# Preparation and Cytotoxicity Study of Polycaprolactone/Polyethyleneglycol-Hydroxyapatite Nanocomposite for Biomedical Application

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*Abstract*— **Polycaprolactone (PCL) is one of the best synthetic and biodegradable polymers used in biomedical applications, while the hydroxyapatite (HAP) is fast emerging as a highly suitable biomaterial for the bone implant/ dental applications. The synthesis of non toxic and biodegradable composite of HAP and PCL is a promising approach in the development of bone tissue engineering (BTE). In the present work HAP nano rods were prepared by using Polyetheleneglycol (PEG) as a shape directing agent. PCL was dissolved in chloroform and HAP-PEG nanorods were added and then it was ultrasonicated for 15 min. In order to prepare HAP-PEG/PCL nanocomposites, the chloroformic solution was poured on to a petriplate and it was dried under vacuum .The prepared nanocomposite was characterized by FTIR, XRD, FESEM, HRTEM and EDX analyses. Biocompatibility of the HAP-PEG/PCL nanocomposite was investicated by MTT assay. The cytotoxicity studies imply that the nanocomposite is nontoxic. Anti microbial activity study on drug loaded HAP-PEG/PCL nano rods was also performed.**

**Keywords-component; Hydroxyapatite , Nanorods , Nanocomposites**

## I. INTRODUCTION

Due to its excellent biocompatibility,bioactivity and osteoconductivity HAP is consider to be a biomaterial. Its chemical formula, $Ca_{10}(PO_4)_6OH_2$, is similar to that of bone and teeth. It has been extensively used in various field such as implant coatings, bone tissue engineering, bone grafting and drug delivery systems[1-4]. Recently, polymer-HAP nanocomposite materials are fast emerging candidate material towards the biomedical applications. These composites are mostly used as scaffolds in bone tissue engineering (BTE) due to the porosity of the polymers. Several methods were developed for fabricating porous HAP scaffolds, such as pore forming, foaming, gel-casting, freeze drying and polymer impregnating method (PIM) [5-10]. Out of these methods employed, PIM is the most suitable method to produce high porous HAP- composites with great structural similarities with natural bone ,which induces osseointegration in implant devices [10-11].Many biodegradable polymers are commercially available and are used for BTE, the Polycaprolactone (PCL) is one of the attractive and commonly used hydrophobic biodegradable polyester12]. PCL plays a major role in biomedical applications [12-14] such as scaffolds in tissue engineering and for controlled release of drug due to its porosity. In the present work a synthesizing a bioactive ceramic HAP in nano scale is attempted using shape directing agent polyethylene glycol. This is further coated with biodegradable hydrophobic polymer PCL to form a nanocomposite PCL/PEG-HAP.

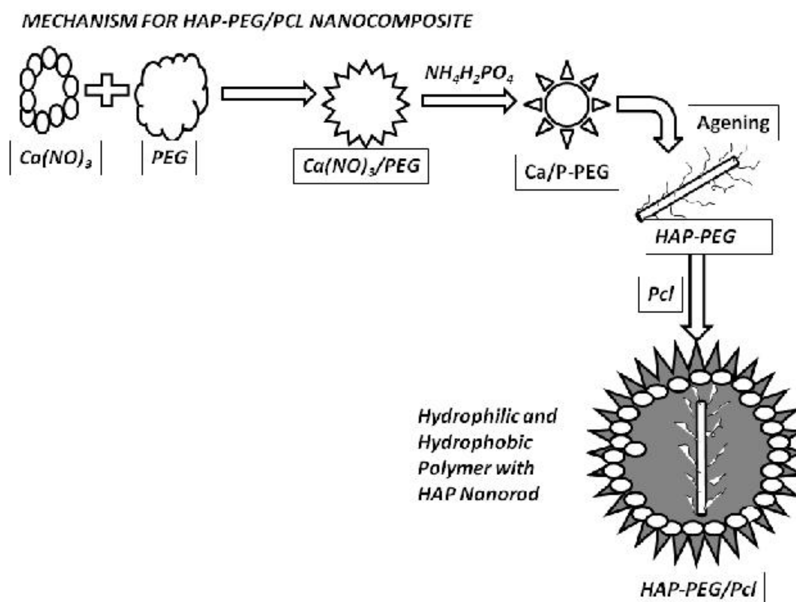
## II. MATERIALS AND METHODS

### A. *Chemicals and Materials*

Reagent grade calcium nitrate tetra hydrate ($Ca(NO_3)_2.4H_2O$), ammonium dihydrogen ortho phosphate, liquid Ammonia, poly ethylene glycol and poly(caprolactone) from Aldrich chemicals were used.

### B. Synthesis of HAP

The Hydroxyapatite was synthesized by direct precipitation method. An aqueous solution of 1M calcium nitrate and 0.67M of $NH_4H_2PO_4$ solution was prepared and both the solution was maintained at a pH 11.0 using ammonia solution. A 5ml of PEG was added to the 1M $Ca(NO_3)_2.4H_2O$ and the solution was continuously stirred for 1 hour in room temperature and subsequently, the pH was adjusted to 11. A drop wise addition of 0.67 M $NH_4H_2PO_4$ solution (pH 11) to the above solution. After addition of $NH_4H_2PO_4$, the final PH was adjusted to 11. The aqueous solution was continuously stirred for about 2 hours. The white precipitate obtained was separated by centrifugation, washed repeatedly with distilled water. The product was dried in an oven at 80ºC for 24 hr. The HAP-PEG powder obtained were ground with a mortar and pestle.




0.5g of PCL was dissolved in chloroform and HAP-PEG nanorods were added and then it was ultrasonicated for 15 min. In order to prepare HAP-PEG/PCL nano composites, the chloroformic solution was poured on to a petridish and dried under vacuum.

### C. Characterization

A Schimadzu FT-IR 8300 series was used for recording IR spectrum for HAp an polymer composites. The samples were scanned at the range of 4000 to 400 cm-1 by KBr pellet technique. Phase analysis was performed using BRUKER D8 advance X-ray Diffractometer (XRD).Synthesized HAp powder were characterized using XRD to determine the fraction of crystallinity, crystallite size, specific surface area. The crystallite size of the sample was calculated from the Scherrer's equation [12],

$$Xs = 0.9 \quad / \quad \cos$$

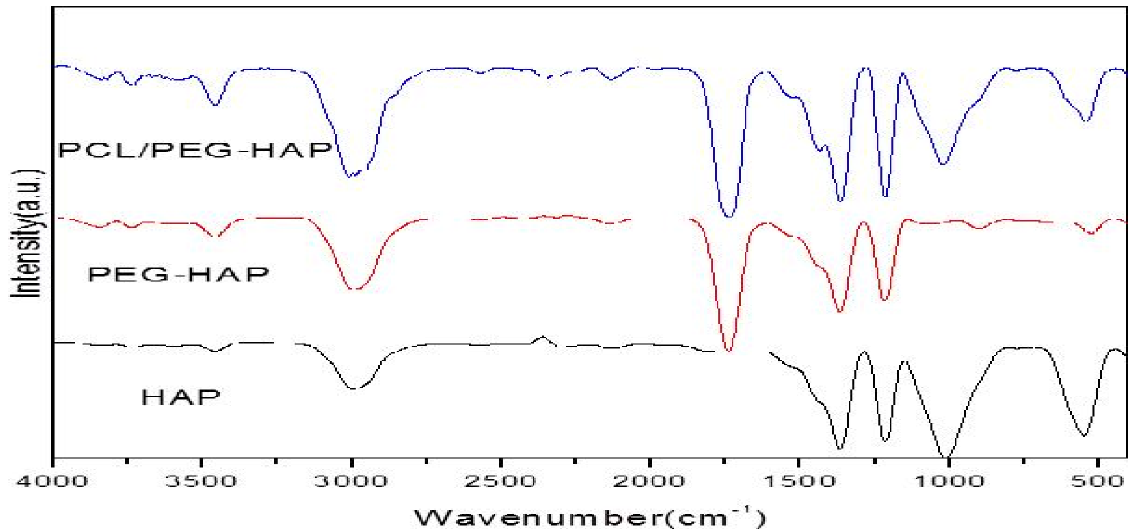
Where, is the wavelength CuK radiation source ( =1.54A°)
is the full width half maximum,
is the angle of diffraction
The morphology of the sample was investigated using FESEM (HITACHI SU6600) and HRTEM (HRTEM-FEI,TECHNAI G2,30s-twin D905).

## III. RESULTS AND DISCUSSIONS

### A. FTIR analysis

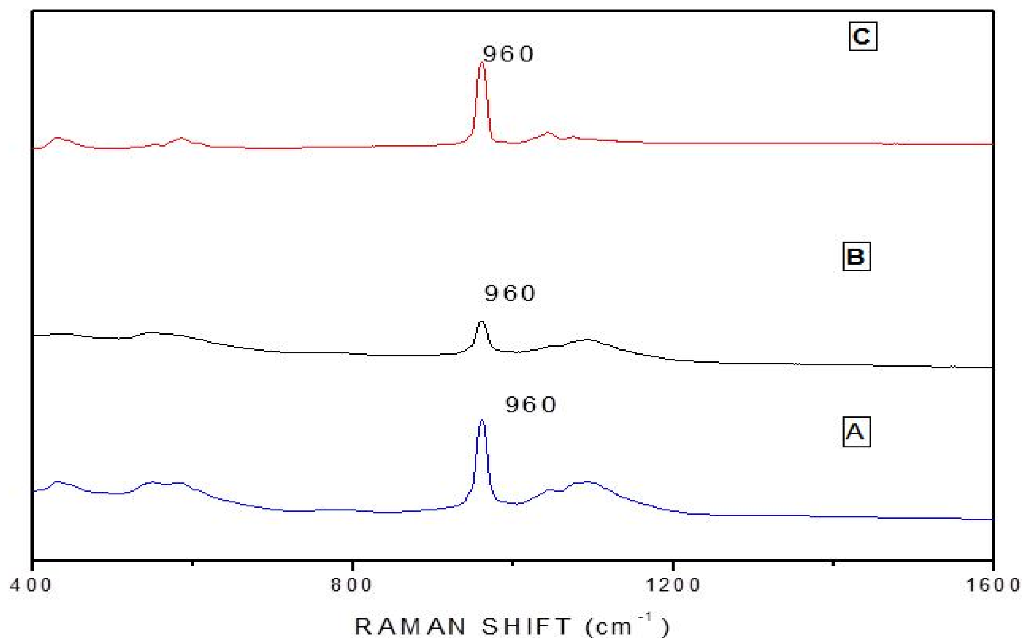
IR spectrum of PEG-HAP and PCL-PEG-HAP were shown in Figure(1.A.). The bands due to C–O stretching mode were merged in the very broad envelope centered on 1268 and 1007 cm−1 arising from C–O, C–O–C stretches and C–O–H bending vibrations of Ca/P nano particles in PEG. The aliphatic C–H stretching, in 1413 and 1344 cm−1 observed were due to C–H bending vibrations [15]. The peak at 1094 cm−1 was due to binding of C–C–O and C–C–H groups present in PEG with nanoparticles [16,17]. The peak at 1752 cm−1 is due to stretching of the carboxylic acid, while the peaks at 3575 cm−1 corresponds to stretching of OH group of carboxylic acid. Most of the phosphate peaks of HAP were found to be merged

with the polymers and therefore, the FT-IR spectra showed the existence of van der Waals interactions between the chain of PEG, PCL and HAP NPs in the polymeric media [18].



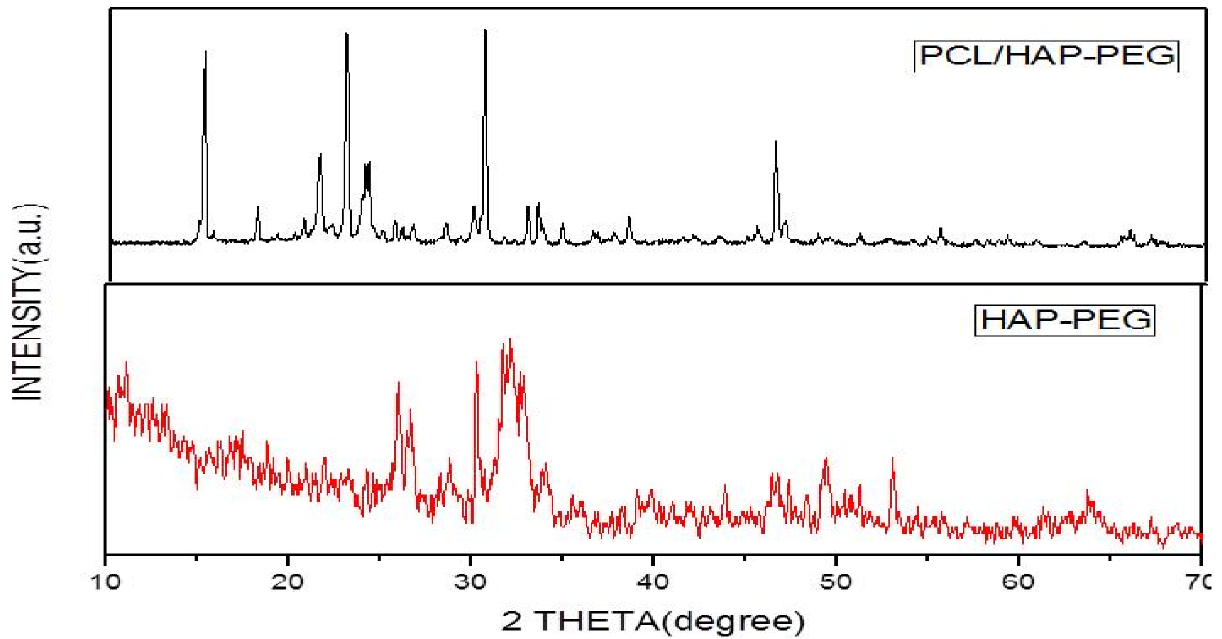

*B.* R**aman Analysis :**

The instrument used in this study was Nanophoton Raman spectrometer equipped with Nd: YAG 1064 nm as an excitation source. The Raman spectra for all the four samples were shown in Fig.(1.B) The sharp peak observed at 959cm-1 shows the strongest 1 P-O stretching mode of HAP The bands observed around the region of 1000-1150cm-1 were attributed to 3 P-O stretching. Splitting of peaks at 426 and 549cm-1 were assigned to 2 O-P-O doubly degenerated bending mode. The presence of polymer on higher concentration resulted in reducing the intensity and increasing the broadness of phosphate peaks since the polymer tend to cover the HAp particles thus minimizing the intensity and as well as crystallinity of HAp.



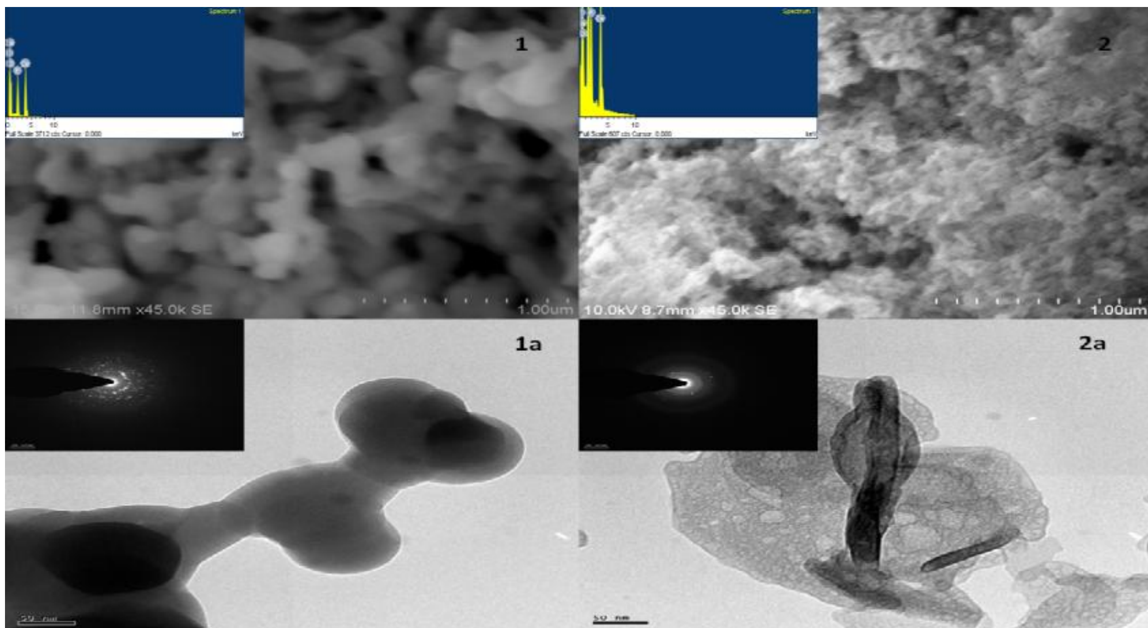

***X-Ray Diffraction:***

*The XRD patterns of samples were shown in Fig.(1.C) . The XRD patterns indicated the presence of crystallized hydroxyapatite. The X-ray patterns collected on the powders after polycaprolactone addition showed a highly crystalline phase of HAP. These patterns are in good agreement with the ASTM data (JCPDS) file (no. 09-0432) for hydroxyapatite. No characteristic peaks of impurities, such as calcium hydroxide and calcium phosphates were observed and thus a phase pure HAP was prepared under the present experimental conditions. The diffraction peaks particularly in the planes (0 0 2), (2 1 1), (1 1 2) and (3 0 0) were of higher intensity which implies that the HAP crystallizes out well.*




**FESEM and HRTEM:**

The Morphological investigation of the samples were presented in Fig.(1.D) The HAP sample yielded an particles of random size and shape. The shape directing agent PEG aided HAP produced nanorods. The PCL is found provide a uniform coverage to the above nano rods. The FESEM and HRTEM images were found correlated well. The nano rod with dimension of  20nm breadth and 300nm length wasshown in TEM micrograph. EDX value matches with the stchimometric Ca/P ratio of 1.67

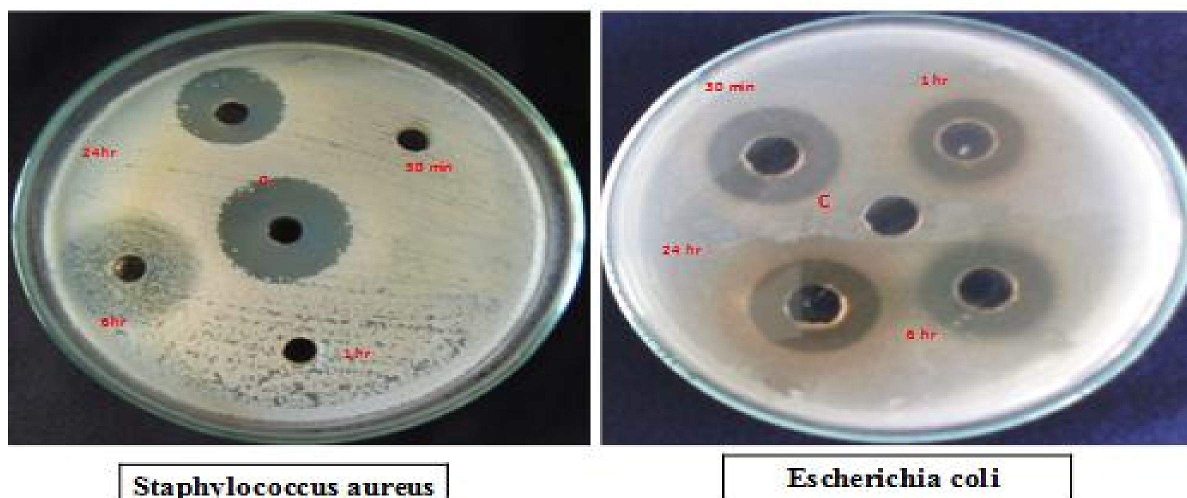



***Antimicrobial studies:***

***Drug loading:***

Standard amoxicillin solution (4 mg/mL) was prepared in tris-buffer solution (pH 7.4). Then, 0.5 g of HAP particles were immersed in 10 mL of antibiotics solution at 370C for 48 h. The drug solution was then decanted and the HAP particles were dried.

**SODIUM PHOSPHATE BUFFER SOLUBLE Drug/HAP USED FOR ANTIMICROBIAL ACTIVITY**




The Drug loaded HAP particles were subsequently immersed phosphate buffer solution (pH 7.4) and thermally agitated. The drug leached out at various time intervals were tested for microbial activity.

***Cell viability:***

***Mtt assay:***

***ADSC isolation method***

The Rat Adipose Derived Stromal Cells (ADSC) isolation procedure was based on a technique previously reported by Yamamoto et al. (2007). Briefly, after weighing the harvested adipose tissue, it was minced and mixed with 20 ml PBS containing 0.2% collagenase type II (Worthington, Lakewood, NJ). All isolation steps were performed under sterile conditions in a laminar flow hood. Digestion was supported by incubating the mixture at 37˚C for 45 min. Upon digestion, the cell suspension was filtered through a 100 lm cell strainer and an equal amount (20 ml) of culture medium was added. Afterwards, the cell suspension was centrifuged (1000g for 10 min), the supernatant removed and the cell pellet re-suspended in fresh cell culture medium. Cell culture was carried out in T25, T75 or T225-flasks with media changes twice a week. Biological assays were performed using Adipose derived stromal cells (ADSC). ADSC were cultured in 75 cm2 cell culture flask in Dulbaco minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic solution (streptomycin 100 lg/mL and penicillin 100 U/mL, Sigma Chem) and 2 mM L-glutamine. For in vitro osteogenic differentiation, ADSC cells were cultured in ‘‘osteogenic induction medium’’ consisting of complete DMEM medium supplemented with 50 lg/mL of ascorbic acid, 10 mM glycerol-2-phosphate and 10-7 M of dexamethasone. ADSCs from passages 4 through 6 passages were used for all the experimental procedures and incubated at 37ºC in a humidified atmosphere with 5% $CO_2$ and 95% air.

***Cell viability (MTT Assay)***

Cell viability was assessed by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) method as described by Yuan et al., 2004.

***Principle***

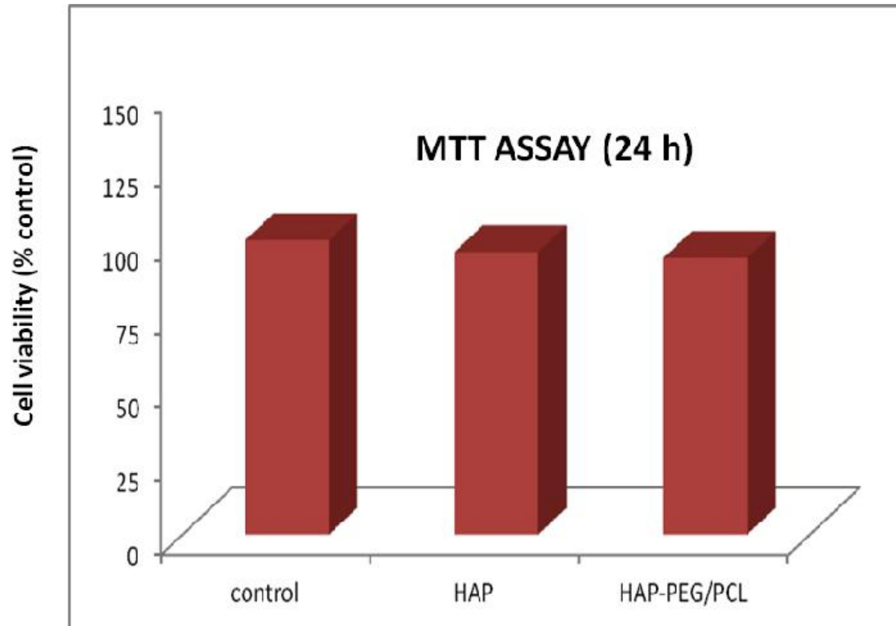
Tetrozolium salts form nonwater soluble formazan crystals within the cells to form a coloured complex in addition to solublizing solution, which can be read at 570 nm in an ELISA reader.

***Reagents***

1. MTT (0.5%):

0.25 g MTT was dissolved in 50 ml of serum free DMEM medium.

2. Solubilizing solution (20% Sodium Lauryl Sulfate (SDS) in 50% dimethylformamide DMF) 5.0 ml DMF was made up to 10 ml with Distilled water and 2 g of SDS were added and mixed well.




Each bar represents ± 3 observations

IV) **Experimental protocol for MTT assay**

20,000 cells were plated in 6 multiwall plates with DMEM medium containing 10% FBS. The cells were incubated for 12 h under 5% CO2, 95% O2 at 37°C. Then the DMEM with FBS was removed and replaced with containing 1% BSA for 12h.Then, BSA medium was removed and the control plates received DMEM medium and treatment plates received , Hydroxyapatite (HAp) and (HAp-PEG)/polycaprolactone(PCL) . The treatment protocol is represented in the experimental protocol.

After 24h of culture, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5diphenyl tetrazolium bromide(MTT) in to an insoluble dark blue formazan reaction product. MTT was dissolved in DMSO and the culture plate was incuabated at 37’C for 24h.

**Conclusion**

In this study, PEG-HAP/PCL composite was synthesised successfully. Functional group analysis by FTIR and RAMAN investigations of the sample showed the incorporation of PEG and PCL. The XRD data showed the formation of phase pure and highly crystalline composites. Morphological study of the sample has been evolved and there is good treaty between the FESEM and HRTEM .Antimicrobial results shows the Pathogens are Inhibited with the drug loaded samples and the inhibition zone increases due to the leachout of the drug in phosphate buffer. The incorporation and leaching out of drug is due to the porous nature of the hydrophilic and hydrophobic impregnated HAP. Cytotoxiciy study shows the sample of HAP and PEG-HAP/PCL were non-toxic and can be capable of used as a scaffold in in-vivo system

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