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Nano bioactive HAP-nano bioresorbable β-TCP-PEG composite scaffolds and their biochemical activity for implant applications

K. Kala[a], M. Sundara Ganeasan[b], V. Balasubramani[b], J. Manovasuki[b], K. Valivittan[a], T.M. Sridhar[b] and M.R. Kuppusamy[c,*]

[a]*Department of Biotechnology, St. Peter's University, Chennai 600054, India*
[b]*Department of Analytical Chemistry, University of Madras, Guindy Campus, Chennai-600025, India*
[c]*Department of Chemistry, RV Government Arts College, Chennai 603001, India*

Recent efforts towards the treatment of bone defects and diseases focus on the development of bone scaffolds. Bioceramics provide strength, osteoconductivity and also imparts flexibility and resorbability. In this study, the biodegradable composites were fabricated using bioactive nano Hydroxyapatite (n-HAP) and bioresorbable nano β-Tricalcium phosphate (n-β-TCP) taken in 1:1 proportion. The nano composite scaffolds were synthesized using PEG (Poly Ethylene Glycol) by wet precipitation method. XRD (X-ray diffraction) confirms the presence of crystalline structure of n-HAP and n-β-TCP within the lattice. FESEM (Field Emission Scanning Electron Microscopy) and EDS (Energy Dispersion X-ray Spectroscopy) confirms the micro porous nature and the phase purity of the composite. Further, biochemical studies were carried out using MG-63 Osteoblast cell line to evaluate their sustainability after implantation. The viability of the cells and proliferation rate is evaluated using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with different concentration and different incubation period. The ALP (Alkaline Phosphotase) test reveals that the composite favours bone regeneration through apatite layer formation. Further studies were carried out to explore the DPPH (1,1-diphenyl-2-picrylhydrazyl) activity on the composites and results reveals that the composite have an ability to trap the free radicals in the biological surroundings. The antimicrobial studies indicate that the composites shows no major inhibitory effects towards the most common bone affecting bacteria *Staphylococcus aureus*. The studies indicate that the concentration range of the composite is ideal for bone growth and can be used as substituents in the scaffold synthesis for normal and cancerous patients.

Keywords: n-HAP, n-β-TCP, Poly ethylene glycol, composite, MTT assay, DPPH assay, ALP activity

Introduction

Treatment of bone diseases and multiple fractures is a great challenge and tissue engineering offers a solution to patch-up and heal the damaged hard tissue by using biomaterials. Among several biomaterials that are available biodegradable polymers have attracted researchers to study its potential as an alternative to metallic implants [1]. These biodegradable polymers are commonly used as the biological substitutes for treatment of damaged bone, drug delivery, repair of tissues and organs of the human body [2]. Biocompatibility is the primary property that any biomaterial should possess during the selection of substituted biomaterials in order to provide a platform for cell growth and proliferation. These biomaterials should actively interact with the cell and activate the osseointegration process leading to new bone deposition [3]. In recent studies, considerable attention has been given to HAP based composite biomaterials. Since, it has specific properties such as biocompatibility, biodegradability, an intrinsic antibacterial nature and the ability to mold into various forms or geometries which is suitable for cell growth and osteoconduction.

Bioactive ceramics (n-HAP) are widely used in scaffold preparation. n-HAP on implantation in the human body has the ability to react with physiological fluids. This bioceramic on further contact with the fluids and tissues for a few days to weeks leads to the formation of new bone with n-HAP layers [4, 5].

The composite materials on implantation provokes immune response in the body which elicits a cascade mechanism in the biological environment. This makes it necessary to evaluate the biological properties of the composites along with their characterization to prove their biological efficacy. This would help us to determine the longevity of the implant [6]. The improper choice of the substituent materials during scaffold preperation limits its biological application [7]. Even the minute level of injury during restoration period affects the performance of the scaffold. This may be due to the leakage of the toxic substances from the scaffold into the biological fluids. This may induce inflammation at the implant site [8]. To avoid such a situation, it is

*Corresponding author:
Tel : +91-44-2743 1257
E-mail: kuppusmr@gmail.com

highly essential to carry out the in vitro biochemical evaluation before the clinical studies. Hence, in vitro biochemical screening is the best choice to evaluate the biocompatibility and toxicity [9].

In bone tissue engineering, the biological behaviour of the composites namely its osteoconductivity, osteoinductivity and degradability would help us to analyse the ability of the implant to induce bone growth in the biological surroundings. This could be accomplished in the lab by carrying out in vitro testing with an appropriate cell line to study the mechanism of interaction. In humans, when the composites are implanted the ions present in the body fluids surround the surface of the materials forming the ionic layers. This is followed by the interaction with protein that would support us to understand the osteoinduction process [10]. In case of osteoconduction, the bone tissue grows around the internal pores of the scaffolds which would aid the conduction process. The evaluation of osteoconduction property and osteoinduction property helps us to assess the behavior of the scaffold after implantation in the biological system. This osteoconductivity can be measured by taking into account the bone coverage area using optical or FESEM analysis [11]. Biodegradability is the another important property by which the materials get degraded once inserted inside the body. The biodegradability of the biomaterials would determine the osteoconductive nature of the surrounding cells that are in contact with the physiological environment [12].

In the view of osteoconduction and osteointegration requirement nano Hydroxyapatite which is a chief mineral component of bone matrix was selected. It is the most appealing inorganic constituents that is widely used for the bone regenerative medicine. n-β-TCP is a bioactive material that posses good biocompatibility and biodegradability. The degradable property of n-β-TCP ensures the release of elements such as calcium and phosphorus at the site of implantation. n-β-TCP has limited direct clinical application due to its brittle nature and difficulty of obtaining various implant shapes. This problem can be overcome by using it as a one of the materials in composites [13]. Bioceramic composites are prepared in nano dimensions by mixing one or more phases of ceramics with mainly polymers or other biocompatible materials. The typical microstructures of nanoceramic composites result in exceptional properties (biological, mechanical, electrical and electronic etc.). These composites can be used as bulk materials for structural applications or can be developed as coatings on different substrates for various applications [14]. The composites can be prepared with Polyethylene Glycol (PEG) which acts as an important additive component and provides hydrophilicity to the composite [15].

Nano form of biomaterials is preferable due to its small grain size and large surface area that promotes the interfacial interaction with bone. Moreover, nano ceramics are widely used in scaffold preparation as they pocess controlled rate of degradation which could exactly match with new bone growth rate. This can be acheived by controlling the crystalline grain size of the nano ceramics and thus improve the bone graft healing without any complication [34]. Further, researchers have reported that nano HAP can suppress the cancer cells without affecting the normal cells [30].

Nano Hydroxyapatite reinforced polyphenylene sulfide biocomposites were reported to have good cell proliferation with MG-63 cells. It shows the osteogenic differentiation with higher ALP activity [16]. In designing the synthetic scaffolds for bone tissue engineering the materials can be prepared in various physical forms such as powders, pastes or granules and porous scaffolds [3]. Scaffolds should be resorbable with time in the human body with gradual replacement by newly formed bone tissue and should ensure a high protein-adsorption capacity. n-HAP is stable against dissolution in body fluids, whereas n-β-TCP has a much higher resorption rate compared to that of n-HAP. One can easily control the resorption rate by varying the ratio of n-HAP/n-β-TCP composites [17, 18]. Selection of polymer is critical for preparing the composite and it should be evaluated for its toxic nature. The systemic screening of scaffolds by invitro method is reported in literature [31]. Invitro cytotoxicity and biochemical tests are most ideal, reliable, simple and help us to evaluate the toxic nature of the scaffolds. These tests aids in screening the bone deposition properties such as osteoconduction, cell viability, cell proliferation and bone regeneration. These tests are carried out using the osteoblast cell line.

Cell viability and cell proliferation can be evaluated using MTT assay. In this study the viable nature of the cell is confirmed by the active presence of mitocondrial dehydrogenase enzyme. This enzyme inturn reduces MTT and promotes cell proliferation. The reduction of MTT is confirmed by the colour change. The cell proliferation is further confirmed using the growth curve [19]. Implant-material related infection is common and this could arise during surgery. This infection may affect not only the surgical sites but also other organs of the body through the migration of pathological microorganisms during blood circulation [20]. The presence of bacterial infection at the implanted site often shows a delayed wound healing and may sometimes result in revision surgery.

The main aim of this study is to prepare n-HAP/ n-β-TCP/PEG nano composite using different ratios of the bioceramics and polymer. This would be followed by the evaluation of the composite for biochemical and biological behaviour to identify the suitable ratio for the scaffold preparation. Since both n-HAP & n-β-TCP are the constituents of bone. In order to impart the hydrophilicity and antifouling property the PEG added the composite. These composites were evaluated using invitro studies which includes cell viability, cell proliferation, antimicrobial activity, alkaline phosphatase

activity and antioxidant activity. The scaffolds prepared using 1:1:5 ratio of n-HAP/n-β-TCP/PEG was found to show good cell attachment with least toxicity.

Materials and Methods

Materials

Calcium Nitrate Tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$], Diammonium hydrogen Phosphate [$(NH_4)_2HPO_4$], Poly Ethylene Glycol 6000 [PEG], Conc. Ammonia, Ethanol were purchased from SRL and Finar. All the chemicals were of analytical grade and used further without purification.

Synthesis of n-HAP/ n-β-TCP/ PEG composites

The n-HAP and n-β-TCP was synthesized based on the earlier established procedure in the lab [21]. The powders were dried and sintered at 800 °C for 2 h in air atmosphere using a muffle furnace. PEG solution was prepared separately with deionized water by using a magnetic stirrer for 2~3 h to obtain a homogenious solution. Synthesized n-HAP/n-β-TCP was added into the solution to make a n-HAP/n-β-TCP/PEG by adding different concentrations of the powders (0.5:1:5,1:1:5 and 2:1:5) mixture. The polymer n-HAP/n-β-TCP mixture was stirred for 10 hours with stirrer until n-HAP/n-β-TCP was completely dispersed in the solution and then this mixture was dried at 100 °C in an hot air oven.

Material Characterization studies of n-HAP/n-β-TCP/PEG composites

The synthesized n-HAP/n-β-TCP/PEG composite materials were characterized using different analytical techniques. The crystal structural properties of n-HAP/ n-β-TCP/PEG composites were characterized using X-ray diffractometer (XRD) (model, Rigaku Ultima IV) with Cu Kα (λ = 0.154 nm) radiation and 2θ scanning range of 10-80°. The morphology of n-HAP/n-β-TCP/ PEG were analyzed using Field Emission Scanning Electron Microscope (FESEM) (model, Carl Zesis Supra 55).

Biochemical study of n-HAP/n-β-TCP/PEG composites

Cytotoxicity assay

The assay was performed based on the Mosmann, 1983 procedure [22]. For this assay MG-63 cell line was obtained from NCCS, Pune. Cells were plated in 24-well plates (Nucleon, Roskilde, Denmark) and incubated at 37 °C with 5% CO_2 under humid conditions. After the cell reached the confluence, the prepared samples at different concentration was added and incubated for 24 h, 48 h and 72 h. After incubation, the samples of each concentration were removed from the wells and washed with phosphate-buffered saline (pH 7.4) or DMEM (Dulbecco's modified eagle medium) without serum 100 µL/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation, 1ml of DMSO (Dimethyl sulfoxide) was added in all the wells. The absorbance at 570 nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The percentage of cell viability was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

The time period is extended to 24 h, 48 h and 72 h in order to determine the cell proliferation rate. Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the cell viability assessments.

Antibacterial activity

Antibacterial activity is done using Kirby-Bauer disk diffusion susceptibility test (2009). [25] *Staphylococcus aureus,* a common bone affecting gram positive bacteria is used. The pathogenic organism is made to grow on Mueller-Hinton agar (MHA) in the presence of anti-microbial impregnated filter paper disks. The growth of the organism around the disk is an indirect measure of the compound ability against the pathogen. Antibacterial activity of the composites was determined by disc diffusion method on MHA medium. The medium is then poured into the petri dishes. The inoculum was spread over the solidified solid plate with the help of sterile swab moistened with the bacterial suspension. Ampicillin (20 µL/disc) is taken as positive control. The disc was placed in MHA plates consisting of 20 µl of sample (Concentration: 1,000 µg, 750 µg and 500 µg). The plates were incubated at 37 °C for 24 h. The diameter of zone of inhibition gives the antibacterial activity.

DPPH (2,2 diphenyl 1 picrylhydrazil) Assay

The DPPH assay was performed based on Molyneux, 2004 method [24]. The main characteristics of an antioxidant are its ability to trap free radicals. Highly reactive free radicals like ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) found in biological systems form a wide variety of sources. A rapid and simple inexpensive method to measure the antioxidant supply of a sample involves the use of free radical 1,1 diphenyl 2 picryl hydrazyl (DPPH). Three test tubes were labeled as blank, standard and test. First 3.7 mL of absolute methanol (Sigma Aldrich) is added to all the 3 test tubes. This was followed by addition of 100 µL of standard Butylated hydroxy toluene (Sigma Aldrich) to the standard test tube. 100 µL of composite mixture is added to the test tube labeled as test. 200 µL

of fresh DPPH solution is added to all the test tubes including blank. The test tubes are allowed to incubate at room temperature under dark conditions and the readings were taken at 1, 5 and 30 min respectively. The results are expressed as radical scavenging activity.

The molecule of DPPH were characterized as a stable free radical by delocalization of electrons. This forms a deep violet colour, characterized by the absorption band in methanol solution centered at 520 nm. The antioxidant property is confirmed by the change from violet to yellow colour on addition of DPPH. This change of colour is occurs due to the reduction of DPPH by picryl group.

• The percentage of antioxidant activity can be measured by,

$$\% \text{ Antioxidant Activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Alkaline Phosphatase (ALP) assay

This assay was performed based on George N.Bowers, Jr., and Robert B. McComb, 1966 procedure [23]. ALP at an alkaline pH hydrolyses p-Nitrophenyl phosphatase to form p-Nitrophenol and Phosphatase. The rate of formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

$$\text{p-Nitrophenyl phosphotase} \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{Phosphotase}$$

The buffer selected for this work is 2-amino-2-methyl-l-propanol (2A2M1P) (Sigma Aldrich). The substrate, p-nitro phenyl phosphate as the disodium salt, tetra hydrate (Sigma Aldrich). To evaluate the ALP activity, the bio-composites were incubated in 0.5 mg/ml p-nitro phenol phosphate in glycine buffer solution every five minutes a 90 µL aliquot of the solution was removed and placed in 96 well plates containing 10 µL of a stop solution. 0.1 M NaOH (Sodium hydroxide) (Aldrich chemical) and 0.1 M EDTA (Ethylene diamine tetra acetic acid) (Aldrich chemical). The absorbance was measured at 405 nm. The enzyme activity was calculated using formula.

$$\text{Activity} = A_{405}(V_{\text{reaction}}/V_{\text{aliquot}})/\beta$$

A_{405}: Absorbance at 405, V_{reaction}: Volume of reaction, V_{aliquot}: Volume of aliquot, β: Extinction coefficient for para nitro phenol, λ -18.5 at 405 nm.

ALP Activity in micromol/min (U/L)
= ΔA/min./18.75 × Test volume/Serum Volume × 1/ Temperature factor

Where, 18.75 is mill molar absorptivity, Temperature at 30°C is consider as temperature factor is 1 ALP in U/L can be calculated as,

$$\text{ALP Activity in U/L} = \Delta A/\text{min.} \times 2754$$

The overall summary of n-HAP/n-β-TCP/PEG composite is presented in Fig. 1 along with the biochemical studies involving – (i) cell proliferation (ii) antimicrobial activity (iii) Free radical trapping (antioxidant) and (iv) cell regeneration (ALP activity) is given in Fig. 1.

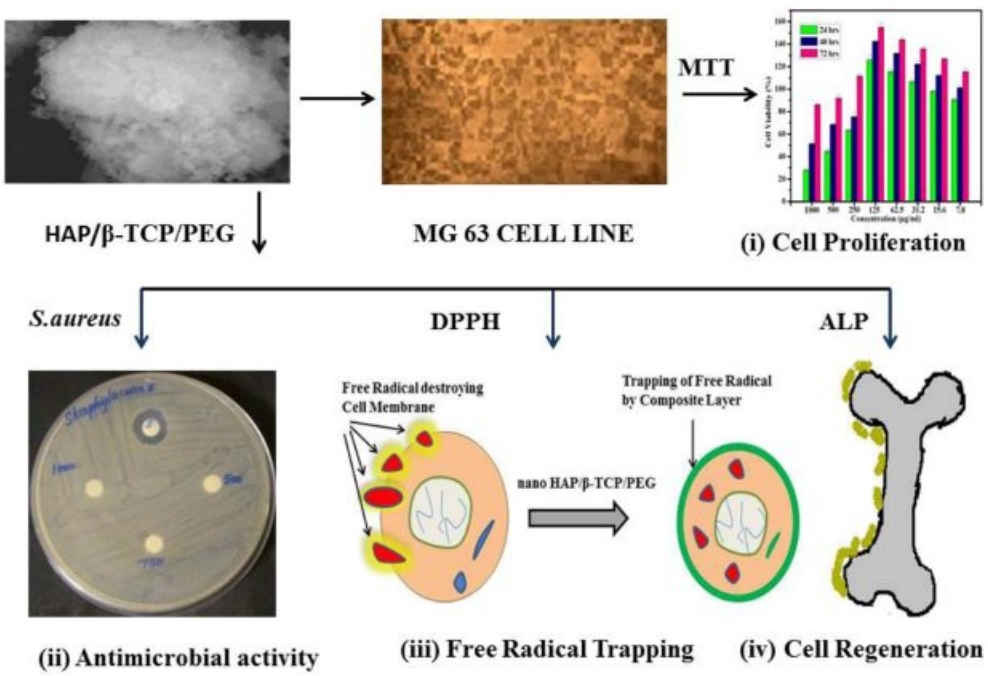

Fig. 1. Schematic representation of n-HAP/n-β-TCP/PEG composite osteoblast cells and bacterial cell culture.

Results and Discussion

Bioceramics such as n-HAP, n-β-TCP are commonly used in new bone generation. But, in order to obtain the specific and desired properties such us porosity, bioactivity, bio resorbability and biocompatibility, researchers have prepared composites in which different biomaterials can be combined based on the specific property depending on the nature of their application [26]. In this concept, the current work focus on the choice of the material composition which includes Nano- Hydroxyapatite, Nano-β-Tricalcium phosphate, and Polyethylene Glycol which posses the basic properties such as bioactivity, bioresorbability and hydrophilicity. The present work focuses on the bone minerlization in scaffold materials, ALP activity which induces the bone mineralization [27] was studied with the prepared scaffolds. The normal value of ALP is 20-140 units per litre. If there is an increase or decrease in the normal values it is termed as abnormality which leads to discomfort to the patient [28]. Since, ALP is needed for the apatite formation the level should be monitored and the values should not cross the maximum level. The report suggests that the ALP can induce hydroxyapatite mineralization [29]. The changes in ALP values of the composite would affect the formation of apatite layers of new bone. Hence, n-HAP can be used in different proportion to maintain the normal ALP activity. The study was further extended to select the proportion of the components present in the composite. To fix the concentration, three different proportions were taken in the composite mixture of n-HAP/n-β-TCP/PEG . The proportion includes 1:1:5, 0.5:1:5, 2:1:5 which has different concentration of n-HAP in n-HAP/n-β-TCP/PEG. The above proportions were tried for different concentration to identify the concentration which can be suitable for further studies. The changes in optical activity with changes in the ratios of n-HAP/n-β-TCP/PEG is given in Fig. 2.

The ALP activity for the composite with n-HAP/n-β-TCP/PEG in 0.5:1:5 ratio shows the ALP activity to be about 89.964. Different ratios of samples were prepared and tested to obtain the highest ALP activity. As the ratio of n-HAP was increased from 0.5 to 2 the 2:1:5 composite shows an ALP activity of about 144.126 which is normally higher than that of the optimum level that can be found in the blood. For a n-HAP ratio of 1:1:5 the ALP was found to be 110.321. Hence, the current study indicates that the n-HAP/n-β-TCP/PEG scaffold with 1:1:5 ratio has the optimum and desired ALP activity and can be considered for further intensive investigations.

Characterization study of n-HAP/n-β-TCP/PEG composites

XRD analysis

Fig. 3 shows the XRD pattern of n-HAP/n-β-TCP/PEG composites which shows that HAP and β-TCP exists as nano form and present together in the composite. The peaks corresponding to nano HAP powder were found at the reflections of (002), (102), (210), (211), (112), (300), (202), (310), (311), (222), (213), (321), (004) and (510). The crystalline nature of nano HAP can be established by the presence of diffraction peaks with high intensities and minimal line broadening after indexing the two theta values with JCPDS file no.9-0432. The observed patterns clearly indicate that no structural transformation of mono phasic nano HAP occurs. This indicate that the bioactivity would be preserved. The intensity peaks are assigned to n-β-TCP were obtained at (024), (1010), (214), (300), (0120), (220), (1016), (3012) and (4010). These, hkl indices were compared with JCPDS file no.09-0169. The intensity peaks for PEG were obtained at the XRD patterns indicates that the addition of polymer to the sample do not affect the crystal structure of n-HAP and β -TCP nanoparticles. PEG is observed to show XRD peaks at 23.328 and a few minor peaks are

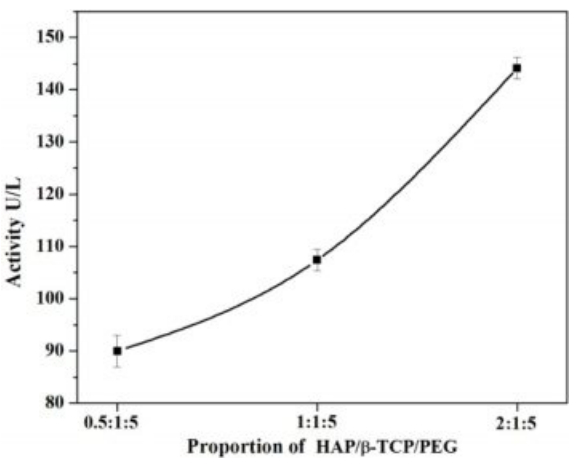

Fig. 2. Alkaline Phosphatase (ALP) activity at different proportion.

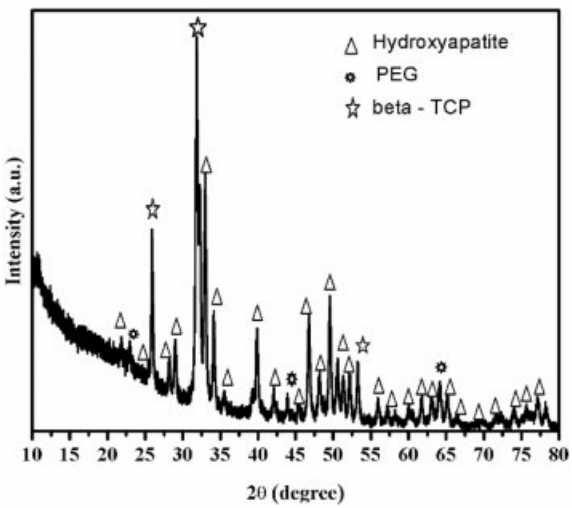

Fig. 3. XRD pattern of n-HAP/n-β-TCP/PEG composites.

observed, which is consistent with data in previous report [33].

Field Emission Scanning Electron Microscope study (FESEM)

The FESEM micrographic images of pure n-HAP/n-β-TCP/PEG compositions are shown in Fig. 4. The FESEM image shows that particles are well agglomerated in the polymer matrix. The images show spherical particles of the ceramic to be embedded in PEG indicating the composite nature of the ceramics. The porosity of the samples were found to vary from 1.3 to 2%. The EDS of n-HAP/n-β-TCP/PEG compositions shown in Fig. 5. This indicates the presence of Ca, O, P and C in the nanocomposite which further confirms the presence of n-HAP/n-β-TCP.

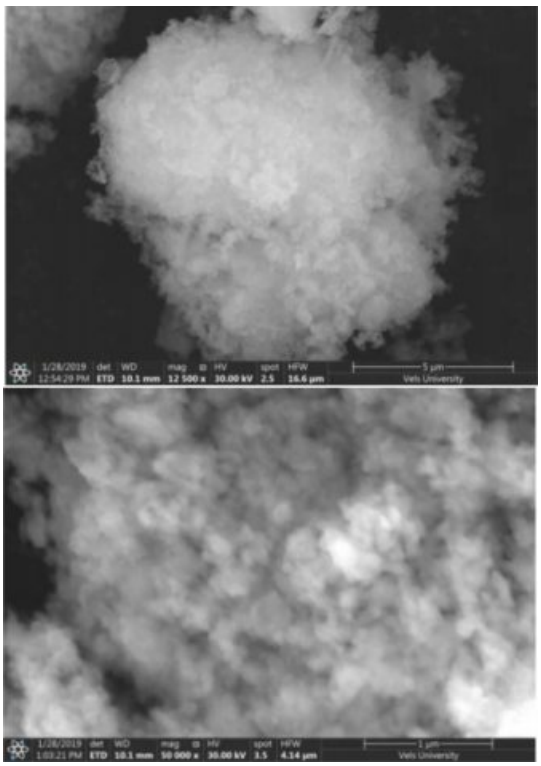

Fig. 4. FESEM analysis of n-HAP/n-β-TCP/PEG composites.

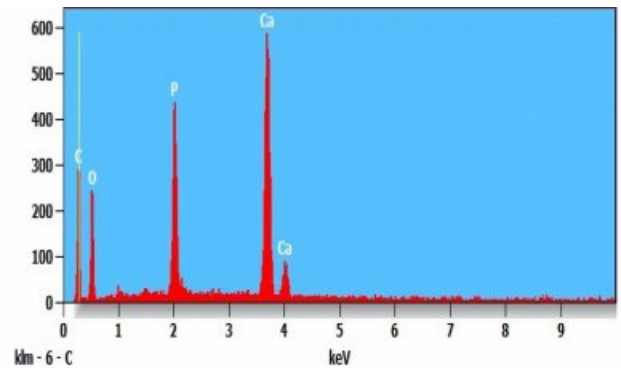

Fig. 5. Elemental analysis of n-HAP/n-β-TCP/PEG composites.

Biochemical study of n-HAP/n-β-TCP/PEG composites

Cytotoxicity assay

The composite mixture prepared with n-HAP/n-β-TCP/PEG (1:1:5) were taken in different dilutions. The linearity of MTT assay in the MG-63 cells were plated out in doubling dilution using 0.1mL growth medium in 96 well plate. The MTT is added to all the cells immediately and the plates were allowed to incubate at 37 °C for 4 h. The graph was plotted between the obtained concentration and the percentage of cell viability. The duration of exposure is usually determined as the time required for maximal damage occurs but is also influenced by the stability of the composites. MTT is the most commonly used yellow colour substrate and this converted into a dark blue formazan product when incubated with the live cells. The viability percentage of the cells were measured spectrophotometrically by the colour obtained after the addition of MTT [32]. The exposure time is prolonged to identify the rate of proliferation of the cell.

Fig. 6 indicates the percentage of cell viability IC_{50} that can be reached by the active cells after 24 h upto 250 μg/ml. This indicates that the percentage of cell viability is found to be 50% and above. The rate of cells were increased when the incuabtion time was extended to 72 h. When the concentration increases the 50% of cell viability reached only in extended period of incubation. When the concentration increases upto 1,000 μg/ml the percentage of cell viability IC_{50} is found only after 48 h of incubation. The proliferation of the cells are identified at different exposure time. In all concentration when the time of exposure increases the rate of proliferation increases The Fig. 6 shows that in each concentration 72 h peak is higher than 48 h which inturn higher than 24 h. This suggests that when the concentration increases to 500 μg/ml and 750 μg/ml, the increase in incubation period is required to get better proliferation at 48 and 72 h respectively.

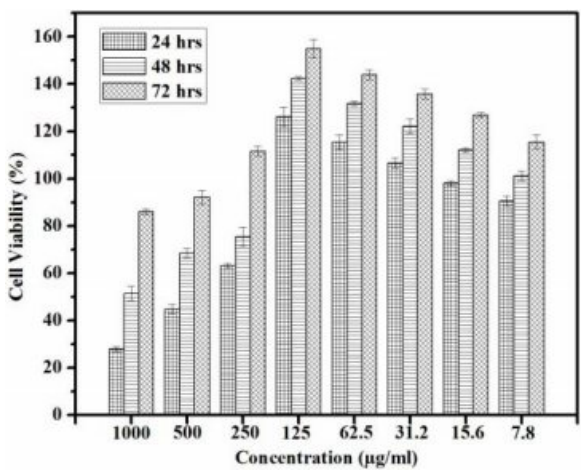

Fig. 6. Cell viability at different concentration and different time period.

These results indicate that the level of cell viability increases with time of exposure and it decreases with concentration. The cells were allowed to proliferate for two to three population doubling times (PDTs) in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable cannot proliferate. The percentage of surviving cells is then determined indirectly by MTT. The amount of MTT formazan produced is directly proportional to the cell viability. The proliferation of the cells were noticed from the concentration 125 µg/ml, 62.5 µg/ml, 31.5 µg/ml where the cell viability percentage is found to be double and that confirms the population doubling through proliferation. Normally IC_{50} represents the concentration of the composite materials that is required for 50% of cell viability. This 50% viability is achieved at 1,000 µg/ml and 500 µg/ml after 48 h of incubation.

The same 50% viability can be achieved in all other concentration (250, 500 and 750 µg/ml) after 24 h of incubation. Fig. 6 shows that IC_{50} can be seen upto 250 µg/ml concentrations with an exposure time period ranging from 24 h, 48 h and 72 h. When the concentration is increased to 500 µg/ml and 1,000 µg/ml the IC_{50} cannot be achieved in the initial time phase of 24 h and this will get elicit only after the period of exposure 48 h and 72 h respectively. This implies that at all the concentration the cells would proliferate indicating that the sacffold is biocompatible. As the concentration range increases above 250 µg/ml it is important to increase the incubation period for cell proliferation to take place. The results indicate that the nano composite of n-HAP/n-β-TCP/PEG can be used maximum of 250 µg/ml as the substituent for the scaffold preparation. The concentration preferable for scaffold is found to be maximum at 250 µg/ml. As the concentration crosses 250 µg/ml the period of incubation should increases.

Thus, it can be inferred that when the concentration exceeds, the period of incubation also need to be increased for finding both the cell viability and proliferation This assay suggest that the choice of materials used in preperation of nano composite is good to provoke the proliferation of cells. The scaffolds prepared indicate the presence of osteoconduction property.

Antibacterial activity

Table 1 shows that no zone of inhibition was found at different concentrations of 1,000 µg/ml, 750 µg/ml and 500 µg/ml and the inhibition zone found only in the antibiotic amphicilline disc. The composite materials were tested with *Staphylococcus aureus* which is the common pathogen that induces the bone infection. The composite materials of different concentration such as 1,000 µg/ml, 750 µg/ml and 500 µg/ml were placed in a disc and the disc placed over the microbial environment. The zone of inhibition was calculated by measuring the diameter of the diffusion around the disc. This confirms that the composite does not show any inhibition towards the *Staphylococcus aureus*. The inhibition is not found with the increase in concentration.

Table 1. Antibacterial activity of n-HAP/n-β-TCP/PEG composites.

Organisms	Zone of Inhibition(mm) Concentration (µg/ml)			Antibiotic (1 mg/ml)
	1000	750	500	
Staphylococcus aureus	-	-	-	13

DPPH (2,2 diphenyl 1 picryl hydrazil) assay

$$\% \text{ Antioxidant Activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Scientific evidence suggests that antioxidants reduce the risk for chronic disease including cancer and heart diseases. The main characteristics of an antioxidant are its ability to trap free radicals. The antioxidant property of the composite n-HAP/n-β-TCP/PEG is measured with the addition of 1,1 diphenyl 2 picrylhydrazyl (DPPH). The formation of reduced form of DPPHH is due to the reaction of free radical with unpaired electron of DPPH become reduced by capturing hydrogen ions

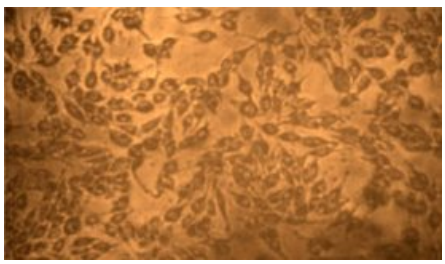

Fig. 7. MG-63- CELL LINE (40x ×100).

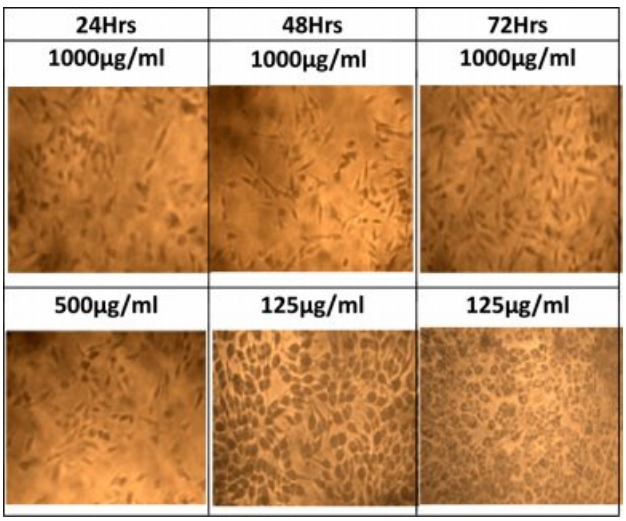

Fig. 8. Cell proliferation image at different concentration and time period.

Table 2. DPPH activity of n-HAP/n-β-TCP/PEG composites.

Sl No.	Concentration (µg/ml)	DPPH Activity %
1	1000	31.70
2	750	51.02
3	500	61.25

Control = 0.880 µg/ml

which was donated by the composite materials. The reduced state of DPPHH would form yellow colour. The increase in the intensity of the colour confirms the higher reducing activity and also more antioxidant property ie. above 50% which is required for implantation inside the biological medium.

Table 2 explains the activity of the DPPH for the different concentration of the composite n-HAP/n-β-TCP/PEG. A decrease in the acitivity of the composite was found with the increase in concentration of the composite. The activity above 50% can be viewed only in 750 µg/ml and also below that concentration. This study concludes that the DPPH property can be good for the composite when the concentration range is less than 750 µg/ml is used for the scaffold preparation.

Alkaline phosphatase assay

The composites were further analysed for the bone formation ability by using ALP activity. The analysis were taken for different concentration of the n-HAP/n-β-TCP/PEG composites such as 1,000 µg/ml, 750 µg/ml, 500 µg/ml and 250 µg/ml respectively. The results were taken after 3 minutes incubation at the standard condition. The results indicate that ALP activity of the composite increases when the concentration increases. This is mainly due to the presence of increased levels of Alkaline phosphatase enzyme in the hemolysis sample. The increase level leads to the breakdown of the proteins in the body. This will convert more pyrophosphate to inorganic phosphate which induces bone mineralization and this would in turn increase the bone growth. Normally, healthy adult should have the ALP level 20-140 units per litre (U/L) below and the above will leads to discomfort in healthy individual. Since, ALP is need for the apatite formation the level should be monitored that it should not cross the maximum level.

$$\text{ALP Activity in U/L} = \Delta A/\text{min.} \times 2754$$

The results of ALP activity are given in Fig. 9 indicate that the concentration up to 750 µg/ml, the ALP level is increased and does not cross the optimum level. These reports confirms that the n-HAP/n-β-TCP/PEG composite can be used up to 750 µg/ml concentration as scaffold for implantation.

Conclusion

n-HAP/n-β-TCP/PEG composites indicate better biochemical and bioactivity performance at 1:1:5 proportion. The presence of n-HAP/n-β-TCP crystalline phases were confirmed from XRD patterns. FESEM analysis of the prepared composites indicates that the n-HAP and n-β-TCP are embedded in the polymeric matrix. The elemental analysis further confirms that the compsotion of nano composites. The biological studies of the composites indicate its biocompatibility, cell proliferation, antioxidant, toxicity and ALP activity. MTT assay confirms the MG-63 cells when adhered to the composite mixture at different concentration shows better viability and also proliferation at exponential phase. The concentration range preferable is 250 µg/ml to 750 µg/ml. To get better proliferation it is suggested that when the concentration range exceeds 250 µg/ml the incubtion period should increases. The DPPH assay confirms that the composite can be used for cancerous patients due to its anti oxidant property that results in the trapping of free radicals. The increased alkaline phosphatase enzyme can be seen in the required concentration for the scaffold preperaed in the ratio of 1:1:5 indicating its osteoconduction and osteoinduction nature.

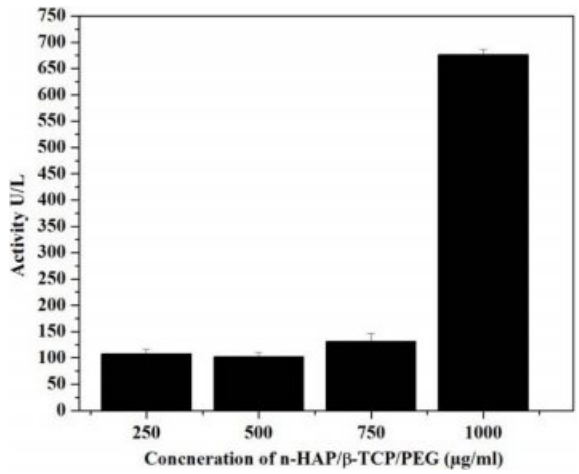

Fig. 9. Alkaline phosphatase activity of n-HAP/n-β-TCP/PEG composites.

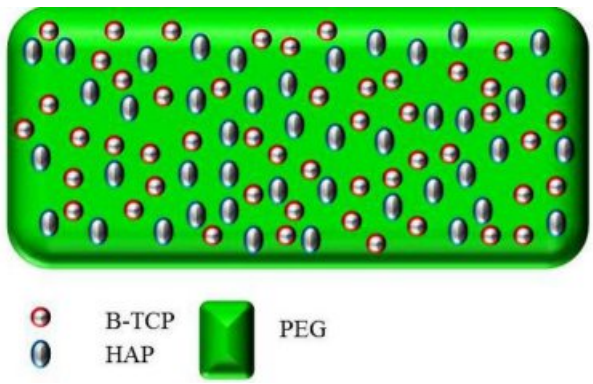

Fig. 10. Porous spongy structure of scaffold.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgments

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