) containing Chitosan (Figure 6) has induced mineralisation when compared to control (Figure 4). Cells devoid of calcium deposits are slightly reddish, whereas cells mineralised with Osteoblasts with extracellular calcium deposits are bright orange-red. The highest increase in calcium deposits observed in MG-63 cells treated with formulation III containing 0.25% Chitosan and 0.5% Fucoidan (Figure 7).

Alkaline phosphatase activity

The ALP activity of chitosan containing fucoidan hydrogel was high in comparison to control and constant, indicating the capability of the effectiveness of hydrogel in bone regeneration. The presence of fucoidan in the hydrogel is known to elevate the bone markers. Fucoidan markedly increased osteoblastic differentiation. The ALP activity of hydrogel is displayed in Table 4.

In this study, we have attempted to fabricate a hydrogel having the potential to induce osteogenic property in bone formation. However, there are certain limitations in this study to name a few like study failed to identify the specific proteins and their role in bone regeneration and as well we have only performed ALP activity, other bone marker genes have not been identified to understand the role of fucoidan in achieving the osteogenic potential in new bone formation.

CONCLUSIONS

Chitosan containing fucoidan nanocomposite hydrogel has proven to be biocompatible and biodegradable with increased bioactivity. The addition of fucoidan to chitosan increased mineralisation as well as increased ALP activity indicating the impending role in tissue engineering.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

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The authors declare that they have no funding support for this study.

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