RESEARCH PAPER

Potential of Glutaraldehyde Cross Linked Graphene-Reinforced-Chitosan/Hydroxyapatite Composite in Regenerative Medicine

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ABSTRACT

In recent years, biodegradable polymers like chitosan have been studied as scaffolds for bone tissue engineering applications. As chitosan suffers from drawbacks such as solubility in acids and poor mechanical strength, there is a need to modify the same. In order to improve the mechanical properties, in the present work, reinforcing graphene with chitosan/hydroxyapatite has been investigated. The novelty of this work consists of the possibility of obtaining porous and highly interconnected scaffolds mimicking the sacrificial component. In the present research work, the glutaraldehyde cross linked graphene-reinforced-chitosan/hydroxyapatite (GCH) composite was synthesized and characterized by FTIR and XRD analysis. The FT-IR spectroscopic results indicated that the graphene had been uniformly dispersed in the chitosan biopolymeric matrix. Broadness of a peak evidenced from XRD studies indicates the partial demineralization of HAP and low crystallinity of the glutaraldehyde cross linked graphene-reinforced-chitosan/hydroxyapatite composite. In order to assess biocompatibility of the composites, the in vitro experiments were carried out using osteoblast cell line and cell viability assays such as MTT, biomineralisation, ALP and LDH activity tests. The overall results of osteoblast growth on composites demonstrated that fabricated novel composites are non-toxic and are favorable to cell adhesion and growth.

Keywords: Chitosan, Hydroxyapatite, Graphene, Composites, Tissue engineering.

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1. Introduction

Tissue engineering (TE) requires more efficient systems that favour local tissue regeneration with minimum cytotoxicity and in order to facilitate appropriate cellular architecture, cell polarization and guidance the regenerative medicine requires adequate physical supports [1]. In the present and in the future too, this tissue engineering process is found to be an important therapeutic strategy to be used in regenerative medicine. Materials based on natural compounds ensure biocompatibility and have better effects for regeneration since it involves the fundamental understanding of structure function relationships in normal and pathological tissues and the development of biological substitutes that restore, maintain or improve tissue function.

Many synthetic polymers demonstrate hydrophobic behavior and hydrophobic surfaces show a lower proliferative and a higher apoptotic rate for osteogenic cells [2][3]. Chitosan is a natural polymer from renewable resources, obtained from the shell of shellfish and the wastes of the seafood industry which mainly composes of both reactive amino and hydroxyl groups that can be chemically or physically modified and hence it has a high potential in tissue engineering applications. Chitosan biopolymer have given the pattern for use in tissue engineering for repair and/or regeneration of different tissues including skin, bone, cartilage, nerves, liver, muscles and so on and this was due to their different biological properties such as being biocompatible, biodegradable and bioactive[4].

Hydroxyapatite (HA), a predominant mineral component found in bones and coral, has been widely used as bone graft since the 1960s and could be a potential candidate for bone tissue engineering [5]. Due to its osteoconductivity, biocompatibility and biodegradability, HA has been highly favored in surgical reconstruction of bones [6]. Novel hydroxyapatite (HA)–

collagen micro carriers (MCs) with different micro/nanostructures were developed for bone tissue-engineering applications by Perez and his coworkers. Results showed that the synergistic effect was found between the presence of collagen and the nanosized hydroxyapatite crystals, resulting in significantly enhanced alkaline phosphatase activity on the collagen-containing nanosized hydroxyapatite (HA)–collagen microcarriers [7].

An improved Bone marrow Mesenchymal Stomal Cells (BMSCs) differentiation into osteoblastic linkage was provided by Mercedes Vila and his coworkers through the combination of the ceramic material (silicon doped hydroxyapatite, Si-HA) and the Elastin like recombinamers (ELRs) polymers in the presence of the integrin-mediate adhesion domain alone or in combination with SNA15 peptide that possess high affinity for hydroxyapatite. The *in vitro* tests showed that there was a total and homogeneous colonization of the 3D scaffolds by bone marrow Mesenchymal Stromal Cells (BMSCs)[8]. Hence to create bone like environment hydroxyapatite material was chosen in preparing the composite material.

Due to its enhanced electrical, mechanical and thermal properties, graphene which is composed of sp^2 hybridized carbon atoms existing in two dimensional single layer sheets has attracted much attention of the researchers in recent years [9][10]. Graphene, a carbon crystal sheet of molecular thickness, shows diverse and exceptional properties ranging from electrical and thermal conductivities, to optical and mechanical qualities. This graphene is used as a cell-contacting biomaterial for tissue engineering and regenerative medicine. In recent years, the biomaterial graphene has excellent mechanical properties (high elasticity, strength, flexibility) which can be tailored for various functionalities on flat surfaces and is recognized as a biomimetic nanomaterial for a number of biomedical applications [11]. The electroactive scaffolds, such as carbon-based nanostructured scaffolds, have emerged as promising candidate materials for tissue engineering applications. These electro active materials enhance the osteoconductivity and thus improve the proliferation and differentiation of cells.

Graphene based nanomaterials were utilized in larger number of projects especially in bone regeneration [12][13] and also in skin regeneration[14] to a small extent, in nerve cell differentiation and also for cardiac tissue or musculoskeletal engineering [15]. Graphene provides a promising biocompatible scaffold that does not hamper the proliferation of human mesenchymal stem cells (hMSCs) and accelerates their specific differentiation into bone cells due to its osteoconductive property. Chiara Gardin and her coworkers reported about the use of graphene in regenerative medicine and it mainly focussed on stem cells and neuronal differentiation [16].

Based on the literature survey, the present study focuses mainly on scaffold design for the evaluation of growth of the seeded scaffolds using graphene-reinforced-chitosan/hydroxyapatite composite. A special design of the scaffold was developed to optimize the seeding efficiency and to observe the cell proliferation into the inner structure. The objective of design was the maximization of the surface, facilitation of the seeding process, to enhance cell adhesion and good supply of nutrients and growth factors to the interior of the of graphene-reinforced-chitosan/hydroxyapatite composite.

2. Experimental Section

2.1. Materials

Chitosan was purchased from Indian Sea Foods, Cochin, Kerala which is 92% deacetylated. Graphene was purchased from Nano beach and hydroxyapatite (HAP) was obtained from Leochem, Bangalore. The solvent glacial acetic acid was procurred from SD Fine Chemicals. All chemicals used in the present study were of analytical grade.

2.2. Preparation of Graphene-reinforced-Chitosan/Hydroxyapatite composite

About 1g of chitosan were weighed and dissolved in 2% acetic acid. Simultaneously 0.5g of hydroxyapatite (HAP) and 0.2g of graphene were weighed and dispersed in minimum amount of de-ionized water. Followed by this process, the dispersed HAP and graphene were slowly added to the chitosan suspension. In order to perform the cross linking process, about 7 ml of glutaraldehyde is added to the above solution mixture and this mixture was then stirred well for a period of 2 hours using magnetic stirrer. After this stirring process was over, the contents were then transferred to the petriplates and allowed to dry in air [17]. Subsequently, the prepared materials were kept in the desiccators for further experimental evaluation.

2.3. Characterization

2.3.1. FT-IR studies

The structure of the prepared glutaraldehyde cross linked graphene-reinforced-chitosan/hydroxyapatite composite was characterized by

FTIR spectroscopic technique using potassium bromide pellets. Measurements were performed on these samples with a Perkin Elmer 200 FTIR Spectrophotometer. The FTIR spectra were obtained in the wave number range from 4000 to 450 cm^{-1} during 64 scans with 2 cm^{-1} resolution.

2.3.2. XRD studies

The X-ray diffraction patterns of the prepared glutaraldehyde cross linked graphene-reinforced-chitosan/hydroxyapatite composite was tested by an X-ray scattering SHIMADUZ XD Diffractometer using Ni filter Cu Kα radiation source (λ=0.154nm), set a scan rate = 10/min, using a voltage of 40kV and a current of 30 mA.

2.3.3. SEM studies

The surface morphology and cross section morphology of the prepared glutaraldehyde cross linked graphene-reinforced-chitosan/hydroxyapatite composite were observed with scanning electron microscopy to verify the compatibility of the polymers. For the analysis, the composite were cut into pieces of various sizes and wiped with a thin gold – palladium layer by a sputter coater unit (VG – microtech, UCK field, UK) and the cross section topography was analysed with a Cambridge stereoscan 440 scanning electron microscope (SEM, Leica, Cambridge, UK).

***2.4. In vitro* assays**

***2.4.1.* MTT assay**

The MTT assay was used for measurement of the cell viability and proliferation. The preosteoblast MC3T3-E1 cells (2×10^4 cells per well) were seeded in 96-well plates. The Graphene-reinforced-chitosan/hydroxyapatite composite was added with final concentrations of 200, 400, 600, 800 and 1000 μg mL^{-1}. Cells without prepared sample treatment were used as control and the wells without cells were used as blanks. Cells were cultured for 24 hrs and 48 hrs and after treatment,100 μL/well of the MTT solution (0.5 mg/mL in cell culture medium without phenol red) is added, then incubated at 37 °C for 3 h. At the end of 3 h incubation, the MTT medium was discarded and 100 μL of DMSO was added to dissolve the purple formazan by shaking for a period of 10 min at room temperature. After incubation and adding DMSO, the viable cells were detected by measuring the absorbance of the cell lysates at 550 nm (A_{550}) for MTT assay respectively. The cell viability was expressed by optical density (OD) of A_{550} of cells cultured on the scaffolds [18] in a Bio-Rad 550 microplate reader.

The cell viability (%) was calculated using the following formula

$$(OD_{treated} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100.$$

***2.4.2.* S-Alizarin Red assay**

The MC3T3-E1 cells were seeded in 24-well tissue culture plates and cultured overnight at 37°C in a 5% CO_2 humidified incubator. The medium was then changed to differentiation medium containing 10 mmol/L –glycerophosphate (BBI) and 50 g/mL ascorbic acid. In the presence of different concentration of samples for 8 days, and then transferred to a medium containing 3.0 mmol/L NaH_2PO_4 for an additional 8 and 12 days, the formation of mineralized matrix nodules was determined by alizarin red S (Sigma) staining. Briefly, the cells were fixed in 70% ethanol for 1 h at room temperature. The fixed cells were washed with D-Hank's and stained with 1% (w/v) alizarin red S at pH 4.2 for 30 min at room temperature. Quantitative analysis of alizarin red S staining was performed by elution with 10% (w/v) cetyl pyridium chloride (Sigma) for 10 min at room temperature and the absorbance was measured at 570 nm. Results were expressed as the mineralized promotion rate (%) and the image was obtained using phase contrast microscope (Nikon, Tokyo).

***2.4.3.* Alkaline phosphatase activity**

In cell toxicity assay, for estimation of alkaline phosphatase (ALP) activity, the cells were seeded on the scaffolds and after the adequate incubation on the respective days, the cells were rinsed with PBS buffer, homogenized in 25 mM carbonate buffer (pH = 10.3) containing 0.1% Triton X-100. Next the ALP activity in the pretreated cells was measured by incubating for 30 min at 37°C in 250 mM carbonate buffer containing 1.5 mM $MgCl_2$ and 15 mM para-nitro phenyl phosphate (p-NPP). In the presence of ALP, p- NPP is transformed to p-nitro phenol and inorganic phosphate and the ALP activity of the prepared scaffolds was determined from the absorbance at 405 nm spectrophotometer (Systronics, India).

***2.4.4.* Lactate dehydrogenase release assay**

Lactate dehydrogenase (LDH) is an abundant intracellular enzyme, which is mainly used for monitoring cell death, disruption and its release in tissue culture supernatants acts as a standard indicator of lytic cell death. LDH in cell-free supernatants diluted 10-fold with PBS was quantified colorimetrically with a coupled enzymatic assay which results in the conversion of a

tetrazolium salt (INT) to a red formazan product, using a kit (Roche applied sciences). A commercially available LDH-activity quantification kit was used to quantify the LDH enzyme activity in cell lysate. After 48 h of incubation the prepared graphene-reinforced-chitosan/hydroxyapatite (GCH) composite scaffold were washed with PBS and lysis buffer was added to the scaffold (1 mL/scaffold) and incubated for 30 min.

Lysates were centrifuged at 2000 rpm for 5 min and 140 µL from the supernatant solutions were transferred to 1 mL cuvettes. 60 mL of master-mix (equal amounts of substrate solution, dye solution, and cofactor solution for LDH assay) were added to each cuvette and incubated for 30 min. The reaction was stopped with 300 µL of 1N HCl, and 500 µL of water were added. The absorbance of each solution was measured at 490 and 690 nm using a UV-Vis spectrophotometer (Specord 40, Analytik Jena AG, Jena, Germany).

3. Results and Discussion

3.1. FT-IR Spectral studies

FTIR spectrum of pure chitosan and glutaraldehyde crosslinked graphene reinforced chitosan/hydroxyapatite composite are shown in **Figure-1a and 1b.** Broad absorption band due to the intermolecular hydrogen bonded OH stretching vibration of chitosan **(Fig-1a)** and graphene reinforced chitosan/hydroxyapatite composite **(Fig-1b)** is observed at around 3400 cm^{-1}. The appearance of the less intense absorption band at around 3400 cm^{-1} in case of composite **(Fig-1b)** indicates that the OH group of the composite was involved in weaker hydrogen bonding. Chitosan showed its C-H, C=C and C-C stretching vibrations at 2926 cm^{-1}, 1435 cm^{-1},1382 cm^{-1} respectively.

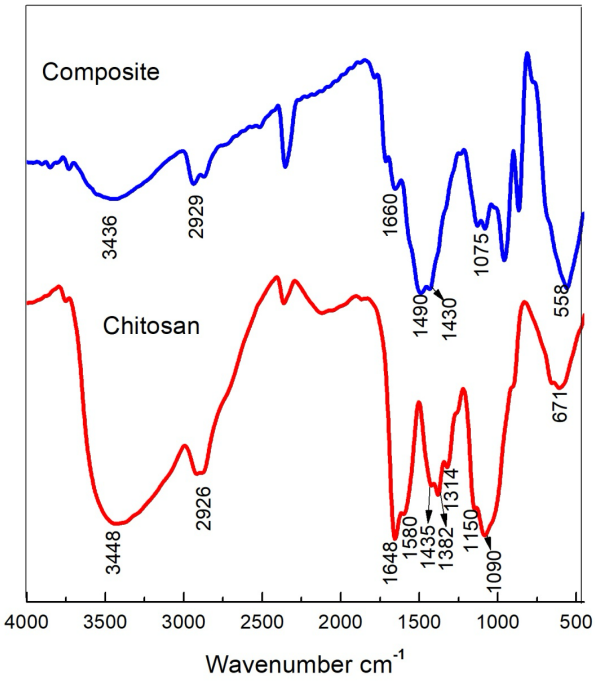


Figure-1: FTIR Spectrum of (a) pure chitosan (b) graphene reinforced chitosan/hydroxyapatite composite

For pure chitosan, the C=O stretching of NHCO (amide I) and N-H bending of NH_2 (amide II) was found to be observed at 1648 cm^{-1} and 1580 cm^{-1} and a similar type of absorption band was obtained at 1660 cm^{-1} for glutaraldehyde crosslinked graphene reinforced chitosan/hydroxyapatite composite **(Fig-1b).** The absorption band observed at 1660 cm^{-1} corresponding to C=N stretching suggests that the glutaraldehyde gets cross linked effectively in graphene reinforced chitosan/hydroxyapatite composite and also in addition, it was observed that the C-O stretching vibrational bands of chitosan was overlapped with phosphate bands of HAP at 1075 cm^{-1} [19][20]. The strong interaction which had taken place effectively between glutaraldehyde, chitosan, graphene and hydroxyapatite during composite formation was evidenced from the shifting of above absorption bands to lower wave numbers [21][22][23].

3.2. XRD studies

X-ray diffraction (XRD) is a versatile, non-destructive analytical technique which provides the detail information about the lattice parameter, lattice defects, lattice strain, chemical composition, crystallite size (in case of nano particles) and the type of molecular bond of crystalline phase. **Figure-2a and 2b** shows the X-ray diffractogram of pure chitosan and the prepared glutaraldehyde crosslinked graphene reinforced chitosan/hydroxyapatite composite. The X-ray

diffractogram of pure chitosan presented in **Figure-2a** shows two characteristic peaks at 10° (Crystalline structure) and 20° (amorphous structure). In glutaraldehyde crosslinked graphene reinforced chitosan/hydroxyapatite composite **(Figure-2b)** both the peaks are shifted to 11.95° and 26.58°. Due to the strong interaction of chitosan with hydroxyapatite and graphene, the broadness of the first peak was decreased and the second peak was shifted and overlapped with graphene (26°) and HAP (26°) peak [24]. In addition, the X-ray diffractogram of glutaraldehyde crosslinked graphene reinforced chitosan/hydroxyapatite composite presented in **Figure-2b** shows (002) diffraction at 26° and the broadened peak at around 30° which are the diffractions of (211), (300), (202) [25][26]. The observed broadness of a peak indicates the partial demineralization of HAP and low crystalinity of the glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite [27].

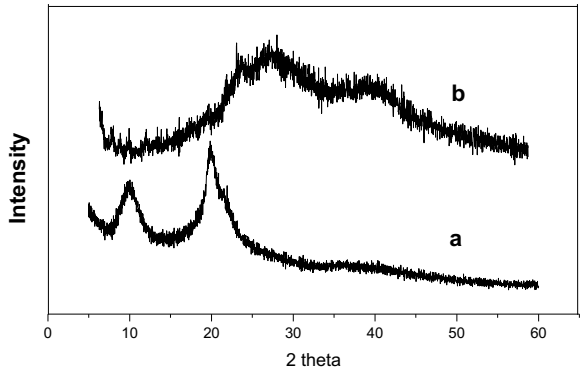


Figure-2: X-ray diffractogram of (a) pure chitosan and (b) glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite

3.3. *In vitro* cytotoxicity and cell proliferation assay

The present project work involves determining MC3T3-E1 osteoblast cell line response to the prepared glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds *in vitro.*

3.3.1. MTT Assay

The MTT assay is a sensitive, quantitative and reliable colorimetric assay that measures cell viability, cell proliferation and cell activation and is a quick and effective method for testing mitochondrial activity, which correlates quite well with cell proliferation. This assay mainly reports the combined effects of proliferation (number of viable cells) and cellular metabolic activity [28] [29]. The cytotoxicity effects and cell proliferation of respective graphene reinforced chitosan/hydroxyapatite composite scaffolds were investigated through MTT assay and the observed results were represented in **Figure-3**.

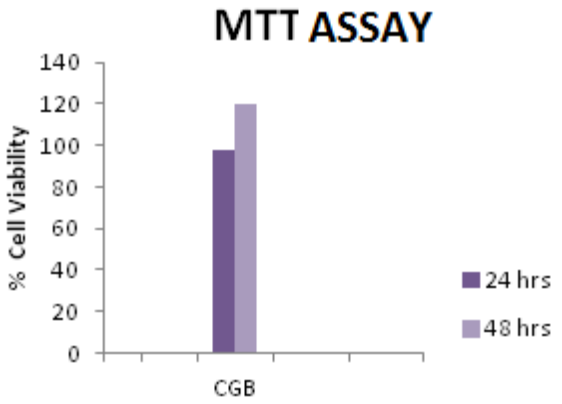


Figure-3: Calculated percentage cell viability of the prepared glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds using MTT assay *(CGB: chitosan/ hydroxyapatite/graphene composite)*

The interaction between cells and the prepared composite scaffold material was potentially stimulated by the biocompatible graphene material which may result in an ideal condition for cell adhesion and regeneration [30]. Results showed that the cell survival was only slightly dependent on the contact time between the growth medium and the material, while it was highly dependent on the extract concentration. The above observed results showed that all the graphene reinforced chitosan/hydroxyapatite composite scaffolds prepared in various ratios with and without the cross linking agent glutaraldehyde were not found to be cytotoxic in MC3T3-E1 cell line. The percentage of viable cells decreased, with a minimum of 70% survival, which still correlates to the non-toxic category [31] and the incubation for 48 hours yielded better cell proliferation behaviour, with more than 90% cell viability. This result agrees very well with the good biocompatibility properties of graphene reinforced chitosan/hydroxyapatite composite scaffolds prepared in various ratios with and without the cross linking agent glutaraldehyde.

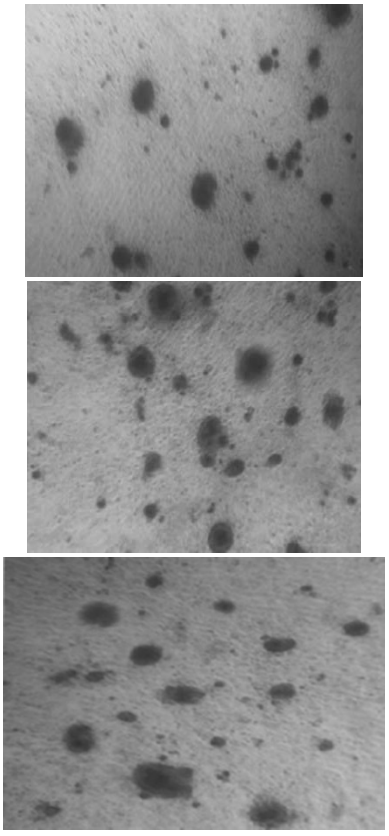
3.3.2. Biomineralization studies

In real time, the information about the calcification kinetics and mechanism was predicted from the description of the biomineralization process and this

could also describe the evolution of the properties of the biomaterial [32]. In this phenomenon proteins such as osteocalcin (OC), bone sialoprotein (BSP), and osteopontin (OPN) are expressed and once mineralization is completed, calcium deposition can be visualized using adequate staining methods.

3.3.2.1. S-Alizarin Red Assay

Alizarin red assay was used to stain the calcium deposits in the extracellular matrix of the pre-osteoblasts [33]. In order to assess the extent of matrix mineralization by osteoblast cells differentiated on composites (with and without cells) during 21 days of incubation the alizarin red assay was performed in this study. The observed results were represented in **Figure-4** and **Figure-5.**



Control **2.5 µg/mL**

20 µg/mL

Figure-4: ARS staining images of MC3T3-E1 cells treated with glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds

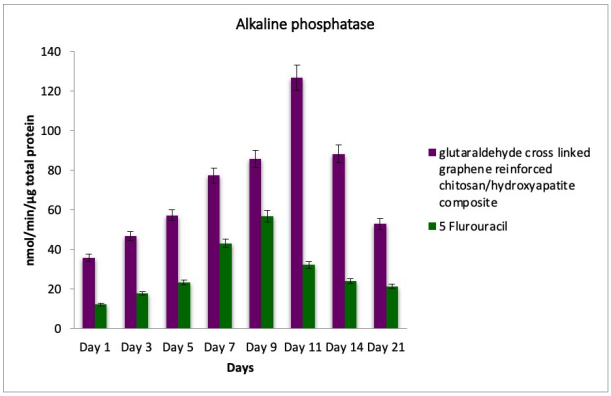


Figure5: ALP activity of seeded glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds

To further explore the synergistic effects of and also to study the deposition of calcium phosphate, considered as a marker for bone regeneration, ARS staining has been used in this study (**Figure-4** and **Figure-5**). As presented in **Figure-5**, the extracellular calcium deposition stained with ARS (red color) was clearly observed in MC3T3-E1 cells treated with graphene reinforced chitosan/hydroxyapatite composites. These above observed results could be due to the fact that the composites can effectively adsorb serum proteins in culture media, results in the promoted and accelerated osteogenic differentiation [34][35]. Therefore, in conclusion, our results suggested that the graphene reinforced chitosan/hydroxyapatite composites can efficiently increase the osteogenic differentiation by the synergistic effects of chitosan, graphene and hydroxyapatite and hence it can be a promising candidate as bone fillers and bone grafts for bone tissue regeneration.

3.3.2.2. Alkaline phosphatase assay (ALP)

The osteogenic differentiation of MC3T3-E1 cells treated with glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composites was evaluated by assessing ALP activity and mineralization of calcium. The alkaline phosphatase activity (ALP) is considered to be an important marker of the differentiation of osteoblast cells at a relatively early bone forming stage. In other words we can say that the ALP is a well-established marker for early osteogenesis, and is also strongly related to the mineralization ability of osteoblasts. In this study, the levels of alkaline phosphatase activity which can be expressed from the pre-osteoblastic cells cultured on the graphene reinforced

chitosan/hydroxyapatite composite scaffold surfaces has been measured with the help of enzymatic activity. ALP assay of (MC3T3-E1) seeded scaffolds was done to evaluate the osteogenic potential of the graphene reinforced chitosan/hydroxyapatite composite scaffolds and the obtained result was shown in **Figure-**6.

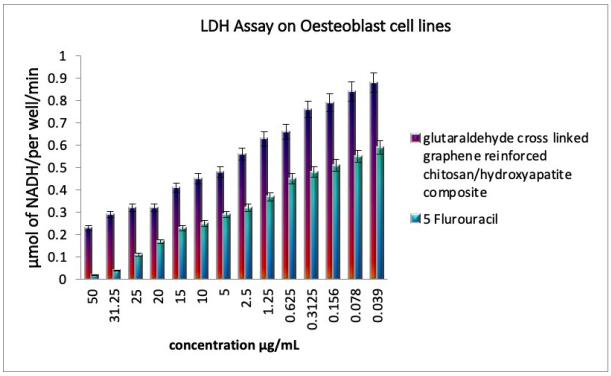


Figure-6: LDH assay of seeded glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds

The above observed results suggest that cells reached a stage of maturation as the ALP was increased in early stages and ultimately decreased by the initiation of calcification. This rapid proliferation was followed by a more moderate increase in cell number for all conditions to day 14 and this indicate that MC3T3-E1 cells responded to this graphene reinforced chitosan/hydroxyapatite composite material by stimulating the proliferative activity.

From **Figure-6**, it was also evident that the cells did not show appreciable increase in ALP activity after 11 days of incubation. However, the ALP activity of cells treated with the prepared glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds was significantly increased after seven days of incubation and these findings indicated that the graphene reinforced chitosan/hydroxyapatite composites can effectively increase the ALP activity by the synergistic effects of graphene, chitosan and hydroxyapatite. On the other hand, interestingly, it was evident that the graphene reinforced chitosan/hydroxyapatite composites increased the ALP activity without hindering the proliferation rate of cells and therefore, this glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite might have biocompatibility and an enhancing effect on the osteogenic differentiation [36].

3.3.2.3. LDH Assay

Based on the conversion of pyruvate to L-lactate and by monitoring the oxidation of NADH, the Lactate dehydrogenase (LDH) activity has been measured. The Lactate dehydrogenase is an enzyme found in all living cells and this LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium [37]. This assay mainly helps in determining the amount of lactate dehydrogenase (LDH) that is leaking through a damaged cell membrane [38] and generally the LDH which is released during tissue damage catalyses the conversion of lactate into pyruvic acid. In this study, the MC3T3-E1 cells were used for the LDH assay of mineralization. Results were expressed as the mineralized promotion rate and the image which was obtained using phase contrast microscope was represented in.

The prepared biocomposite scaffolds are known to be involved in bone metabolism. LDH assay showed that the cells were significantly multiplied, growing exponentially over the entire period of contact [39]. Due to the conversion of accumulated pyruvate into lactate, transported through muscles to liver and regenerated glucose and glycogen, the LDH activity has been increased. As a result of this study, the composites were proved to induce cell proliferation. From the observed tissue culture studies using osteoblast MC3T3-E1 cell line it was evident that the glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite scaffolds have promising clinical potential as cell scaffold in tissue engineering applications.

4. Conclusion

The present project work thus enabled us the efficient usage of exoskeleton of sea animal (source of chitosan) as one of the component of ternary composite and from the observed results it was found that the prepared glutaraldehyde cross linked graphene reinforced chitosan/hydroxyapatite composite was to be a very effective biomedical agent. From the tissue culture studies using osteoblast MC3T3-E1 cell line it was identified that the fabricated novel composites have promising clinical potential as bone defect filler in regenerative medicine or as a cell scaffold in tissue engineering applications and in the near future, this work can be extrapolated in other biomedical applications with expected success.

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